Physiological responses of developing Port Jackson sharks to predation and elevated temperatures

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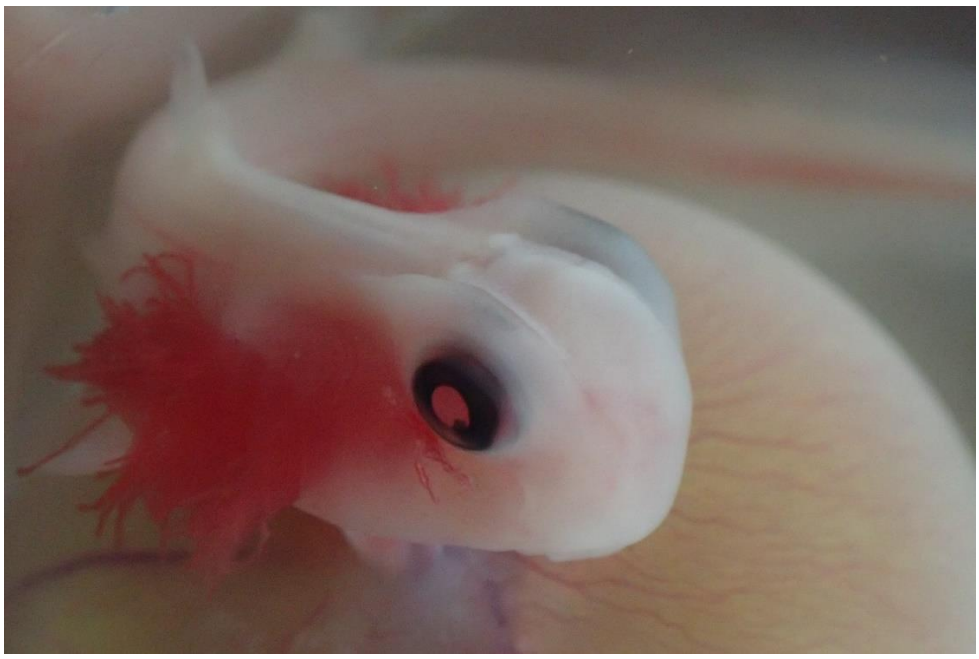

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Statement of originality

I submit this thesis "**Physiological responses of developing Port Jackson sharks to predation and elevated temperatures**" as a wholly original work, not submitted for any other higher degree.

This thesis has been written by me and any external contributions to the planning, execution, and editing are credited and acknowledged in an appropriate manner. I ensure that I have referenced all of the literature used in this thesis and accurately included them in the references.

Furthermore, the research and experimental protocols utilized in this thesis were conducted with approval by Macquarie University's Animal Ethics Committee under protocols 2014/003 and 2016/027.

Connor Robert Gervais

April 10, 2019

List of original publications

I. Friend or foe? Odour detection, differentiation, and anti-predator response in an embryonic elasmobranch, *Heterodontus portusjacksoni*

II. Fight, Flight, or Freeze? Impact of conspecific necromones on the oxygen uptake rates of a benthic elasmobranch

III. Developing in hot water: Populations vary in the physiological cost of embryonic development in Port Jackson sharks, *Heterodontus portusjacksoni*

IV. Phenotypic plasticity or local adaptation: thermal and spatial effects on the physiology and behaviour of an oviparous elasmobranch, *Heterodontus portusjacksoni*

Contribution of others

Contributions from each collaborator during each chapter are listed.

Collaborators: Connor Gervais- CG; Culum Brown- CB; Charlie Huveneers- CH; Jodie Rummer- JR; Tegan Fuchert- TF; Tiffany Nay

Thesis chapter	*Planning/ experimental design*	*Data collection*	*Analysis*	*Writing & editing*	*Overall responsibility*
I.	**CG, CB**	**CG**	**CG, TN**	**CG, TN, CB**	**CG**
II.	**CG, CB**	**CG**	**CG**	**CG, CB**	**CG**
III.	**CG, JR, CB**	**CG, TF**	**CG**	**CG, CB, CH, TF**	**CG**
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Abstract

Elasmobranchs are especially vulnerable to perturbation given their life history characteristic including slow growth rates and relatively long gestation time. Species which develop in eggs are thought to be at a heightened risk to predation and the most vulnerable to changes in environmental conditions, such as increased temperatures, given their lack of capacity to move and evade these situations. Relative to other taxa, elasmobranchs are relatively under studied and lack much of the baseline information regarding their embryonic development and responses to these pervasive conditions. In this thesis, I investigated the physiological and behavioural implications of predation and elevated temperature over embryonic and juvenile life stages of the Port Jackson shark, *Heterodontus portusjacksoni.* Specifically, the first two data chapters explored the respiration strategies of embryos in response to chemical cues from a known predator during development, and how conspecific necromones influence these traits in juvenile sharks. Secondly, the final two data chapters investigated how rearing temperature impacts the physiology and development of embryos collected from two distinct populations, and the knock-on effects this may have on the behaviour and physiology after hatching. Intermittent flow-respirometry techniques were utilized to measure the physiological changes in oxygen uptake rates with exposure to different stimuli (predator cues or temperature). This methodology can be useful at determining changes in an individual's energetic need (proxy for metabolic rates), as well as determining strategies linked to changes in respiratory function. Furthermore, to determine upper thermal limits and activity patterns across temperatures, standard critical thermal methodologies and a step-wise thermal stress methodology were utilized.

Predation can shape marine communities, influencing behaviour and physiology of prey. Even without the physical presence of a predator, predator-associated cues can elicit anti-predator strategies. While most species simply show enhanced or pre-emptive escape responses to evade predatory situations, young life stages may have limited options. Specifically, during embryonic development species are restricted within their egg capsule and may be limited in their sensory capabilities. Therefore, different strategies may be utilized during these early life-history stages to allow them to evade predator detection. In Chapter I, I investigated the capacity of developing embryos to detect and respond to predatory odours. Embryos were exposed to predatory cues (a horn shark) and non-predator cues (a teleost fish) while in a respirometry chambers to measure changes in oxygen uptake rates. Embryos displayed variable responses both dependent on odour and on developmental stage. During development their response was strongest to non-predatory cues, in which embryos depressed oxygen uptake rates, similar to that of 'crypsis' responses. However, this response diminished over development which may indicate that embryos have the capacity to assess predation risk and thus regulate their response based on prior experience. At hatching, the embryos responses where highest

towards predatory cues in which they increased oxygen uptake which may be similar to that of 'fight or flight' responses in which sharks were pre-emptively trying to relocate away from predation cues.

Unlike embryonic individuals, hatchlings are able to move and utilize different anti-predator strategies to that of embryos. No longer are they restrained to one place, but they can use energetically costly escape responses to evade capture. The scent of a dead conspecific (necromones) is often enough to elicit anti-predatory strategies in other species such as teleost fish and amphibians; however, it is unknown if these cues would elicit any response from sharks. Given that juvenile Port Jackson sharks are found within loose aggregation in the wild, in Chapter II I explored the anti-predatory strategies of juvenile sharks and their response to conspecific necromones. Juveniles were exposed to necromones, chased (to simulate a predator attack) or both. Sharks depressed their oxygen uptake rates by 18% in response to necromones, indicative of 'crypsis' response. Unsurprisingly, when chased, regardless of if exposed to necromones, oxygen uptake rates increased to meet the energetic demand of exercise, and any other associated processes.

Temperature is often viewed as the most influential environmental factor shaping development, influencing the physiology and thus behaviour of ectotherms. As such, the development of ectotherms is often significantly impacted by changes in environmental temperature. As the climate continues to change and ocean temperatures rise, ectotherms will be exposed to warmer waters. Those without the capacity to behaviourally thermoregulate, such as embryos, will be forced to develop in conditions that may be suboptimal and thereby experience deleterious effects (e.g., elevated metabolic rate, depressed body condition, or increased developmental abnormalities) which may not just impact embryonic development, but cause knock-on effects after hatching. Furthermore, local environmental conditions are likely to influence the strategies and sensitivity of different populations to changing conditions. In Chapter III, I reared embryos collected from two populations (Jervis Bay, warmer water population, and Adelaide, cooler water population) and reared them under current day temperatures, predicted end-of-century temperatures, and reciprocal cross-population temperatures to examine the influence of provenance and temperature across the development of Port Jackson sharks. Rearing temperature significantly influenced the growth rate of embryonic sharks; however, there was no difference in oxygen uptake rates between rearing temperatures. Instead, developmental stage was the strongest driver of changes in energetic need, most likely reflecting changes in developmental processes. When populations were reared under similar conditions, populations displayed innate differences, with those from Jervis Bay requiring a greater oxygen uptake rate than those from Adelaide. Furthermore, provenance had significant influences on several morphometric traits; Those from warmer population (Jervis Bay) were larger than those from cooler locations (Adelaide). Given their long developmental period and thus exposure to both summer and winter conditions, Port Jackson shark embryos appear to rely on developmental acclimation and may be relatively robust to changes in rearing conditions.

Juvenile sharks are thought to more effectively behaviourally thermoregulate than during their embryonic stages. In theory this may enable later life stages to live within a narrower thermal niche and conserve energy, otherwise required to maintain a broad thermal tolerance (i.e., thermal window). While embryos may inhabit a thermal window that is dictated by environmental conditions, juvenile can potentially select more favourable conditions. Therefore, in chapter IV, I explored the influence of temperature on the physiology and behaviour of juvenile sharks. Specifically, I investigated three traits, oxygen uptake rates (proxy for metabolic rates), the upper thermal limits and the swimming activity with acute exposure to elevated temperatures. Elevated temperatures significantly influenced each population differently, yet overall, shark thermal limits reflected rearing temperature (i.e., higher rearing temperature indicated higher upper thermal limit). While sharks from Jervis Bay showed no difference in resting oxygen uptake rates between rearing temperatures, those from Adelaide reared under elevated temperatures displayed double the resting rates to those under ambient conditions. In contrast, when exposed to progressively warmer conditions, Adelaide sharks displayed no decline in swimming traits, while those from Jervis Bay displayed a reduction in swimming speed and swimming distance. However, sharks from both populations reared under similar temperatures displayed similar resting oxygen uptake rates and swimming performance, indicating that these traits may not display any local adaptations between populations. As temperatures continue to rise, those from the warmer range boundaries (here Jervis Bay) will be exposed to relatively more challenging conditions than that of cooler water populations (here Adelaide). Understanding the physiological and behavioural costs associated with warmer conditions may elucidate the strategies that potentially sensitive populations (or life-history stages) may utilize in the future.

The response of young, developing sharks is thought to reflect the highly stressful conditions that they experience; however, in this thesis, Port Jackson sharks may be relatively robust. Rather than utilize escape responses, an energetically costly tactic, embryos and juveniles appear to preferentially depress their oxygen uptake to try and "blend in" to their environment. Additionally, as embryos, this species displays an incredible capacity to acclimate their energetic needs with exposure elevated rearing temperatures in order to persist until hatching. Given that many elasmobranchs are both the prey and predator, future studies should aim to investigate the relationships and trade-offs between predator responses and temperature. Here we display that sharks respond to predator cues; however, this species displays significant changes in their metabolic rates and/or depressed activity as a result of warmer rearing temperatures. If these traits work antagonistically with each other, the overall health and fitness of these species, and potentially others, could be at risk. Early life stages are highly vulnerable to a suite of different factors. The strategies that these species use in response to predation and temperature ultimately influence the fitness and survival of the individuals, but also populations and ecosystems. Sharks help maintain different ecosystems in which they are present, understanding

the effects that a changing climate play, can help us predict future distributions and relative vulnerabilities.

Introduction

Aquatic predators play a crucial role in maintaining healthy ecosystems including such impacts as, controlling food webs and trophic cascades, aiding in nutrient cycling and even promoting climate change mitigation, among a suit of other ecological and even socioecological benefits (Hammerschlag *et al.*, 2019). Elasmobranchs (subclass *elasmobrancii*)—sharks, skates, and rays— are a group of marine predators that are known to promote the health of many ecosystems (Heithaus and Vaudo, 2012; Heithaus *et al.*, 2008). For example, in Australia, sharks limit seagrass herbivory either through consumption of herbivores or merely influencing herbivore behaviours through predation risk (Heithaus *et al.*, 2012). This in turn aids in the growth and health of seagrass meadows which in turn leads to increased CO_2 sequestration (Atwood *et al.*, 2015; Atwood *et al.*, 2018). Without many of these aquatic predators we may lose essential ecosystem services.

Over one quarter of all elasmobranchs are either threatened or endangered due to increasing anthropogenic impacts and nearly half are classified as data deficient (Dulvy *et al.*, 2014). Population declines over all species are correlated with increased anthropogenic effects, primarily overfishing and habitat degradation (Dulvy *et al.*, 2014). However, concomitantly, elasmobranchs are influenced by a range of other factors, both natural and anthropogenic in origin. Importantly, as our climate continues to change, species are being progressively exposed to increasing environmental pressures, such as elevated temperatures (Chin *et al.*, 2010). Elasmobranchs display life-history characteristics that make them especially vulnerable to overexploitation and environmental perturbation, placing them at a higher risk of extinction (Beddington *et al.*, 2005). Given that most elasmobranchs are slow-growing, take many years to reach sexual maturity, have a relatively low fecundity and low reproductive rates, population recovery within these species takes a long time, and given the rate of exploitation, many populations are declining faster than they can be replenished (Robbins *et al.*, 2006). Given the range of species life-history characteristics that elasmobranchs display, some species will be inherently more at risk than others. However, across all species, younger life stages are inherently vulnerable (Heithaus and Vaudo, 2012).

Development

Early development, especially embryogenesis, sets the stage for an organism's life. During this time individuals are most at risk either because they are small and relatively defenceless, or they are unable to select optimal conditions for normal development and performance (Johnson *et al.*, 2016). Stressful conditions during the early development can have potential deleterious impacts not only on embryos and juveniles, but potentially later in life as impacts on young can have knock-on effects,

including decreased growth, activity and/or reproductive output, all of which have the potential to affect survival and fitness. However, certain developmental modes play a strong role in the relative vulnerability of young during early development.

As a group, elasmobranchs display perhaps the most diverse array of developmental modes. Each mode is associated with different benefits and costs (either to the developing embryo or to the mother), which influences the relative risks to either (Conrath and Musick, 2012). With over seven general reproductive modes in sharks, each varies primarily in the kind of foetal nutrition provided (Conrath and Musick, 2012). Foetal nutrition can be classified as either lecithotrophic, wherein embryos receive nutrition from developed yolk reserved, or matrotrophic, in which embryos receive additional maternal nutrition over development. The amount of energy stores to which embryos have access to largely influences the total mass and vulnerability upon hatching/birth. Therefore, species which utilize matrotrophic development modes are significantly larger and less vulnerable at birth (Conrath and Musick, 2012). In contrast, those which rely on lecithotrophy are relatively smaller given they are constrained by a finite yolk source and to a lesser extent, the size of their egg capsule (Carrier *et al.*, 2004).

In addition to the degree of maternal input, the location of embryonic development varies and with it the exposure of developing young to potentially deleterious conditions. During internal development modes (matrotrophic and retained oviparity), embryos are relatively buffered from external conditions, both abiotic and biotic factors, as females constantly move, and are thought to adjust their behaviour and habitat use to aid embryonic growth and/or reduce their own thermal risk during gestation (Hight and Lowe, 2007). However, these modes are thought to be at greater risk to factors that influence the health and survival of the mother (i.e., predation, capture)(Adams *et al.*, 2018). For instance, capture-induced premature parturition is regularly observed in these species (Adams *et al.*, 2018). This is highly likely due to stress associated with capture (e.g., elevated hormone production, exhaustive exercise, increased energetic needs), following which females expel their young, possibly to aid their own survival as the cost of different physiological and/or behavioural processes may be high and thus females cannot support embryo growth (Adams *et al.*, 2018; Guida *et al.*, 2017) . Conversely, those which develop in the open environment are left on their own following oviposition (external oviparity) (Amsler *et al.*, 2015; Compagno, 1990; Conrath and Musick, 2012). These species are immobile and directly exposed to environmental conditions throughout gestation, which can be up to four years in some species (Hoff, 2008; Luer and Gilbert, 1985; Rodda and Seymour, 2008; Wourms, 1977). This makes oviparous species highly vulnerable to a suite of environmental stressors (e.g., water quality, predators, water movement, dislodgement, sedimentation), which individuals need to endure, given their immobility within an egg. Oviparous species need to be more resilient to environmental

changes and display a range of strategies to deal with conditions throughout development (Johnson *et al.,* 2016).

Oviparity

Extended oviparity occurs in around 42% of all *chondrichthyans* (subclasses *elasmobranchii* and *Holocephali*), most of which are relatively small-bodied, demersal species (Compagno, 1990). These species lay leathery egg cases directly into the environment that remain among the benthos until the embryos hatch. While gestation is largely dependent on external temperatures (Rodda and Seymour, 2008), embryos are exposed to the ambient environment for relatively long durations, (Conrath and Musick, 2012; Hoff, 2008) much longer than most oviparous marine species (e.g., teleosts, and invertebrates). These capsules are relatively self-contained during the first portion of embryonic development with minimal exposure to external water conditions (baring the passive conduction of temperature). However, the egg capsules open around one-third to a half way through development. This is likely to remove metabolic waste build-up, re-oxygenate the surrounding water, and/or promote water flow over respiratory organs throughout the remainder of development (Rodda and Seymour, 2008). During this developmental stage, embryos are introduced to a myriad of sensory cues and changes in water quality such as novel chemical cues (e.g., predators, non-predators, pollutants) and fluctuating water quality (e.g., fluctuating dissolved oxygen, carbon dioxide, water movement).

The immobility of oviparous species is a significant limitation and poses increased risk to developing embryos. During development, embryos' energetic budget is largely devoted to somatic growth in order to grow quickly to limit their time within their egg and to exceed the gape-width limitations of many predatory species (Rombough, 2011). Even upon hatching, much of a juvenile's energetic uptake is devoted to somatic growth. Therefore, deviation from the ideal growth trajectory in which essential energy stores are reallocated away from developmental processes can decrease the fitness and likelihood of survival upon hatching. Moreover, exposure to certain stimuli (e.g., detection of predatory cues and/or prolonged exposure to suboptimal temperatures) may require the use of strategies (such as, predator-escape responses or use of temperature mediated enzymes [i.e., heat shock proteins]) which can elevate an organism's energetic needs. Here, I focused on the energetic impact of two ever-present sources of stress across species –predation and developmental temperature— and the various strategies (physiological and/or behavioural) that different life-history stages may rely upon to survive.

Predation

Despite the predatory influence that elasmobranchs have across marine ecosystems, many are meso-predators and are subject to predation events themselves (Mourier *et al.*, 2013; Reyes and García-

Borboroglu, 2004; Vaudo and Heithaus, 2013). However, as most studies view sharks as the predator, very little work has explored their role as prey. Nonetheless, some work has focused on predation of elasmobranch eggs. Relative to older life history-stages, elasmobranch embryos are energy-rich and targeted by many species (Bor and Santos, 2003; Cox and Koob, 1993). They are found in the diet of both aquatic and terrestrial species and in some cases are the primary nutrition source for elasmobranch egg specialists, such as the Angular roughshark (O*xynotus* centrina) and prickly dogfish (*Oxynotus bruneiensis*) (Finucci *et al.*, 2016; Guallart *et al.*, 2015).

In oviparous species, the presence of the tough egg capsule can protect the developing embryo from a range of factors; however, embryos can still subject to significant predation events. Given the immobility of eggs across relatively long development periods, slow-moving invertebrates are able to bore through the thick protective egg case and are thus thought to be important predators of some species given (Cox and Koob, 1993; Cox *et al.*, 1999; Lucifora and García, 2004). The placement and oviposition of elasmobranch eggs can strongly determine the relative protection/vulnerability to predation as exposure increases the risk of being detected and consumed. In a caging experiment, *L. erinacea* egg placed in close proximity to soft sediments experienced around 45% gastropod predation, however, only 3% of eggs placed 0.5m above the substrate were predated upon (Cox and Koob, 1993). Similarly, Port Jackson shark (*Heterodontus portusjacksonii*) and red-spotted catshark (*Schroederichthys chilensis*) are thought to exhibit decreased predation given viable eggs are found secured within rocky reef matrix and adhered to kelp holdfasts (respective of egg morphology) (Powter and Gladstone, 2008a; Trujillo *et al.*, 2019). Embryonic development in oviparous species is demarcated by potentially high predation pressure, as they are situated in one position over long periods of time and unable to move to avoid predatory events. Even upon hatching, however, young are still at high risk from predation but are no longer restricted by an egg case.

Upon hatching, juveniles can move and actively respond to predation. Many smaller species, especially benthic sharks and rays are thought to be under significant predator pressure, which can drive their behaviour, and/or habitat usage (Heithaus and Vaudo, 2012; Vaudo and Heithaus, 2013). For example, increased predator abundance in more optimal habitats during low-tide periods is thought to drive shovelnose rays, (*Glaucostegus typus*), reticulate whiprays (*Himantura uarnak*), and pink whiprays (*H. fai*) habitat selection (Vaudo and Heithaus, 2013). Rather than risk exposure to predation during low tides, rays relocated to very warm, nearshore habits, which possibly offer decreased foraging opportunity and increased metabolic costs. While elasmobranch species do appear to recognize and respond to predators, their capacity to detect and differentiate cues is largely unknown. This capacity is especially important in young species which may have limited prior exposure to potential predators (Brown *et al.*, 2011).

What little is known of embryonic predator response, is inferred from embryo reactions to generalized electrical stimuli (Ball *et al.*, 2016; Kempster *et al.*, 2013; Sisneros *et al.*, 1998). However, given the number of species which are known to actively predate on elasmobranchs and/or their eggs (Bor and Santos, 2003; Cox and Koob, 1993; Cox *et al.*, 1999; Lucifora and García, 2004; McIntosh *et al.*, 2006; Mourier *et al.*, 2013; Powter and Gladstone, 2008a; Reyes and García-Borboroglu, 2004; Vaudo and Heithaus, 2013), it would stand to reason that this group of species would display effective anti-predator responses early into development. The capacity to utilize anti-predator strategies is well conserved across both vertebrates and invertebrate taxa (Creel, 2018; Hawlena *et al.*, 2010) and it is thought that these groups may rely on similar physiological mechanisms during predation events, especially typical 'fight-or-flight responses' (Hawlena *et al.*, 2010). Given that predator detection can occur across a range of sensory cues (e.g., chemical, electrical, visual, etc.) the necessary sensory organs need to be functional to enable embryos and juveniles to detect cues. Some species, such as rainbowfish, *Melanotaenia duboulayi* (Oulton *et al.*, 2013) and woodfrogs, *Rana sylvatica* (Chivers and Ferrari, 2009), are able to detect and respond to predator cues during embryonic development. Overall, it is unknown if elasmobranchs, regardless of development stage, have innate predator recognition and/or rely on prior experience to learn and associate different cues with predation. Only one study has suggested that juvenile lemon sharks (*Negaprion brevirostris*) may innately recognize the odour of a predator, the American crocodile (*Crocodylus acutus*) (Rasmussen and Schmidt, 1992). Regardless of the mechanism, the response of a species to a predatory cue ultimately aims to decrease the relative risk (Fuiman and Magurran, 1994). By promoting movement away from potential predatory influence or decreasing activity by relying on passive avoidance strategies such as crypsis, 'prey' species try to prevent predator detection, despite any associated cost (Brown *et al.*, 2011; Fuiman and Magurran, 1994). Even without the direct risk of consumption, the costs associated with predation can either come as the direct energetic requirements associated with activity and/or decreased opportunity for essential processes (e.g., decreased foraging) (Creel, 2018). Predation risk is a natural and present influence across most species' lives, but how sharks responds to predation is largely unknown, as are the strategies with which they may decrease relative risk.

Environmental temperature

Temperature is thought to be the most important abiotic factor, driving the physiology and behaviour patterns of many species (Angilletta, 2009). Ectothermic species, such as most elasmobranchs, rely on their external temperatures to regulate the rate and efficiency of many processes, including growth rate, digestion efficiency and swimming performance (Angilletta, 2009). These processes and traits are influenced by temperature, with performance peaking at an optimal temperature (T_{opt}) and subsequently declining on either side of the optimum, until performance is no longer feasible (Pörtner and Farrell, 2008). This response curve, a species' thermal window, is generally

skewed to the left, indicating that species performance is more quickly impacted at temperatures above a trait's T_{opt}. The width of a species thermal window reflects the temperature variation in which the species has evolved (Tewksbury *et al.*, 2008). For example, temperate species are thought to have a large thermal range given they are exposed to wide temperature shifts associated with annual seasonal changes, while tropical species live within a relatively stable thermal environment throughout the year (Pörtner and Farrell, 2008; Tewksbury *et al.*, 2008). In addition, some species can shift their thermal curves (and thus their thermal optimum) when exposed to potentially costly temperatures, via acclimation processes. These processes are typically defined as short-term non-genetic biochemical, physiological, and/or morphological changes which can permit species to live at temperatures above (or below) their typical thermal limits (Angiletta, 2009). While useful, this strategy is costly and is species specific (Portner and Farrell, 2008). Given the rapid rate of warming associated with contemporary, anthropogenic climate change, as species' approach temperatures beyond their optimum, they will need to rely on strategies (adaptation, acclimation, behaviour) to mitigate the thermal effects. However those unable to rely on physiological mechanisms (specifically adaptation or acclimation processes) will likely need to relocate or else perish under warming conditions (Habary *et al.*, 2017). Indeed, already a number of species in Australia have shifted their ranges in the face of warming temperatures (Pitt *et al.*, 2010; Sunday *et al.*, 2015). Marine teleosts and many invertebrates with pelagic larvae may easily redistribute because ocean currents can rapidly transport larvae to new locations. However, elasmobranchs and other species which do not have larval dispersal mechanisms, exhibit strong site fidelity, and/or are unable to move long distances must utilize other strategies to cope with rising temperatures.

Embryonic stages are a highly vulnerable to shifts in temperature through ontogeny. Embryos that develop within their mother are buffered from deleterious thermal conditions given that thermoregulating females can select beneficial conditions for herself as well as the developing embryos. Furthermore, it is thought that pregnant female sharks preferentially select warmer waters to increase embryo growth and decrease gestation rate (Hight and Lowe, 2007). However, oviparous species lack the ability to move and effectively thermoregulate during embryonic development. Therefore, developing embryos are exposed to potentially extreme conditions across daily and/or seasonal scales. However, upon hatching, juveniles can regulate their own temperatures through various strategies. Despite the capacity to move, many species reside within the relative safety of nurseries during juvenile development and may not initially make large scale movements (Heupel *et al.*, 2012; Knip *et al.*, 2011). Both environmental conditions and movement potential, can constrain access of young life-history stages to optimal temperatures. Given that early life stages may be exposed to variable temperature conditions, they may display a wide tolerance to thermal conditions.

To date the literature suggests that elasmobranchs appear to experience a plethora of impacts from elevated temperatures. Embryos and/or juveniles display declines in post-hatching growth rates,

digestive efficiency, and body condition, as well as increased mortality, food uptake, and developmental abnormalities (Di Santo, 2015; Gervais *et al.*, 2016; Gervais *et al.*, 2018; Pistevos *et al.*, 2017; Rosa *et al.*, 2014; Rosa *et al.*, 2016). If individuals are unable to meet the increased energetic demands associated with warmer waters this can have potentially deleterious knock-on effects as energy required to live is reallocated away from processes such as growth (Gervais *et al.*, 2018). Despite the information known regarding the potential impacts of warm water on shark embryonic development, few have quantified the impacts of temperature on the costs of development. To date there are only two elasmobranch studies which have been conducted in this area. Rosa *et al.* (2014) reared tropical bamboo sharks (*Chiloscyllium punctatum*) under predicted end of century conditions and measured oxygen uptake rates (as a proxy for metabolic rate) at three developmental stages and Di Santo (2015) reared temperate skates from two different populations under different temperatures and measured the oxygen uptake of near term embryos.

Outline and aims

In this thesis I used the Port Jackson sharks, *Heterodontus portusjacksoni*, as the model species to investigate the impact of predation and temperature on development. Two studies have previously used one population of Port Jackson sharks as a model to explore the effects of ocean warming and acidification (Pistevos et al. 2017, 2015). They found that warming significantly increased late term development (eggs were collected nearly 80% through development) and upon hatching had increased growth rates and increased motivation for food (Pistevos et al. 2017, 2015); however, acidic conditions depressed the ability to young sharks to locate prey. Concomitantly, both temperature and acidic conditions worked antagonistically to impair the increased growth rates associated with warmer water (Pistevos, et al. 2015). However, these studies collected very late into development and suggest that there are very significant impacts that temperature alone has on developmental processes, both of which I expand upon in this thesis. Port Jackson sharks are an ideal study species given they have known embryonic predators (Powter and Gladstone, 2008a), are widely distributed throughout the southern half of Australia and are thus exposed to a wide range of developmental temperatures (O'Gower and Nash, 1978). As an oviparous species, Port Jackson sharks are small, and relatively easy to maintain in captivity. They display distinct breeding seasons during which adults can undergo relatively long migrations (some up to 1000km round trips), exhibiting extremely strong site fidelity to distinct breeding sites (Bass *et al.*, 2017; Day *et al.*, 2019; Powter and Gladstone, 2009). Eggs are deposited within the rocky reefs (McLaughlin and O'Gower, 1971) and are secured in cracks and crevices via water movement (Powter and Gladstone, 2008b) where they remain for the duration of development (~10-12 months depending on temperature)(Rodda and Seymour, 2008). Upon hatching, juveniles move off of the rocky reefs to nearby nurseries comprised of sandflats and seagrass beds (Powter and Gladstone, 2009),

where they are found in loose aggregations (Powter and Gladstone, 2009). Juveniles remain close to oviposition sites for around 2-3 years. This exposes both embryos and juveniles to fluctuating temperatures; both the summer highs and the winter lows.

Predation

Aim 1: Predation is a driving influence across ecosystems and taxa, with most species considered prey at some point during ontogeny. The early detection and response to predatory cues enhances the likelihood of survival. Embryonic elasmobranchs are generally accepted to detect electrical stimuli from predators and respond by decreasing activity and ventilatory activity as a form of crypsis to avoid detection (Ball *et al.*, 2016; Kempster *et al.*, 2013; Sisneros *et al.*, 1998). Yet, in an aquatic environment, risk assessment is often facilitated via chemosensory cues (Chivers and Smith, 1998; Wisenden, 2000). Little is known about how, or if, juvenile sharks rely on chemosensory cues to detect predators. Moreover, the functional capacity of the olfactory system during embryonic development in sharks is largely unknown. In *Chapter I,* embryonic sharks were exposed to both horn shark (i.e., known predator) and teleost fish odours (i.e., theoretically non-threatening species) across key developmental stages to determine their capacity to detect olfactory cues during embryonic development, discriminate between different odours, and utilize anti-predatory strategies in response.

Aim 2: While embryonic sharks generally lack the capacity to move away from predatory cues, juveniles are released from these constraints and are free to utilize a wider array of anti-predatory strategies. The strategies that young individuals utilize can incur high costs either energetically through the use of generalized stress responses and increased activity (i.e., predator evasion), or it can come at the indirect cost of missed opportunities (e.g., foraging) through decreased activity and crypsis strategies (i.e., predator avoidance). Even without direct predatory cues (e.g., predator odours, visual presence) many species will elicit anti-predatory strategies in response to predator-associated cues such as conspecific necromones (Brown *et al.*, 2011; Yao *et al.*, 2009). In *Chapter II* I measured the oxygen uptake rates of juvenile sharks exposed to the scent of dead conspecifics to measure anti-predator responses. Furthermore, sharks were exposed to conspecific necromones coupled with a simulated predator attack (chase) to determine the energetic, non-consumptive effects of a predator "attack" on young sharks.

Temperature

Aim 3: Developmental temperatures in oviparous species are regulated by environmental condition which embryos are unable to control. Given that developmental time can be relatively long in elasmobranchs, it may be that embryos utilize different strategies (i.e., adaptation or acclimation) to help mitigate or endure suboptimal conditions. However, living under suboptimal conditions may still be costly, potentially impeding growth rates and developmental processes, which can have knock-on

effects later in life. In *Chapter III* I reared embryos under temperatures reflective of current day and end-of-century to investigate the impact that rearing temperature has on the oxygen uptake rates (i.e., proxy for metabolic rate), cost of development, and growth over development. Furthermore, given that Port Jackson sharks rely on distinct oviposition sites, eggs were collected from two distinct populations and reared under reciprocal temperature regimes to investigate the role that provenance plays on the plasticity (or lack of) on the physiology and growth of this species.

Aim 4: Theoretically, juvenile sharks should be able to mitigate the effects of temperature much more so than embryos. As the climate continues to change, species and population will need to utilize physiological and/or behavioural strategies in order to persist. Specifically they are thought to adapt, acclimate, or move (e.g., thermoregulation)(Angilletta, 2009; Habary *et al.*, 2017). The strategies used are largely thought to reflect the local conditions populations are exposed to (i.e., cold and warm exposed population may respond differently) (Angilletta, 2009). In *Chapter IV* I examined how rearing temperature impacts the physiology and behavioural performance of juvenile Port Jackson shark; and furthermore, the role that provenance has on these traits. Specifically, upon hatching, I investigated three traits, oxygen uptake rates (proxy for metabolic rates), the upper thermal limits, and the swimming activity with acute exposure to elevated temperatures.

Chapter I: Friend or foe? Odour detection, differentiation, and anti-predator response in an embryonic elasmobranch, *Heterodontus portusjacksoni*

Friend or foe? Odour detection, differentiation, and anti-predator response in an embryonic elasmobranch, *Heterodontus portusjacksoni*

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Abstract

Predation risk is ever present. Young animals, especially those still developing within egg capsules, are extremely vulnerable given their immobility, small size and potentially limited functionality of their sensory systems. Embryos from a range of taxa can detect predatory cues and utilize anti-predator responses to reduce risk; however, little is known of this capacity in oviparous elasmobranchs, especially in regard to olfactory detection. Furthermore, the capacity to discern between cues and identify potential threats can prevent wasting limited energy reserves on unwarranted anti-predator responses during development. Embryonic Port Jackson sharks, *Heterodontus portusjacksonii*, were exposed to shark (horn shark) and teleost (sand whiting) odours across key development stages to investigate the capacity for embryonic elasmobranchs to discern between cues and the associated energetic needs (here oxygen uptake). Oxygen uptake rates were measured to assess the capacity for embryos to detect, differentiate and respond to predatory olfactory cues. Embryos displayed variable responses to cues, which depended on both the type of odour (teleost or shark) and developmental stage. Earlier developmental stages displayed limited responses, regardless of the odour, likely associated with the poor development of the necessary sensory organs. Later into development, however, Port Jackson shark embryos depressed their oxygen uptake rates (i.e., crypsis responses) when exposed to teleost cues, but showed little response to shark cues. This suggests that embryos could discern between different odours yet were not able to appropriately associate them with predation threat. Unlike embryos, hatchlings displayed a significant increase in oxygen uptake rates when exposed to shark odours, which is associated with physiological processes occurring during general stress responses (e.g., increased cardiovascular output, upregulation of hormones), in preparation of escape responses. Collectively our data suggests that embryonic sharks can differentiate between odour cues and elicited various responses, but this ability is likely limited by the developing sensory capacity.

Keywords: Oxygen uptake; Shark; Physiology; Predation; Embryonic development; Olfaction, Cognition

Introduction

Predation is a strong driver of many behavioural patterns in nature with potentially lethal endpoints for prey species, and as such, minimizing predation risk is key (Brown et al., 2011). Reducing predation risk is essential for survival, such that many species respond to predatory cues without the physical presence of a predator and solely in response to chemosensory cues (Brown et al., 2011; Creel, 2018; Hawlena & Schmitz, 2010). Species utilize a range of strategies to limit the likelihood of being detected and/or consumed. Some species have physical/chemical defences (e.g., spines or toxins) which deter predators and prevent predation (Arbuckle et al., 2013; Hodge et al., 2018; Price et al., 2015). However, when a prey species detects a predator, many other species attempt to pre-emptively prevent interaction with the predator (i.e., predation avoidance strategies), or upon detection by a predator, employ more reactive strategies to evade capture and consumption (i.e., predator evasion strategies) (Fuiman & Magurran, 1994). While more passive, avoidance strategies are not as energetically costly, but species lose time and opportunities (i.e., foraging time, social interactions) in favour of utilizing crypsis, sheltering, or relocating to low-risk areas (Creel, 2018). Conversely, evasion responses are often much more costly, in that they can be associated with general stress responses (e.g., "fight or flight") (Hawlena & Schmitz, 2010). These stress responses often incur a large energetic debt (e.g., increased metabolic rate), given the suite of physiological mechanisms and aerobic activities which occur to drive successful anti-predator responses (Creel, 2018; Hawlena & Schmitz, 2010). The cost of using any of these strategies is variable, but unnecessary (e.g., false alarms) and frequent use can impact important traits such as development, growth, and reproduction, as energy is reallocated to anti-predator processes. Therefore, an individual's capacity to refine its recognition and response to predators can significantly reduce costs (Brown et al., 2011). This is particularly important for young animals which are subject to heightened predation risk.

Embryonic organisms are immobile and are an easy and high source of energy for predators (Orians & Janzen, 1974). However, despite the apparent disadvantage of encapsulation and lack of experience with predators, embryos can and do respond to predator cues (Atherton & McCormick, 2015; Chivers & Ferrari, 2009; Oulton et al., 2013). Embryonic amphibians change their hatching time if they detect predators, hatching earlier or later in response to varying threats (Moore et al., 1996; Warkentin, 1995, 2005). Some species even have the capacity to distinguish between predatory cues and display a gradient of anti-predator responses according to the predator type and/or degree of risk (Brown et al., 2011; Ferrari & Chivers, 2006). Rainbowfish (*Melanotaenia duboulayi*) embryos, for example, detect and differentiate between chemical cues during development and elicit increased heart rates when they detect native predator cues and conspecific alarm cues but not to novel predators (Oulton et al., 2013). Increased heart rates are observed in a suite of other taxa and life stages, indicative of stress responses to predation (Hawlena & Schmitz, 2010). Other species opt for less

energetically expensive strategies and given their inability to move, relying on passive crypsis responses to try to "blend-in" to their environment. Individuals can evade detection by relying on cryptic egg colouration patterns, remaining still, or depressing activity to mask their bioelectric signature (Bedore et al., 2015; Sisneros et al., 1998).

Elasmobranchs are often viewed as top predators; however, many species are subject to predation, especially during early developmental stages. This is particularly true for embryonic oviparous elasmobranchs, which are contained within leathery eggs. These eggs are extremely vulnerable as they are directly exposed to their environment for long periods of time; from months to years (Hoff, 2008; Luer & Gilbert, 1985; Wourms, 1977) and have large energy-rich yolks, which make them prime targets for predators (Cox & Koob, 1993). Indeed, predation on elasmobranch eggs and embryos is widespread ranging from opportunistic feeding by terrestrial species such as baboons (*Papio ursinus*), to general predators such as gastropods, pinnipeds, teleosts, and other elasmobranchs (Bor & Santos, 2003; Cox & Koob, 1993). Interestingly, there are some elasmobranch species that predate exclusively on elasmobranch eggs (Finucci et al., 2016; Guallart et al., 2015).

During early development elasmobranch eggs are sealed from external conditions; however, as early as a third into development (Ball et al., 2016) egg capsules open either through a thinning of the egg case, or dissolution of a mucous plug (Rodda & Seymour, 2008). This marks a period during which embryos are in direct contact with the external environment (Conrath & Musick, 2012; Hamlett, 2005) including predatory cues. Embryonic activity can be associated with an increased bioelectric field. Thus, during early development embryos appear to display anti-predatory responses such as the suppression of activity, to reduce and mask their bioelectric field (Ball et al., 2016; Kempster et al., 2013; Sisneros et al., 1998). When exposed to electrical pulses that simulate predators, skates and sharks displayed "freeze" responses (Ball et al., 2016; Kempster et al., 2013; Sisneros et al., 1998) coinciding with ventilatory depression and decreased heart rate—thus depressing oxygen uptake rate. Indeed, Southern rock lobsters reduced their activity with exposure to elevated predatory risk, which coincided with depressed oxygen uptake rates for up to three hours (Briceño et al. 2018). Crypsis strategies such as this are thought to diminish a predator's ability to detect an embryo's bioelectric field (Bedore et al., 2015; Kempster et al., 2013). While research has focussed on electrosensory cues and their involvement in anti-predatory responses, there are a plethora of other predatory cues (e.g., olfactory or mechanosensory cues) which embryos are exposed to, especially upon the opening of the egg capsules.

Predatory chemical cues (e.g., predator odours, alarm cues, necromones) instigate responses in a wide range of species, including elasmobranchs (Chivers & Smith, 1998; Rasmussen & Schmidt, 1992; Stroud et al., 2014; Yao et al., 2009). While evidence suggests embryonic teleosts can detect and respond to predator olfactory cues, (Brown et al., 2011) this has never been investigated in elasmobranch embryos. Here, we use embryonic Port Jackson sharks, *Heterodontus portusjacksoni*, to

explore the physiological response to varied chemical cues (horn shark and teleost). Port Jackson sharks are a medium-small benthic elasmobranch found throughout the temperate waters of Australia. Port Jackson sharks suffer very high mortality rates at the onset of development, as successful development requires the capsules to be secured within cracks and crevices over their relatively long development (10-12 months) (O'Gower & Nash, 1978; Powter & Gladstone, 2008). While many oviparous species are thought to have high mortality due to boring invertebrates, large vertebrate species are thought to be the primary predators of Port Jackson shark embryos (Powter & Gladstone, 2008). Horn sharks spp. such as the crested horn shark (*H. galeatus*) as well as adult Port Jackson sharks have been observed crushing egg capsules and consuming the embryos (Powter and Gladstone, 2008a; Pers. observation). While adult Port Jackson sharks are a seasonal predator to embryos, crested horn sharks are present throughout the year and likely a primary predator. We hypothesised that the response of embryonic Port Jackson sharks to horn shark cues would be characterised by a depression of oxygen uptake rate, concurrent with "freezing" responses that have observed in other species including several embryonic elasmobranchs. Conversely, embryos are thought to display no response to teleost cues given the negligible associated predation risk. Here we used intermittent-flow respirometry to measure the changes in oxygen uptake during periods of rest and exposure to odours (shark and teleost odours).

Methods

Collection and holding facilities

Port Jackson shark eggs were collected by hand from Sydney Harbour (n = 8) and from rocky reefs along the southern side of Jervis Bay, NSW (-35.068°S, 150.684°E) (n = 6), and transported, by car, to Macquarie University's sea water facility (Sydney, New South Wales, Australia). Eggs were in transit, no longer than 3 hours and were supplied with constant aeration. Throughout development, eggs were reared under 20.6 ± 0.5°C (ambient temperatures during May–June), and maintained under a 12:12 photoperiod. Eggs were housed in four, 40 l aquaria with three or four egg capsules per aquaria.

Following the dissolution of the mucus plug (i.e., around four months into development), embryos were removed from egg capsules (developmental stage 10; see Rodda and Seymour 2008) and placed into 160 ml containers and shaded. This was done to allow continuous, direct monitoring throughout development, eliminate potential build-up of microbes on egg capsule, and facilitate respirometry trials.

Eggs were collected under NSW department of Primary Industries permit P080010.42 and animal care and experimental protocols were approved by Macquarie University animal committee under research permit ARA 2014/003.

Odour preparation

As crested horn sharks consume Port Jackson shark eggs (Powter & Gladstone, 2008a; Pers. observations), shark odours were collected from an adult shark housed at Manly Sea Life Sanctuary,

Sydney, Australia. We collected teleost odours from sand whiting (*Sillago ciliata*), a common, non-threatening species at oviposition sites, as they consume small, benthic polychaetes and crustaceans (Hadwen et al., 2007). Odour cues were prepared following procedures similar to Oulton et al. (2013). Odours were collected by placing one crested horn shark (total length ~0.70m) and three whiting (total length ~ 0.17-0.25m) in separate aquaria (1000 l and 500 l respectively), filled with oxygenated, filtered seawater. Fish were allowed to "soak" for 24hrs during which water flow was turned off, to ensure the production of concentrated chemical cues (see Ferrari et al. 2008). The difference in fish numbers were selected so as to minimize the difference in size between the two species. Following soak time, water was collected from each aquaria and shark and teleost odours were stored in 1.25 l aliquots and frozen until needed.

Intermittent-flow respirometry

Intermittent-flow respirometry was used to measure the response of embryos to predator odours across key developmental stages. The system consisted of four respirometry chambers, of which the chamber size depended on developmental stage/size of the individual (0.68 l chambers prior to hatching or 1.9 l upon hatching). Chambers were divided between two 50L aquaria, maintained at 20.6 ± 0.2°C, and each chamber was connected to a recirculating in-line pump (200 l h^{-1}, AP200LV; Aquapro) and a flush pump (EHEIM compact 600; EHEIM GMbH & Co. KG, Deixisau, Germany). The recirculating in-line pump was on throughout the trial, mixing water and ensuring homogenous O_2 levels in each chamber, while the flush pump was set on an automated timer (5 min on[flush cycle], 5 min off [O_2 measurement period]) and "flushed" the chambers with well aerated, filtered seawater to remove any build-up of CO_2 and metabolic waste. The duration of the measurement period was long enough to ensure O_2 did not fall below 80% air saturation (Clark et al., 2013). During the measurement period, temperature-compensated O_2 concentration (mg l^{-1}) were continuously recorded (every 2 s) via 2 mm contactless spots with O_2 sensitive REDFLASH dye in-line with each recirculating pump. Fibre-optic cables were connected to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany). Background oxygen uptake rates (microbial respiration) were measured prior to trials and again after the shark was removed following the trial. Background O_2 consumption was linearly interpolated and subtracted from each slope (Clark et al., 2013). Microbial build-up in chambers was mitigated by rinsing chambers in a 10% bleach solution, rinsed in freshwater, and then sun-dried, following each trial.

At each distinct developmental stage (i.e., stage 11, 12, 13, 14, and 15 [hatching]; see Rodda and Seymour, 2008), embryos (n = 14) were gently placed into a respirometry chamber to rest and habituate for 12 h. Following, this period, water flow into the aquaria was stopped and the first treatment odour (1.25 l of shark or teleost odour) was added to the sump at the start of a "flush" period. Embryos were randomly exposed to one of the two odours for 20 min, while oxygen uptake rates were recorded over two measurement periods. After the exposure period, water flow to the aquaria was resumed and the aquaria was partially drained and refilled four times to remove lingering odours. Water was drained up

to 3 cm above the top of the flush pumps to ensure that chambers remained full and air was not introduced into the system. Embryos were then allowed to recover for one hour and then exposed to the remaining odour for 20 min, until the end of the trial. $\dot{M}O_2$ values were averaged over the 20 min prior to exposure ($\dot{M}O_2$ baseline values) and again during odour exposure ($\dot{M}O_2$ chemical cue response). The order of odour exposure, for each individual, was randomised at each developmental stage. The relative response of embryos to chemical odours was measured as the difference between baseline oxygen uptake rates (i.e., prior to exposure to odours) and the oxygen uptake rate during each odour exposure. Following each trial, embryos mass was measured (nearest 0.1 g) and they were returned to holding conditions.

Analysis

The oxygen uptake rates were calculated by taking the linear least square regression of oxygen concentration, during each measurement period, in LabChart version v8.1v9 (AD Instruments, Colorado Springs, CO, USA). To calculate mass specific uptake oxygen uptake rates across developmental growth (mg O_2 $kg^{-0.67}$ h^{-1}), the mass exponent of 0.67 (Sims, 2000) was used to correct for the allometric relationship between oxygen uptake rate (i.e., proxy for metabolic rate) and mass (Sims, 2000). The 'mean of the leftmost normal distribution' method (MLND) was used to establish the resting oxygen uptake rate ($\dot{M}O_{2Rest}$), as described in Chabot *et al.* (2016). The MLND approach is widely used as it acknowledges that the frequency distribution of oxygen uptake rates is often bimodal. Here, fitting two normal distributions across oxygen uptake measurements can account for the handling activity of embryos (rightmost normal distribution) until the embryos settle and resume resting oxygen uptake values (leftmost normal distribution). The mean of the leftmost distribution is thus determined to be MO_{2Rest}.

A generalized linear mixed model (GLMM) was used to compare MO_{2Rest} (mg O_2 $kg^{-0.86}$ h^{-1}) values across developmental growth. A linear mixed effect model (LMM) was used to compare the change in embryo oxygen uptake rates to odour cues (fixed variable), from $MO_{2Rest,}$ across developmental stage (fixed variable). Model selections were carried using LMM with and without the inclusion of the random factor to determine relative importance of individual shark response over time. As individual embryos were used across developmental stages, embryo ID and tank ID was incorporated as random factors to account for individual differences. Tukey post-hoc analysis were performed when significant differences were observed. Linear mixed models were generated using S-PLUS for Windows Version 8.0 (Insightful Corp.).

Results

Resting oxygen uptake rates

Resting oxygen uptake rates ($\dot{M}O_{2Rest}$) increased over development concurrent with embryonic growth (i.e., smaller embryos required less O_2 per kg than hatchlings) (Fig. 1; Supplementary materials, Table S1). However, intermediate stages (developmental stage 12, 13, and 14) displayed similar respiratory needs (Fig. 1; Supplementary materials, Table S1). The $\dot{M}O_{2Rest}$ value, specific to each shark, represents the baseline uptake rates from which embryonic response to odours is compared.

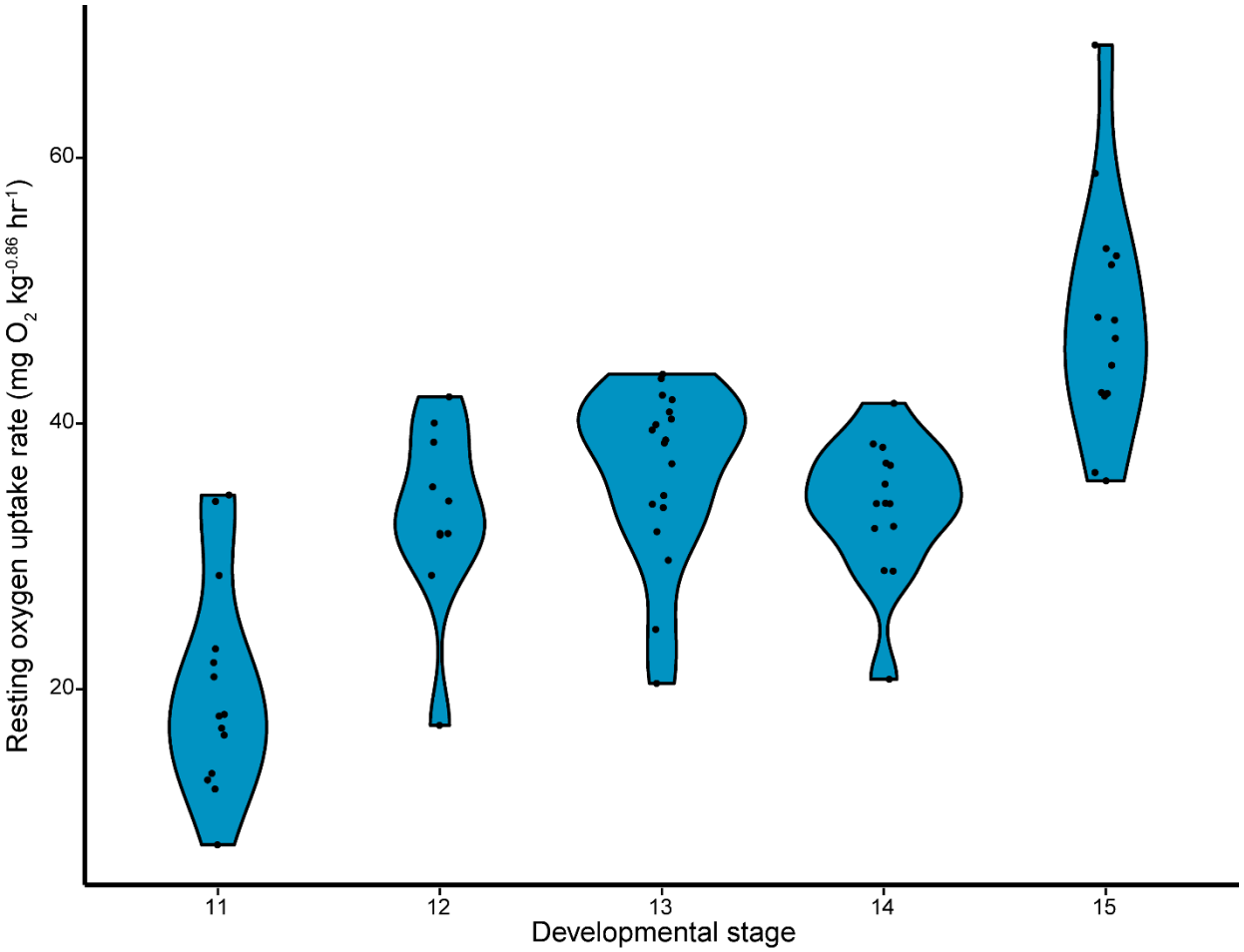

Figure 1: Violin plots depict the resting oxygen uptake rate (mg O_2 $kg^{-0.67}$ h^{-1}), for embryonic Port Jackson sharks (n=14) across developmental stages (see Rodda and Seymour, 2008).

Chemical odour responses

The change in embryonic shark $\dot{M}O_2$ values, from $\dot{M}O_{2Rest}$, were significantly different depending on the chemical odour treatment (LMM, $F_{1,117} = 11.62$, $p < 0.001$), with exposure to teleost cues eliciting a decrease in oxygen uptake rates. Furthermore, oxygen uptake also depended on embryo developmental stage (LMM, $F_{4,117} = 2.73$, $p < 0.05$). However, the interaction effect between both chemical odour and developmental stage was significant (Fig. 2, LMM, $F_{4,117} = 2.94$, $p < 0.05$). Specifically, embryos displayed limited-to-no response to either chemical cue during stages 11 and 12, but displayed

different responses depending on chemical cue within later stages. Embryos displayed strong negative responses (decrease in oxygen uptake rates by 3.61 ± 1.15 mg O_2 $kg^{-0.86}$ h^{-1}) to teleost cues at stage 13 (Fig. 2) followed by a decreasing trend in response until hatching (stage 15). Conversely, embryos displayed limited responses to shark cues until hatching (stage 15), at which point embryos responded to shark cues by increasing their oxygen uptake rates by3.40 ± 1.76 mg O_2 $kg^{-0.86}$ h^{-1}.

Comparing the model with and without the random factors (individual ID and Tank) indicated that there was no statistical difference between the models (ANOVA, L-ratio = 19.60, p = 0.72). Therefore, all sharks regardless of holding tank displayed similar responses to the chemical cues.

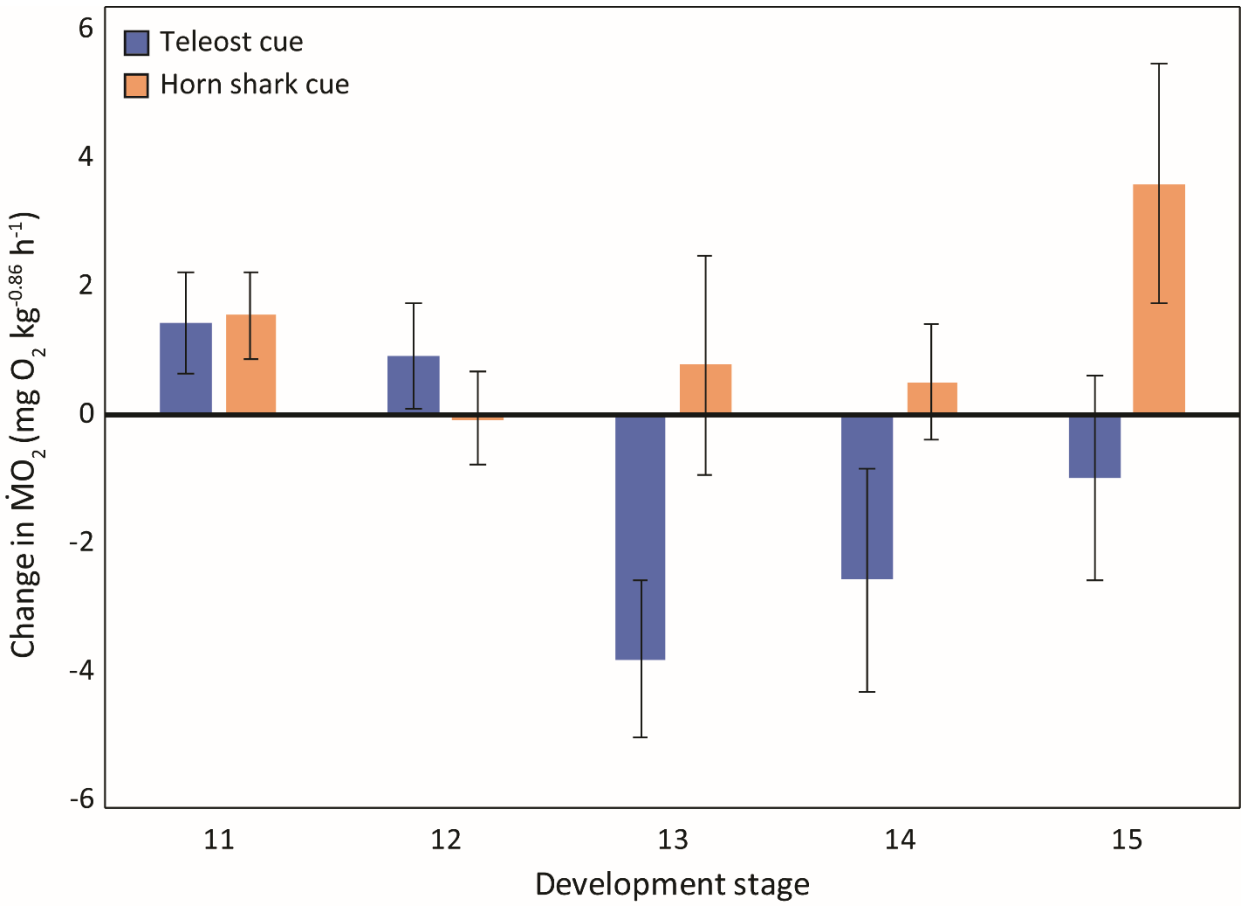

Figure 2: Embryonic Port Jackson sharks (n=14) relative response of oxygen uptake rate (mg O_2 $kg^{-0.67}$ h^{-1}), from stage/mass dependant baseline resting rates (see Fig. 1), to chemical cue exposure across developmental stages (see Rodda and Seymour, 2008). Response to shark cues are displayed as orange (lighter) bars, while response to teleost cues are displayed as blue (darker) bars. Values are mean± SEM.

Discussion

Anti-predator responses enable organisms to decrease predation risk. During embryonic development, however, the capacity to detect and respond can be limited by the inherent vulnerabilities during early life stages, relative naivety to chemical cues, and ongoing development of sensory capabilities. Port Jackson shark embryos showed changes in oxygen uptake rates (up to an 18%

change) to crested horn shark odours at hatching; however, responses varied across development and between cues. Interestingly, embryos only displayed moderate responses to shark cues by increasing oxygen uptake rates by 14.5-15.4% during stage 11 and upon hatching (stage 15), suggesting a 'fight or flight' stress responses. In contrast, embryos during the latter half of development (stage 13-15) depressed oxygen uptake rates when exposed to teleost odour, consistent with the use of "crypsis". Reliance on anti-predatory strategies influences the physiological responses of developing organisms; however, the capacity to detect and react to cues is likely linked to ongoing developmental processes and may depend on additional cues to reinforce predatory associations, for example by associative learning (Brown et al., 2011). The responses that embryos displayed herein may reflect developmental changes, relative predation risk, or even innate neophobia to certain odours.

During embryonic development, there are significant limitations for most species to be able to respond to predatory cues. Clearly embryos cannot flee from predators, but they may respond in other ways. It was apparent that embryonic Port Jackson sharks displayed varying responses to olfactory cues. Indeed, they utilized two different strategies, each with its own benefits and disadvantages. Similar to skates and other embryonic elasmobranchs (Ball et al., 2016; Kempster et al., 2013; Sisneros et al., 1998), embryos here displayed a form of crypsis in response to the cues emanating from a teleost, which we initially hypothesised would not present a threat. By decreasing their oxygen uptake, via reduced ventilatory movements, embryos may be able to mask their bioelectric signature from predators (Ball et al., 2016). This strategy is widely used by a wide range of taxa ranging from mammals to cephalopods and teleost fish (Bedore et al., 2015; Briceño et al., 2018; Cooke et al., 2003; Sisneros et al., 1998; Smith et al., 1981). Reducing predation risk in this manner pre-empts the need for organisms to actively evade capture. Crypsis strategies are energy conservation strategies as individuals decrease metabolism and may and associated physiological processes. For post-embryonic life stages, this may come at the cost of decreased opportunities (i.e., reduced foraging time, social interactions, etc.) (Creel, 2018); however, embryonic organisms may experience relatively few impacts, such as delayed embryonic development. Rather than use up energetic resources, which are limited during development (e.g., finite yolk-source), crypsis strategies may conserve energy by limiting unnecessary yolk depletion.

In contrast to the teleost chemical cues, embryonic Port Jackson sharks displayed increased oxygen uptake rates, reminiscent of 'fight or flight' responses, with exposure to horn shark odours (Hawlena & Schmitz, 2010). During potentially life-threatening situations—here elevated predation risk— a suite of physiological processes are implemented to aid an individual's survival (Hawlena & Schmitz, 2010). For example, these processes can enhance the capacity for aerobic and/or anaerobic activities required for predator evasion (Hawlena *et al.*, 2010). While this response may not immediately aid embryonic organisms, pre-conditioning to these chemical cues may pre-empt predatory detection post-hatching and enhance their survival (Atherton & McCormick, 2015). As with crypsis responses,

increases in oxygen uptake rates in response to predator detection are observed in many different taxa (Cooke et al., 2003; Hawlena & Schmitz, 2010; Steiner & Van Buskirk, 2009) and life-history stages, including embryos (Atherton & McCormick, 2015; Oulton et al., 2013). This strategy is much more energetically expensive and can limit the allocation of available resources away from processes such as growth, development, and reproduction (in mature individuals) (Palacios et al., 2016). While post-embryonic organisms can compensate for increases in metabolic rate by increasing food intake, embryonic organisms are ultimately limited by a finite-yolk supply. While crypsis can be an energetically "safe" strategy, active anti-predator strategies such as this can have potentially critical consequences (e.g., decreased body condition) especially if utilized frequently. The use of these strategies and the magnitude of response change over development may reflect the behavioural or cognitive abilities of embryos.

Port Jackson shark embryos displayed varied responses over developmental stages. Initial responses during stage 11 were similar (both in magnitude and direction) towards both shark and teleost odours. Just prior to the earliest developmental stage (less than 2 weeks) embryos were contained and isolated within a sealed egg capsule. During this time, embryos are thought to receive limited cues from the external environment. Upon opening of the egg case, however, embryos are exposed to a suite of novel stimuli and may be eliciting a response to novel cues they encounter (e.g., neophobic response, see Sneddon *et al*. 2003). These responses may pre-empt predation and are utilized by many individuals in the face of novel cues, because the cost of responding far outweighs the cost associated with failing to respond to predators (i.e., consumption) (Brown et al., 2011; Hirvonen et al., 2000). Following this stage, embryos may be able to differentiate between odours, given that the average responses to predatory cues were in the opposite direction (i.e., embryos increased oxygen uptake rates). Interestingly, at stage 13, Port Jackson sharks responded strongly to teleost (sand whiting) odours, despite the fact that sand whiting are invertivores and pose no risk to embryonic sharks. However, repeated exposure resulted in a decline in this response until hatching, at which point there were negligible differences between embryo response and resting values. In the absence of any negative reinforcement, embryos may learn that these cues are non-threatening and consequently decrease their response over subsequent exposures (Ferrari & Chivers, 2011).

Developmental changes, specifically at hatching, may also influence how individuals perceive predator risk (Ferrari et al., 2010). Some species rely on multiple cues to reinforce predator risk, and without these associations, species may not recognize odours as threatening (e.g., pairing predator odour with that of an injured/dead conspecific) (Brown et al., 2011). Larval woodfrogs, *Rana sylvatica*, fail to recognize tiger salamander, *Ambystoma tigrinum*, odours as a predatory influence; however, exposure to both the scent of the salamander and injured conspecifics odours, which warn larvae of elevated predatory risk, elicited anti-predator responses (Ferrari & Chivers, 2009). Repeated exposed to

these cues during early ontogeny can lead to enhanced fitness later on as embryos learn to associate certain cues and/or situations as non-risky, thereby reducing the usage of unnecessary anti-predator responses and increasing potential predatory rates (Ferrari & Chivers, 2011). While neophobic responses initially aid in reducing any potential species interactions (positive and/or negative), as individuals grow and acquire new information, learned experiences can help reduce the number of costly 'false responses'.

Ontogenetic changes can further influence anti-predation responses. Specifically, upon hatching (stage 15) embryos display a significant shift away from the sedentary immobile lifestyle within the egg. Port Jackson hatchlings changed their response towards predator odours by increasing their oxygen uptake rate, in a manner similar to teleost fish in the presence of a predator (Huuskonen & Karjalainen, 1997; Oulton et al., 2013; Palacios et al., 2016). This may reflect changing individual priorities, how hatchlings perceive risk, as well as the strategies available upon hatching. While embryos responded similarly at stage 13 (5 months prior), they were constrained within an egg capsule and unable to move. Upon hatching however, this enhances their capacity for aerobic and/or anaerobic activities to evade capture. Whilst the cost of this strategy is high, during post-hatching stages individuals are especially vulnerable to predation as they begin to forage and will encounter novel interactions with both predators and non-predators alike. Failure to respond accordingly, can quickly lead to lethal results, before individuals have had time to familiarize themselves with and differentiate predator and non-predator cues (Brown et al., 2011).

Throughout embryonic development, embryos are undergoing significant changes, and different sensory systems develop and become functional at different stages. Olfactory senses are thought to arise later in development, and in teleost fish, embryos are able to respond to olfactory cues by at least ~60-75% through development (Atherton & McCormick, 2015; Oulton et al., 2013). It is suggested that embryonic development significantly alters and potentially limits the response of embryonic skates and sharks to electrical stimuli (via electroceptory system) (Kempster et al., 2013; Sisneros et al., 1998). However, despite significant research on the olfactory system of elasmobranchs, very little is known regarding its development, and even less (if anything) is known about its functional capacity during embryonic development (Gardiner et al., 2012). How the olfactory system develops and its functionality during embryonic development, specifically following the opening of the egg capsule requires further investigation. Nonetheless, we show that embryonic Port Jackson sharks display the capacity to detect and potentially differentiate between chemical odours quite early in development.

Anti-predator strategies provide embryos with a fighting chance by masking detection or enabling evasion strategies. However, the strategy or strategies species use is reliant upon the capacity for species to detect, differentiate, and appropriately assess predation risk, which varies based on sensory capabilities, innate or learned responses, and even ontogenetic stages. Here, Port Jackson

sharks displayed a range of responses to odour cues during embryonic development. While the magnitude and response to odours varied, this may reflect trade-offs between the costs of anti-predator strategies, the perceived risks, and the dynamic influence of embryonic development. Elasmobranchs have some of the longest developmental times of any vertebrates, and long periods of immobility makes them extremely vulnerable to predation events, it also provides opportunities for learning. Relying on prior experiences to assess predation risk, mitigates the unnecessary utilization of costly anti-predator strategies, as embryos refine their responses to different odours. This capacity to detect, differentiate, and respond to threatening condition *in ovo* enhances survival during development and likely post-hatching.

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Compliance with Ethical Standards Conflict of Interest:

Declarations of interest: none. All animal care and experimental protocols used in this study were approved by Macquarie University Animal Ethics Committee under ARA 2014 and conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

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Supplementary Materials

Table S1: Mean values (± SEM) for developmental oxygen uptake rates ($\dot{M}O_{2Rest}$ [mg O_2 $kg^{-0.86}$ h^{-1}]) for each developmental stage are in shaded cells. Tukey post-hoc analyses between each stage are provided in non-shaded cells. (*) denote significant differences.

Stage	11	12	13	14	15
11	92.21±4.74				
12	p< 0.001* z= 5.34	104.99±3.52			
13	p< 0.001* z= 7.73	p= 0.61 z= 1.43	89.93±3.26		
14	p< 0.001* z= 6.42	p= 0.98 z= 0.56	p= 0.86 z= -0.98	52.67±1.72	
15	p< 0.001* z= 10.51	p= 0.001* z= 4.22	p< 0.01* z= 3.33	p< 0.001* z= 4.08	74.71±3.49

Chapter 2: Fight, Flight, or Freeze? Impact of conspecific necromones on the oxygen uptake rates of a benthic elasmobranch

Fight, Flight, or Freeze? Impact of conspecific necromones on the oxygen uptake rates of a benthic elasmobranch

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Abstract

Given the vulnerability of early life stages to predation, many species elicit anti-predatory responses to predator-associated cues (e.g., alarm cues, necromones). However, the response used may vary between predatory stimuli. Anti-predator responses, whilst enhancing survival, are costly, either inducing missed opportunities (e.g., foraging) through decreased activity and crypsis strategies (i.e., predator avoidance) or incurring high energetic costs through the use of generalized stress responses and increased activity (i.e., predator evasion). Juvenile Port Jackson sharks, *Heterodontus portusjacksoni*, were exposed to the scent of dead conspecifics to measure anti-predator responses. Furthermore, sharks were exposed to conspecific necromones coupled with a simulated predator chase to determine the energetic, non-consumptive effects of a predator "attack". When just exposed to necromone cues juvenile sharks depressed their oxygen uptake rates for up to three hours following exposure, indicating that juveniles use crypsis to avoid detection by predators. However, when forced to swim and evade a predator, sharks elicited increased oxygen uptake rates, regardless of whether or not they were exposed to predatory cues. Juvenile sharks exposed to conspecific necromones may primarily rely on strategies which depress activity to avoid detection by predators, however, if forced to evade capture sharks they elicit energetically costly escape strategies similar to typical 'fight or flight' responses.

Keywords: Predation; Crypsis; Fight or flight; Respirometry; Olfaction; Escape; Exercise

Introduction

Predation is a ubiquitous presence in any ecological community and can help shape the behaviour of prey, influence community structure, and promote the overall health of an ecosystem (Hammerschlag *et al.*, 2019). While the physical presence of a predator can elicit anti-predator responses, species respond to a suite of different sensory cues which indirectly signal the presence of potential predators or predation events (Brown *et al.*, 2011). For example, the tropical goby (*Asterropteryx semipunctatus*) decreased their activity and feeding behaviour when exposed to the chemical scent of an injured or dead conspecific (McCormick and Larson, 2007). While small teleost fish are often preyed upon and therefore need to rely on these cues to signal danger, larger species, should also maintain the capacity to elicit anti-predation strategies especially during vulnerable, early life stages since there is always a bigger fish in the ocean (Brown *et al.*, 2011). Sharks are often viewed as predatory influences in marine ecosystems, however, juveniles are inherently vulnerable to predation (Heithaus and Vaudo, 2012). Because we tend to think of sharks as top predators, there is very little understanding of how they respond to predation risk. Regardless of species, the behavioural and physiological responses an individual utilizes ultimately aim to reduce the risk of being predated upon. Nevertheless, different predator stimuli can prompt different anti-predator responses such as hiding or fleeing and may be explicitly tailored to suit the context.

Most prey species utilize specific strategies to reduce their risk of predation (Creel, 2018; Fuiman and Magurran, 1994; Hawlena and Schmitz, 2010). These strategies can be varied and are often dependent on a variety of factors (e.g., physiology, ontogeny, morphology, environmental conditions) (Fuiman and Magurran, 1994). Some species may rely on anti-predator defences (e.g., chemical deterrents, spines and armour) which can allow movement through the environment with fewer constraints (Arbuckle *et al.*, 2013; Hodge *et al.*, 2018; Price *et al.*, 2015). However, if a species does not have these anti-predatory defences they will often rely on other strategies. Chief among them is a change in behaviour.

Behavioural responses to predatory cues are typically aimed at either limiting/eliminating predator interactions (i.e., predator avoidance responses) or utilize behaviours to reduce capture following a predator interaction (i.e., predator evasion responses) (Fuiman and Magurran, 1994). Predator avoidance responses can be pre-emptive and may not rely on strenuous aerobic activity (i.e., low energetic cost strategies) (Fuiman and Magurran, 1994). Indeed, some species decrease their activity levels and energetic needs by sitting and waiting for the predation risk to abate (Fuiman and Magurran, 1994). These species may be trying to blend in with the background to avoid discovery, which is facilitated by shutting down nonessential processes, reducing their oxygen uptake rate (i.e., metabolic rate), decreasing heart rate and thus a reduction in electrical signals (Bedore *et al.*, 2015; Briceño *et al.*, 2018; Kempster *et al.*, 2013; Sisneros *et al.*, 1998). For example, Southern rock lobsters, *Jasus edwardsii,*

decrease their oxygen uptake rates for up to three hours in situations of elevated predator risk (Briceño *et al.*, 2018). It is thought that by suppressing these processes—thus depressing oxygen uptake rates—predators may be unable to detect immobile prey, given that bioelectric signatures are suppressed (Kempster *et al.*, 2013). Conversely, predator evasion responses are utilized if species have been detected by a predator and urgently need to evade capture (Creel, 2018; Fuiman and Magurran, 1994). In fish, these responses are typically characterized by energetically costly behaviours such as "fight or flight" responses (Hawlena and Schmitz, 2010). While these strategies are immediately beneficial over short-time frames, there may be long-term costs associated, especially if individuals are unable to restore homeostasis.

Both predator avoidance and evasion responses have immediate fitness benefits; however, utilizing these strategies can come at a cost which may change depending on the context. Even if prey utilize lower energetic anti-predator behaviours, predator avoidance strategies can impede on other essential processes such as foraging or mating (Creel 2018). This can arise as individuals either decrease activity for periods of time or pre-emptively seek shelter or move away from potential predation interaction. While the cost associated with avoidance strategies is likely primarily based on missed opportunities (indirect), there are more apparent direct energetic costs associated with predator evasion responses (Creel, 2018). These responses are driven primarily by physiological mechanisms which occur in response to stressful situations, such as a predator interaction. This response prompts an increase in hormone levels (e.g., glucocorticosteroids) and heat shock proteins (to promote cellular function). The increase in hormone levels is associated with elevated cardiovascular activity, blood pressure, and respiration as well as gluconeogenesis (Hawlena and Schmitz, 2010). These responses ultimately can enhance aerobic pathways, to allow individuals to effectively overcome their stressor, or fight back. However, these physiological processes are energetically costly and can present ecologically relevant consequences (e.g., reduced growth, impaired immunocompetence)(Hawlena and Schmitz, 2010). This can be especially costly to the individual when coupled with aerobic activities (e.g., being chased by a predator). To compensate for the increased energetic demands associated with these general stress responses and increased activity, individuals increase their oxygen uptake (Hawlena and Schmitz, 2010). Regardless of the anti-predator strategies and the behaviours involved, utilizing any strategy may come at a cost to the individual and the benefits of using one strategy or the other must offset the potential costs of the other.

Here we examined the response of juvenile sharks to predatory cues using Port Jackson sharks, *Heterodontus portusjacksoni* (PJs) as a model species. PJs are a relatively small-bodied and abundant, oviparous shark species found throughout temperate Australia (Last and Stevens, 2009). As adults, these sharks make relatively predictable, annual migrations (up 1000km return trip), despite their benthic

nature (Bass *et al.*, 2017; McLaughlin and O'Gower, 1971; O'Gower, 1995; O'Gower and Nash, 1978; Powter and Gladstone, 2008b; Powter and Gladstone, 2009). Furthermore, there is little plasticity in breeding sites with adults displaying extremely high site fidelity (Bass *et al.*, 2017). This species deposits egg capsules into cracks and crevices within rocky reefs, where they remain throughout development (up to 11 months) (Rodda and Seymour, 2008). During development oviparous species such as this are particularly vulnerable to predation and environmental conditions as they are unable to actively respond (Lucifora and García, 2004; Powter and Gladstone, 2008a). Upon hatching, juveniles move away from rocky reefs, towards sand flats and seagrass beds, but remain relatively close to their oviposition sites for two to three years after hatching (McLaughlin and O'Gower, 1971; Powter and Gladstone, 2009). During this time, they are often observed in loose aggregations (McLaughlin and O'Gower, 1971). Necromone cues are released by dying and decaying tissues, which can alert other individuals to a recent or ensuing predation event (Yao et al. 2009). There is some evidence that shark necromones decreases feeding activity in some mobile, predatory sharks (Stroud *et al.*, 2014); however, there is relatively little research exploring how small, benthic species are influenced by these chemical cues as well as the energetic response(s) elicited. Given the vulnerability of young Port Jackson sharks during early life-stages and their proximity to conspecifics, this study aimed to measure the metabolic response of juvenile sharks to conspecific necromones as well as a simulated predator chase. Furthermore, we examined the synergistic effect of these two stimuli (odour and chase), since this simulates a natural predation event. Given the sedentary nature of these sharks it is thought that juveniles may decrease their oxygen uptake as cardiac and ventilatory functions "freeze", similar to that of embryonic elasmobranchs. However, upon being chased, passive "freeze" strategies would likely be ignored in favour of escaping and thus energetic uptake would likely increase and incur an energetic debt following exercise.

Methods

Collection and holding facilities

Port Jackson shark eggs (n=24) were collected, by hand, from Jervis Bay, NSW (-35.068°S, 150.684°E) in May 2016 and transported via car to Macquarie University's seawater facility (Sydney, New South Wales, Australia). Three to six eggs were distributed into four 60 l or two 100 l aquaria respectively.

One-week post hatching, sharks (23.06 ± 1.43 cm) began consuming exogeneous food and were fed a mixture of prawn meat, basa filets and market squid (*ad libitum*) daily. Throughout development, temperature was monitored daily and maintained at 20.6°C ± 0.5 (ambient temperatures in Jervis Bay during May–June) and under a 12:12 photoperiod. Prior to any experimentation, sharks were fasted for 48hrs to ensure a post-absorptive state (Heinrich *et al.*, 2014; Niimi and Beamish, 1974).

Eggs were collected under NSW department of Primary Industries permit P080010.42 and animal care and experimental protocols were approved by Macquarie University animal committee under research permit ARA 2014/003.

Necromone preparation

Necromones were prepared following procedures similar to Oulton *et al.* (2013). Shark skin, muscle, and organs (excluding the stomach) were collected from dead juvenile Port Jackson sharks that were originally sourced from the wild. Once collected, for every 1 g of shark, 25 ml of filtered seawater were added to the tissue and emulsified in a blender. The resulting mixture was then passed through 50-micron filter paper and frozen in 50 ml aliquots until needed.

To ensure the sharks were not simply responding to a novel chemical cue, a control cue (Tasty, herbal rosehip caffeine-free tea) was used for comparison. Control cues were prepared by soaking one teabag per 500 mL of filtered seawater for five minutes.

Four chemical cue protocols were used (randomly assigned) to measure the effect of conspecific necromone cues on both resting and/or maximum oxygen consumption rates. Sharks (n=24) were placed in an intermittent-flow respirometry system four times (repeated measures), using each of the protocols. Two of the protocols measured the changes in $\dot{M}O_2$ values with exposure to only the cues (control or conspecific necromone cue), while the other two protocols incorporated a chase period after the addition of the chemical cues (control or conspecific necromone cue) to simulate the effect of a predator attack on $\dot{M}O_2$ values.

Intermittent-flow respirometry

To measure the response to chemical cues, sharks were placed in respirometry chambers. Specifically, maximum ($\dot{M}O_{2Max}$) and resting oxygen consumption ($\dot{M}O_{2Rest}$) rates were measured using intermittent flow respirometry techniques. Resting respirometry chambers have been used extensively to reliably estimate standard metabolic rates (SMR) of both teleost and elasmobranch fishes (Clark *et al.*, 2013; Heinrich *et al.*, 2014; Roche *et al.*, 2013; Rummer *et al.*, 2014). Resting O_2 consumption rates represent the oxygen required while at rest, whereas the $\dot{M}O_{2Max}$ is the maximum rate of oxygen consumption that is needed either for maximum aerobic performance or to recover from exhaustive exercise (Clark *et al.*, 2013; Roche *et al.*, 2013; Rummer *et al.*, 2016). The time to recover was calculated as the length of time required to return to $\dot{M}O_{2Rest}$ following $\dot{M}O_{2Max}$, and the total, or net, aerobic scope, the excess oxygen available after basic maintenance costs was calculated as $\dot{M}O_{2Rest}$ subtracted from $\dot{M}O_{2Max}$.

Rummer *et al.* (2016) found that following exhaustive exercise, teleost fish took less than 3hrs to recover and reach SMR. Therefore, the intermittent-flow respirometry set-up was utilized over a three-hour period to determine the maximum and resting O_2 consumption rates ($\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$) to estimate SMR and MMR.

During each trial, individual sharks were placed into a 68 l aquaria, filled with 20 l of aerated, filtered seawater, prior to chemical cue exposure. Following 10 mins, 50 ml of concentrated chemical cue was added via airline tubing. During protocols not involving a chase portion, sharks remained undisturbed for a further five minutes before being immediately placed in respirometry chambers, to limit handling effects. During protocols involving an exhaustive chase, however, following the introduction of the chemical cue, the shark was left alone for one minute before chasing the shark, via hand, for 3 min followed by 1 min exposure to air (Clark *et al.*, 2012; Clark *et al.*, 2013; Roche *et al.*, 2013; Rummer *et al.*, 2016). Air exposure was only added to the chase protocols to aid in ensuring that sharks were sufficiently exercised (similar to that of an extensive predator chase) (Clark *et al*., 2012; Clark *et al.*, 2013). Immediately after air exposure, sharks (up to four per trial) were placed into individual resting respirometry chambers, which were sealed and covered. Oxygen measurement started within 10 s and continued for 3 h, during which time O_2 consumption was measured for 5 min. every 10 min.

The system consisted of four 1.8 l Perspex, respirometry chambers that were placed in a temperature-controlled water bath (20.6°C). Each chamber was attached to both a recirculating pump (AP200LV; Aquapro) and a flush pump (EHEIM compact 600; EHEIM GMbH & Co. KG, Deixisau, Germany). The former was set at a flow rate of 200 l h^{-1} to keep water circulating around inside each chamber and ensure homogenous O2 levels. The latter was set at a flow rate of 600 l h^{-1} which was manually set to flush each chamber with well aerated, filtered seawater water on a 5 min cycle (5 min on, 5 min off). The period during which the flush pump remained off (i.e., O_2 consumption measuring period) was determined such that that O_2 did not fall below 80% air saturation (Clark *et al.*, 2013), and the time required to flush each chamber was determined such that it would be long enough to remove accumulated CO_2 and restore the oxygen to return to 100% air saturation. Temperature-compensated O_2 concentration (mg l^{-1}) was continuously recorded (every 2 s) using contactless spots (2 mm) with O_2-sensitive REDFLASH dye attached to the inside of glass tubes in line with each recirculating pump. The spots were linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) via fiber-optic cables. Upon conclusion of each trial, sharks were removed, re-weighed (nearest 0.1 g), and returned to holding conditions.

Oxygen consumption rates were established using Lab Chart version v8.1v9 (AD Instruments, Colorado Springs, CO, USA). The $\dot{M}O_{2Max}$ was calculated by segmenting the initial three slopes (each

segment encompassing 30s) to determine the highest rate of change (i.e., $\dot{M}O_{2Max}$) (Nay *et al.* 2018). The 'mean of the leftmost normal distribution' method (MLND) was used to establish the resting oxygen uptake rates ($\dot{M}O_{2Rest}$) (Chabot *et al.*, 2016). This approach is used to determine $\dot{M}O_{2Rest}$ as it acknowledges that the frequency distribution of oxygen uptake rates is often bimodal. Fitting two normal distributions across oxygen uptake measurements can account for the increased activity associated with handling and/or chase protocols (rightmost normal distribution) until shark resume resting oxygen uptake values (leftmost normal distribution). Recovery time was defined as the time from $\dot{M}O_{2Max}$ until the first measurement of $\dot{M}O_{2Rest}$. Background O_2 consumption was determined prior to the shark being placed into the chambers and again following the trial after shark were removed. The background O_2 consumption was linearly interpolated and subtracted from each slope (Clark *et al.*, 2013). Following each trial, all equipment was rinsed in a 10% bleach solution, rinsed in freshwater, and then sun-dried to reduce microbial background oxygen consumption.

Analysis

To access the impact of chemical cues on oxygen uptake rates, generalized linear models were utilized. Individual shark and order of chemical cue protocol were random variables while chemical cue protocol was the fixed factor to investigate $\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$, and the time required to recover following treatment. Tukey post-hoc tests were used to identify relationships between protocols. Analyses were performed in R (Version 3.4.1, R Core Development Team 2013) and all values are reported as mean± standard error of mean (SEM).

Results

Maximum oxygen uptake rates

Sharks exposed to conspecific necromones alone exhibited significantly reduced $\dot{M}O_{2Max}$ values (56.486 ± 2.557 mg O_2 kg^{-1} hr^{-1}) relative to when they were exposed to necromone cues in combination with being chased for three minutes (Fig. 1a, GLM, z= 2.62, p< 0.05). However, exposure to only conspecific necromones was similar to control chemical cue treatments, both exposure to control cue alone (GLM, z= 2.28, p= 0.10) and exposure to control cue followed by a chase protocol (GLM, z= 2.07, p= 0.16). Similarly, other measures $\dot{M}O_{2Max}$ were not different between treatments (e.g., control vs. control chase) (Fig. 1a, Supplementary material, Table S1).

Resting oxygen uptake rates

Exposure to conspecific necromone cues significantly depressed shark resting oxygen uptake rates (56.486 ± 2.557 mg O_2 kg^{-1} hr^{-1}) relative to when they were exposed to control chemical cues (66.442 ± 4.633 mg O_2 kg^{-1} hr^{-1}) (Fig. 1b, GLM, z= 2.90, p< 0.05). Furthermore, $\dot{M}O_{2Rest}$ values with

exposure to only conspecific necromone cues were significantly lower than when exposed to the combination of control cues and being chased (76.062 ± 3.242 mg O_2 kg^{-1} hr^{-1}) (GLM, z= 5.64, p< 0.001) as well as when exposed to conspecific necromones and chasing (71.679 ± 2.471 mg O_2 kg^{-1} hr^{-1}) (GLM, z= 4.60, p< 0.001). Sharks exposed to control chemical cues and chased had significantly elevated $\dot{M}O_{2Rest}$ than those just exposed to control cues (Fig. 1b, GLM, z= 2.74, p< 0.05).

When exposed only to control chemical cues, however, sharks displayed a $\dot{M}O_{2Rest}$ similar to when exposed to conspecific necromones and chased (GLM, z= 1.68, p= 0.33). There was no difference in $\dot{M}O_{2Rest}$ values between either chase treatment. Sharks displayed similar values despite exposure to either control or conspecific chemical cue (GLM, z= 1.06, p= 0.72).

Aerobic scope

Aerobic scope was not different between treatments (Fig. 1c, supplementary material, Table S3). Aerobic scope values were between (160.94- 183.10 mg O_2 kg^{-1} hr^{-1}) for all sharks.

Time to recovery

The time required for all sharks to return to resting values within each trial was not significantly different between treatments (Fig 2, supplementary material, Table S4). It took 1.20-1.35 hrs for $\dot{M}O_2$ values to reach $\dot{M}O_{2Rest}$.

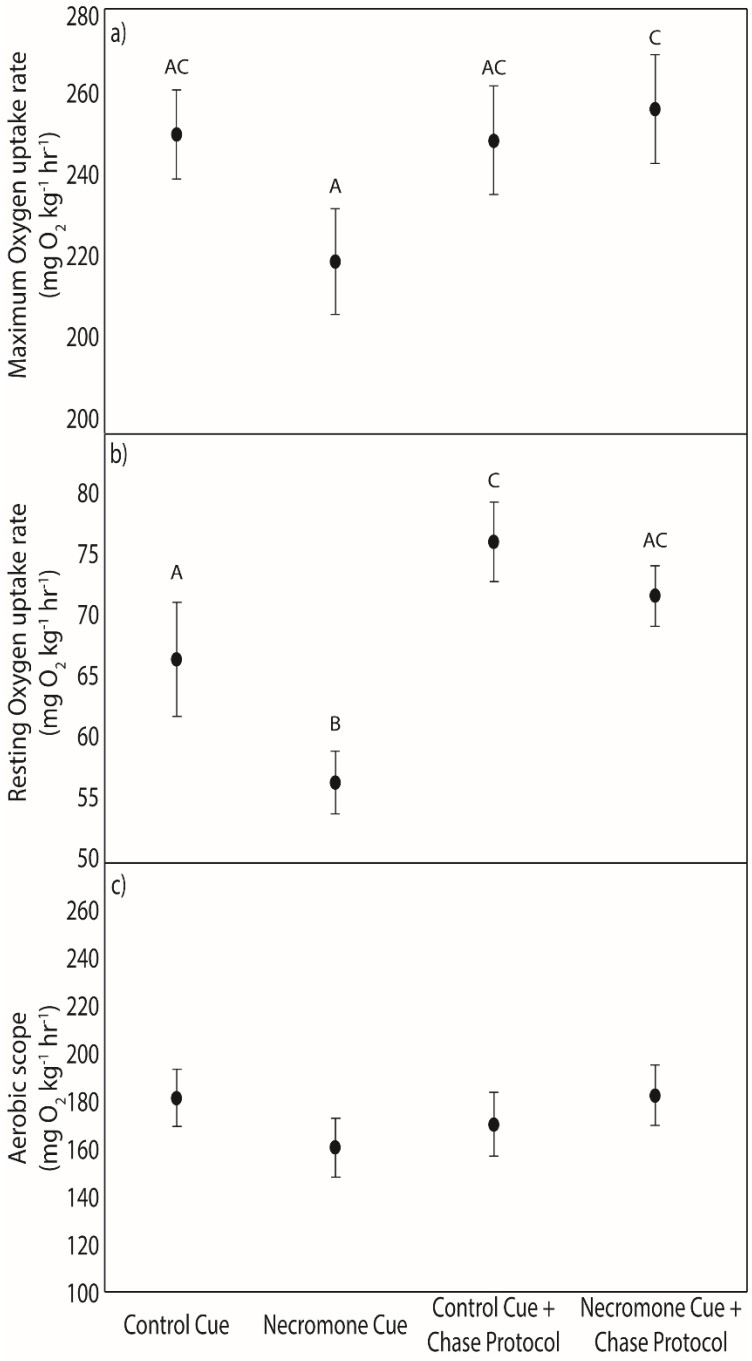

Figure 1: (a)Maximum oxygen uptake rates (mg O_2 kg^{-1} h^{-1}), (b) resting oxygen uptake rates, and (c) aerobic scope of juvenile sharks (n=14) across chemical cue treatments. All points are represented as means with standard error of the mean. Significant values with α=0.05 are indicated by letters.

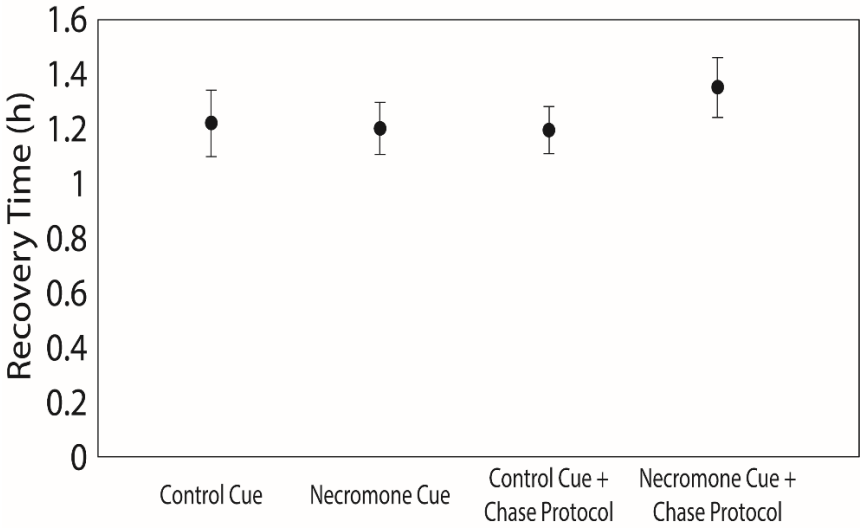

Figure 2: Recovery time (h) of juvenile sharks (n=14) across chemical cue treatments. All points are represented as means with standard error of the mean.

Discussion

Exposure to predator stimuli significantly influenced the oxygen uptake rates of juvenile Port Jackson sharks; however different stimuli prompted different responses. Simple exposure to conspecific necromones elicits a response in which juveniles depress their oxygen uptake rates for up to three hours following exposure. However, if sharks were chased following control chemical cues, they elicit an increase in their oxygen uptake rates, which is typical following exercise. While both stimuli on their own elicit different responses, when exposed to both conspecific necromones and then chased, individuals elevated their oxygen uptake rates, and the overall effect was no different than their response when exposed to control chemical cues, both without and with a chase component. This may suggest that young sharks primarily rely on more sedentary predator avoidance strategies (i.e., crypsis), depressing their oxygen uptake rates and subsequently their respiration function. However, they utilize typical fight or flight responses if forced to evade a predator regardless of whether they detect a dead conspecific or not.

Young Port Jackson sharks should be at high risk from predation, they are small, have a large internal energy store and live in relatively exposed habitats (i.e., sand flats and seagrass beds) (Powter and Gladstone, 2009). However, like most organisms they can potentially utilize multiple predator avoidance strategies. When juvenile sharks detected a dead conspecific, they decreased their oxygen uptake rates, which was still detectable even after three hours following exposure to the cue. This may indicate that anti-predator responses are sustained for prolonged periods of time and may delay sharks return to true resting uptake rates. By decreasing oxygen uptake rates, other processes such as

ventilation and heart rate inherently decrease, which in turn can decrease an individual's bioelectric field (Sisneros *et al.*, 1998). Predators that rely on electroreception to hunt may find it more difficult to detect individuals that are supressing these traits as they may be able to effectively "blend" into their environment (Bedore *et al.*, 2015). Crypsis strategies similar to the one displayed here have been observed in a range of both invertebrate and vertebrate species. Cephalopods (Bedore *et al.*, 2015), crustaceans (Briceño *et al.*, 2018), arachnids (Okuyama, 2015), teleost fish (Cooke *et al.*, 2003; Huuskonen and Karjalainen, 1997), amphibians(Steiner and Van Buskirk, 2009), and mammals (Smith *et al.*, 1981) may decrease their metabolic rates and/or heart rates in response to predation. Additionally, young Port Jackson sharks may rely on a morphological defence in the form of extremely sharp dorsal spines which can puncture skin and their coloration patterns. Such spines have proven anti-predator benefits in a range of species, such as three-spined sticklebacks (Ab Ghani *et al.*, 2016; Reimchen, 1992), while colour patterns can break-up a species' outline thus making them difficult to see (Ruxton *et al.*, 2004). Collectively our data suggests that juvenile PJs primarily utilize crypsis as their first line of defence to avoid predation. They reduce oxygen uptake rates, for prolonged periods of time, and rely on their cryptic coloration and morphological defences to avoid predator detection and conserve energy by limiting activity. Nonetheless, young sharks will elicit active strategies such as fleeing if they are if encountered by a predator and need to evade capture.

Juvenile Port Jackson sharks elicit a more typical stress response if they are chased by a predator. Undergoing aerobic and/or anaerobic activity, such as those experienced during a chase, incurs a greater energetic demand than strategies utilized to avoid detection. While maximum oxygen uptakes rates were not different between control and chase protocols, the cost of using activity incurred an energetic debt which, elicited elevated resting rates. This response is similar to that observed in other species eliciting a "flight" response (Hawlena and Schmitz, 2010). However, sharks exposed to conspecific necromones and chased did not display as strong of a response relative to just being chased. Indeed, there was no suggestion that oxygen uptake was depressed in the presence of necromones at all when chased. Nonetheless, despite the increased energetic demand, "flight" responses fuels processes which aid in aerobic and anaerobic activity (i.e., sustained and burst swimming); essential strategies fish rely on to evade predation. Despite detecting conspecific necromones, once an individual is detected, strategies such as crypsis are no longer a viable option, thus individuals must flee from the predator or risk being consumed (Creel, 2018).

As a stressor, predation risk can have strong impacts on a species physiological processes and can drive different responses. Here, juvenile Port Jackson sharks appropriately altered their anti-predator response according to the type of stimuli they encountered. The patterns expressed may reflect the behaviours these young sharks use in the wild. Limiting their detection via metabolic

depression in combination with their cryptic colouration allows them to remain stationary and conserve energy; however, and if ultimately that is unsuccessful, they will most likely elicit a typical "flight" response, increase their oxygen uptake to meet the increased energy demand. These strategies, and the energetic trade-offs associated with each, may be especially important during these early post-hatching stages. The higher energetic costs associated with "flight" and/or general stress responses can result in depressed growth, impaired immunocompetence, and/or altered body composition (Hawlena and Schmitz, 2010).

Stressful stimuli, such as predation, are common and can pose serious and lethal impacts to individuals if they are ill equipped to mitigate these risks. The capacity to utilize different strategies, depending on the situation can be vital in aiding prey's escape and survival. While, all strategies aim to effectively mitigate predation risk, each strategy relies on different physiological processes and may have different energetic requirements, which can have knock-on effects if conditions fail to improve. However, these responses do not exist in isolation. Juvenile sharks are inherently vulnerable and must respond to a plethora of other stressors (e.g., social, environmental) many of which can influence the physiology and/or behaviour of the individual. These stressors may compound or work antagonistically with anti-predator responses. For example, exposure to elevated temperatures can incur energetic costs and lead to an increase in oxygen uptake rates (Johansen and Jones, 2011) that can undermine strategies, such as escape responses, as other processes may be prioritized to maintain life. However, mitigating predation risk is important in the long-term survival of individuals and the implementation of these strategies and how they may change in the future in response to human-induced climate change is crucial for continuing research. If these strategies are compromised or conditions fail to improve, populations utilizing these strategies may experience declines in health. However, utilizing and relying on different strategies, such as those observed in Port Jackson sharks, may offer species some flexibility. Being able to utilize different strategies such as anti-predator defence, predator avoidance, and predator evasion, species such as juvenile Port Jackson sharks have alternate means of effectively reducing their predation risk in a myriad of different scenarios.

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Compliance with Ethical Standards Conflict of Interest:

No conflict of interests exists. All animal care and experimental protocols used in this study were approved by Macquarie University Animal Ethics Committee under ARA 2014-003 and conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

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Supplementary Materials

Table S1: Mean values (± SEM) for maximum oxygen uptake rates ($\dot{M}O_{2Max}$ [mg O_2 kg^{-1} hr^{-1}]) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

	Control cue	Necromone Cue	Control cue + Chase Protocol	Necromone Cue + Chase Protocol
Control cue	248.153±10.980			
Conspecific Necromone	p= 0.103 z= 2.279	217.425±12.877		
Control cue + Chase	p= 0.997 Z= -0.206	p= 0.163 z= 2.069	247.021±13.306	
Conspecific Necromone + Chase	p= 0.987 z= 0.334	p< 0.05 * z=2.616	p= 0.949 z= -0.539	254.778±13.315

Table S2: Mean values (± SEM) for resting oxygen uptake rates ($\dot{M}O_{2Rest}$ [mg O_2 kg^{-1} hr^{-1}]) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

	Control cue	Necromone Cue	Control cue + Chase Protocol	Necromone Cue + Chase Protocol
Control cue	66.442±4.633			
Conspecific Necromone	p< 0.05 * z= 2.899	56.486±2.557		
Control cue + Chase	p< 0.05 * Z= 2.735	p< 0.001 * z= 4.601	76.062±3.242	
Conspecific Necromone + Chase	p= 0.3325 z= 1.683	p< 0.001 * z=4.601	p= 0.7153 z=1.058	71.679±2.471

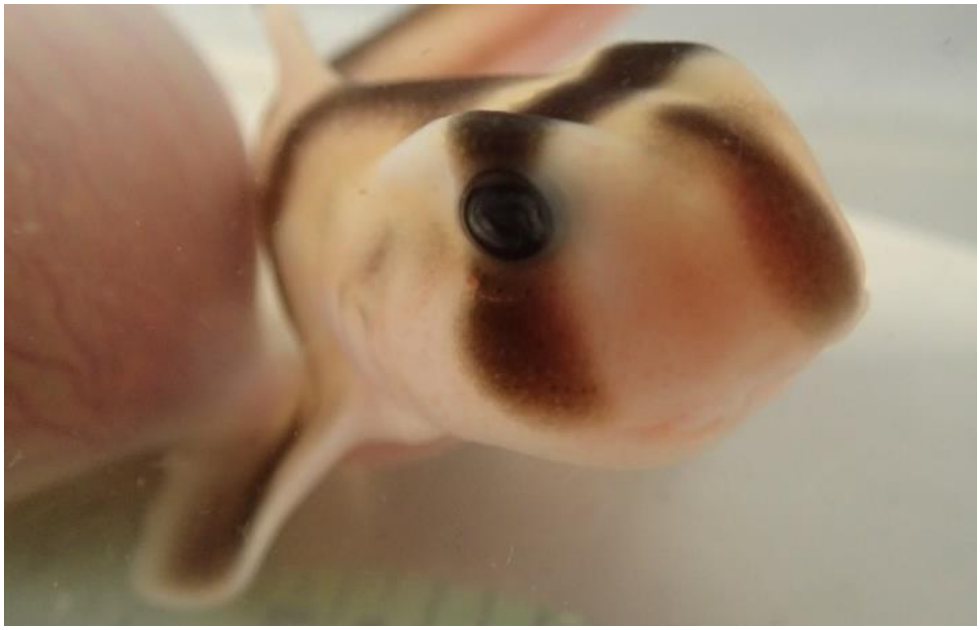
Table S3: Mean values (± SEM) for aerobic scope (AS [mg O_2 kg^{-1} hr^{-1}]) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

	Control cue	Necromone Cue	Control cue + Chase Protocol	Necromone Cue + Chase Protocol
Control cue	181.712±12.034			
Conspecific Necromone	p= 0.468 z= 1.450	160.939±12.325		
Control cue + Chase	p= 0.816 Z= -0.878	p= 0.941 z= 0.571	170.960±13.330	
Conspecific Necromone + Chase	p= 1.000 z= -0.003	p= 0.470 z=1.447	p= 0.816 z= -0.878	183.099±12.784

Table S4: Mean values (± SEM) for recovery time (hr) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

	Control cue	Necromone Cue	Control cue + Chase Protocol	Necromone Cue + Chase Protocol
Control cue	1.216±0.122			
Conspecific Necromone	p= 1.000 z= 0.085	1.202±0.095		
Control cue + Chase	p= 1.000 Z= -0.037	p= 1.000 z= 0.048	1.196±0.086	
Conspecific Necromone + Chase	p= 0.788 z= 0.931	p= 0.739 z=1.017	p= 0.766 z= -0.971	1.352±0.109

Chapter 3: Developing in hot water: Populations vary in the physiological cost of embryonic development in Port Jackson sharks, *Heterodontus portusjacksoni*



Developing in hot water: Populations vary in the physiological cost of embryonic development in Port Jackson sharks, *Heterodontus portusjacksoni*

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Abstract

During embryonic development oviparous species are inherently vulnerable to a wide range of external threats given they are small, immobile and therefore exposed to conditions without the capacity to mitigate their influence. As sea surface temperatures continue to rise, species will be developing under increasingly warmer conditions which can have deleterious effects. Within a species, however, different populations may be inherently more vulnerable or tolerant to changes in environmental conditions given their exposure to local conditions and the relative capacity for adaptation or acclimation strategies. Here we examined the influence of rearing temperature and population plasticity or adaptation on the physiology and development of embryonic Port Jackson sharks. *Heterodontus portusjacksoni*. Eggs were collected from two distinct populations (Jervis Bay, NSW and Adelaide, SA) and reared under ambient temperature, predicted end-of-century temperature, or reciprocal cross-population temperature. Intermittent flow respirometry was utilized to measure the physiological costs over development (via oxygen uptake rates) and the total energetic costs associated with different rearing conditions. Elevated rearing temperatures significantly decreased development time across both populations; however, at each developmental stage, there was no influence of temperature on oxygen uptake rates. Given that temperature did not affect energetic needs, but did influence developmental durations, the overall cost of development was highest for Adelaide sharks reared under ambient conditions due to longer development times relative to other treatments and populations. Across all populations and treatments, oxygen uptake rates were significantly influenced by developmental stage, indicating that ontogenetic changes strongly influence the energetic needs of this species, and as such, Port Jackson sharks may exhibit a wide thermal tolerance during development. This species also displays local population-specific differences in size and there was no plasticity between temperatures and the size at hatching, with all Jervis Bay sharks hatching much larger than those from Adelaide. Furthermore, populations reared under similar temperatures displayed different overall costs of development. As temperatures continue to rise, species constrained within eggs for relatively long periods of time are unable to use behaviour to mitigate thermal effects, however, our data suggest that some species and/or populations, may be relatively robust and tolerant of temperature changes during early ontogeny.

Keywords: Climate change; Temperature; Respirometry; Acclimation; Growth; Elasmobranch

Introduction

Embryonic development is a crucial period for all living organisms and complications during key developmental stages can have potential knock-on effects throughout ontogeny. Changes in incubation temperature during early development of ectotherms influences biochemical, physiological, and/or behavioural processes, as ectotherm internal body temperatures mirror the conditions of their external environment (Pörtner and Farrell, 2008; Tewksbury *et al.*, 2008). For instance, developing in warmer conditions can benefit development by increasing growth rate and decreasing gestation/developmental rates (Pankhurst and Munday, 2011; Pistevos *et al.*, 2015; Rombough, 1997); however, there are often other deleterious impacts, unequal sex-determination, skeletal deformities, abnormal colouration pattern expression, and increased mortality (Billerbeck *et al.*, 2001; Gervais *et al.*, 2016; Janzen, 1994; Rosa *et al.*, 2014; Vinegar, 1974). It is thought that each physiological and/or behavioural trait has an optimal temperature or range of optimal temperatures; therefore, as temperatures move away from the optimum, performance in these traits may decline (Pörtner and Farrell, 2008). Furthermore, as global sea surface temperatures are predicted to increase 2.6-4.8°C by the end of the century, species will be exposed to, and develop in, unfavourable conditions and as such they may need to utilize a combination of strategies to persist (Collins *et al.*, 2013).

As conditions change, species utilize different strategies to cope, such as adaptation, acclimation, and/or changes in behaviour. These strategies work on different timescales and may rely on certain traits (e.g., the capacity to move or the capacity for phenotypic plasticity) to be effective (Angilletta, 2009). Over multiple generations individual species and/or populations adapt to the specific conditions that they inhabit. These adaptations are often underlined by genetic changes, which can influence specific traits to promote long-term survival and persistence of a population in their contemporary environment. One extreme example is that of Antarctic fishes which live in conditions lethal to most life. These fish flourish by utilizing unique adaptations(e.g., “anti-freeze” proteins), which have arisen via mutations over time, to enable them to survive at temperatures beyond which most fishes would freeze (DeVries, 1983). Over shorter timescales, from days to months and even over different generations (transgenerational acclimation), acclimation can enhance the performance of different traits (e.g., resting oxygen uptake rates and swimming performance) by shifting the thermal range at which the traits can operate, thus allowing species to persist in the new conditions (Angilletta, 2009; Angilletta *et al.*, 2006). This strategy has been widely utilized by many species that experience seasonal temperature changes (Gervais *et al.*, 2018; Guderley and St-Pierre, 2002; Huey and Hertz, 1984; Sharma *et al.*, 2015; Vinagre *et al.*, 2016). For example, during summer months, the Indian hill trout (*Barilius bendenlisis*) increase their maximum critical and lethal thermal limits, as well as increasing their oxygen uptake rates to compensate for the increased temperatures (Sharma *et al.*, 2015). While

some species may rely more heavily on acclimation in response to changing conditions, others may be less inclined to use this strategy, and use behavioural strategies instead. That is, when faced with unfavourable conditions an organism can utilize behavioural strategies (e.g., behavioural thermoregulation or sheltering; (Angilletta *et al.*, 2006; Gervais *et al.*, 2018; Nay *et al.*, 2018) to actively regulate their internal body temperature. Regardless of the strategy or strategies utilized, they all enable species to persist and react to environmental changes. However, the conditions and selection pressures that populations, within a species, experience may be different, and thus may promote localized changes at the population level.

Species distributed over a large spatial range have seemingly adapted to a large gradient of environmental conditions, however, different populations may display different phenotypic responses and/or utilize different strategies to cope with their local environment (Pörtner, 2002). As such, different populations may exhibit traits more suited to their specific conditions. Consequently, there may be trade-offs as other traits are selected against or direct costs associated different environmental conditions. For example, Atlantic silversides, *Menidia menidia,* from northern latitudes consumed more food, grew faster, and produced more eggs than their southern conspecifics (Billerbeck *et al.*, 2001). However, the slow-growing southern population excelled in swimming performance; they were faster and had a greater capacity for prolonged and burst swimming. Similarly, elasmobranchs from different populations display shifts in overall morphology, life history characteristics and diet with latitudinal changes, which are thought to reflect localized thermal differences (Bethea *et al.*, 2007). Embryonic little skates, *Leucoraja erinacea*, display variable responses to elevated temperature and ocean acidification based on their population provenance (Di Santo, 2015). Those that naturally develop in relatively cooler waters (northern population) were more sensitive to warmer temperatures, showing decreased survival, growth and increased metabolic rates, when exposed to elevated temperatures during development. These populations appeared to be primarily relying on localized adaptation; however, both populations appear to also utilize acclimation to mitigate some of the effects of elevated temperatures. These differences can be indicative of the relative vulnerability of certain populations to thermal changes. Specifically, populations which display greater plasticity and/or less sensitivity to thermal changes may be better equipped as temperatures continue to rise in future. Unless increased energetic needs are met, energy may be reallocated away from non-vital processes, such as growth and/or reproduction, in favour of maintaining basic maintenance costs (Angilletta, 2009). While populations are specifically adapted to their current environmental conditions, the rapid rate of climate change may outpace the rate of adaptation, particularly for slow-growing species with relatively long generation times, which includes most elasmobranchs (Crozier and Hutchings, 2014; Pörtner and Farrell, 2008).

The life history characteristics of most elasmobranchs may suggest that they may rely more heavily on behaviour (particularly movement) and acclimation processes rather than adaptive responses to cope with shifting environmental conditions (Crozier and Hutchings, 2014; Pörtner and Farrell, 2008; Rummer and Munday, 2017). However, around 43% of all elasmobranch species are oviparous (i.e., develop in external egg capsules; Compagno, 1990), and thus, throughout embryonic development, are unable to move if water conditions become unfavourable. The inability to move and the relatively long developmental time of some oviparous elasmobranchs (some up to 3.5 years; Hoff, 2008) is thought to make developing embryos particularly vulnerable to changes in water conditions (Di Santo, 2015; Johnson *et al.*, 2016). Therefore, these species likely rely on innate adaptations and/or acclimation processes to buffer the impact of environmental conditions and enable embryos to withstand variable rearing temperatures (Carrier *et al.*, 2012; Di Santo, 2015; Hamlett, 2005; Johnson *et al.*, 2016; Pörtner and Farrell, 2008; Rodda and Seymour, 2008).

Port Jackson sharks (*Heterodontus portusjacksoni*) are found throughout the southern, temperate coasts of Australia. Adults undertake relatively long migrations every year and show extremely strong site philopatry to oviposition sites (Bass *et al.*, 2017; Powter and Gladstone, 2008). There are distinct populations separated by space (i.e., oviposition site), size, reproductive timing, and potential migrations patterns (Day *et al.*, 2019; Powter and Gladstone, 2008; Tovar-Ávila *et al.*, 2007). Port Jackson shark populations along the New South Wales coast grow the largest (females up to 910mm at maturity) and reproduce in late winter (late August-early November; Powter and Gladstone 2008), while the South Australia populations are considerably smaller (females up to 750mm at maturity) and breed in spring (late October-early December) (Izzo and Rodda, 2012; Powter and Gladstone, 2008). During the oviposition seasons, females deposit spiral egg capsules (two at a time) at specific rocky reefs, which, through water movement, become securely wedged within cracks and crevices (Powter and Gladstone, 2008). Embryonic development can take up to 11 months, during which embryos are naturally exposed to different thermal regimes based on oviposition sites (Rodda and Seymour, 2008).

Here, we use Port Jackson sharks as a model oviparous species to investigate the role that developmental temperature plays on oxygen uptake rates— as a proxy for metabolic rate— and how it influences growth during early development. Embryonic development should be significantly influenced by elevated temperatures as the rate of different physiological and developmental processes are regulated by temperature. Furthermore, given the wide distribution and thus wide exposure to different rearing conditions, we also included a population effect to explore local adaptations and/or plasticity between distinct breeding grounds. Egg capsules from a warmer-water population (Jervis Bay, NSW) and a cooler-water population (Adelaide, SA) were collected and reared under their average ambient

hatching temperatures, temperatures representative of predicted end-of-century conditions, as well as conditions representative of the alternate location (e.g. Jervis bay embryos reared at Adelaide temperatures). Following the natural opening of the egg capsules to the external environment, oxygen uptake rates were measured across developmental stages (see Rodda and Seymour, 2008) until hatching. Elevated rearing temperatures typically incur greater energetic requirements which is commonly associated with increased oxygen uptake rates and embryos may display altered growth rates. Concomitantly, development time is decreased in many species when reared at elevated temperatures.

Methods

Animal collection and husbandry

Port Jackson shark eggs from New South Wales were collected on snorkel from Orion's reef, Jervis Bay, NSW (-35.068°S, 150.684°E) (n=47, average egg total length± standard error of means [SEM], 157.36± 1.42 mm; egg volume 182.71± 3.7ml) on October 10 and November 2, 2016 and eggs from Adelaide were collected from Christies reef, Adelaide, SA (35.141°S, 138.465°E) (n=45, average egg total length 135.57± 1.31mm; egg volume, 127.65± 3.68ml) on November 10, 2016. Freshly laid eggs are clean, soft, pliable and olive green in colour and become more rigid and brown around 3-5 weeks (Rodda and Seymour, 2008). Therefore, based on egg colour and pliability, eggs from Jervis bay were estimated to be no older than 6 weeks post oviposition at the time of collection, while eggs collected from Adelaide were determined to be no older than two weeks post oviposition (Fig. 1a).

Eggs from Jervis Bay were transported via car to aquarium facilities at Macquarie University, Sydney Australia. Eggs from Adelaide were flown to the aquarium facilities at Macquarie University in plastic bags filled with seawater to cover the eggs and the void was filled with pure oxygen. In both cases eggs were in transit no longer than 5 hours.

Eggs were held in 40L aquaria (up to nine per aquaria) containing aerated, naturally filtered seawater at collection temperatures for one week before slowly transitioning eggs to treatment temperatures at a rate of±0.5°C day^{-1}. Eggs from each location were randomly assigned one of three developmental temperatures. The temperature treatments were:

1) Annual average hatching temperatures of the collection site (i.e., Jervis Bay eggs at annual Jervis Bay hatching temperatures [20.6°C; n=16] (Bureau of Meteorology, 2016) and Adelaide eggs at annual Adelaide hatching temperatures [17.6°C; n=14]) (Gaylard, 2004)

2) Annual average hatching temperature of the alternate collection site (i.e., Jervis Bay eggs at Adelaide annual temperatures [17.6°C; n=14] and Adelaide eggs at Jervis Bay annual temperatures [20.6°C; n=16])

3) Predicted end-of-century (EOC) temperatures; +3.0°C from collection site's annual ambient hatching temperatures to simulate (i.e., Jervis Bay eggs at Jervis bay predicted conditions [23.6°C; n=17] and Adelaide eggs at Adelaide predicted conditions [20.6°C; n=15] (Collins et. al 2013, RCP 8.5))

Around four months into development (stage 10; Fig. 1b), Port Jackson shark egg capsules opened to the environment (Rodda and Seymour, 2008). Jervis Bay embryo development under EOC temperatures was captured from stage 12 onward and those reared under ambient conditions were measured from stage 11 onwards due to egg capsules opening unexpectedly. All embryos from Adelaide and Jervis Bay embryos reared under Adelaide conditions were measured from stage 10 onwards. Embryos were removed from their egg capsule and placed into individual 160mL food-safe containers to continuously monitor embryo development. The aquaria were covered with a tarp to simulate low-level light conditions in eggs.

Figure 1: Observable Port Jackson shark developmental stages, adapted from Rodda and Seymour (2008). a) Port Jackson shark eggs collected from Adelaide (left) and Jervis Bay (right), displaying differences in size between populations. b) Stage 10 at the opening of the mucous plug, c) Stage 11, d) Stage 12, e) Stage 13, f) Stage 14, g) Stage 15 (hatching).

Treatment temperatures were controlled using an automated mixing system (Simex multicon, CMC99), which mixed water from a warm and cold-water sump to achieve treatment temperatures prior to entering the aquaria. Developmental temperatures were held constant throughout development.

Eggs from Jervis Bay, NSW were collected under NSW fisheries permit P08/0010-4.2. No permits were required to collect eggs in South Australia. All animal husbandry and experimental protocols were approved by the Macquarie University Animal Ethics Committee (ARA 2016-027).

Respirometry

Starting from the opening of the egg capsule, oxygen uptake values (as a proxy for metabolic rate) were measured once a month until hatching (stage 15). Prior to the capsule opening (stage 11), no detectable oxygen uptake rate was recorded. Embryos were gently transferred into custom built 0.68L intermittent-flow respirometry chambers inside a temperature-controlled water bath, for a minimum of four hours (Roche *et al.*, 2013; Rummer *et al.*, 2016). The chambers were connected to a flush pump (690 L hr^{-1}) to provide oxygenated water throughout the system at set intervals and a recirculating pump (200 L hr^{-1}) to continuously ensure homogenous oxygen levels within each chamber. A digital relay timer set the flush pump intervals to a flush cycle set to 5 minutes off, 5 minutes on. This time frame was established to ensure each chamber was fully re-oxygenated with filtered seawater and to remove any build-up of CO_2 throughout the trial. It also ensured that O_2 levels did not fall below 80% air saturation (Clark *et al.*, 2013). In-line with each recirculating pump, O_2-sensitive REDFLASH dye contactless spots (2mm) sat inside of glass tubes to record oxygen concentration every 2s. The spots were linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) via 2m fiber-optic cables.

Oxygen uptake rates were analysed in LabChart v.6.1.3 (AD Instruments, Colorado Springs, CO, USA) using a linear least square regression of oxygen concentration over time. This method was deemed appropriate as R^2 values for the closed respirometry phases were above 0.95. While the methods utilized analyse resting oxygen uptake levels, embryonic organisms may be completely rest at certain developmental stages. Specifically, prior to the development of buccal pumping, embryonic sharks rely on tail movements to circulate water over their external gill filaments (Leonard *et al.*, 1999; Pelster and Bemis, 1992; Rodda and Seymour, 2008). Therefore, oxygen uptake rates (hereby defined as $\dot{M}O_{2Dev}$) were calculated as the average O_2 over the trial. The first hour of the trial was not analysed to remove

any handling influence. Following each trial, photographs were taken to establish the developmental stage and total yolk volume, to predict the mass of the embryo to calculate $\dot{M}O_{2Dev}$.

Microbial background oxygen uptake rates were measured both before the embryos were placed into the chambers and again after the embryos were removed at the end of the trial. The background O_2 uptake rate was linearly interpolated and subtracted from each slope. Following all trials, equipment was cleaned with a weak bleach solution (10%), rinsed in freshwater, and allowed to sun-dry, to minimize microbial background O2 uptake.

Morphometrics

Embryos morphometrics (i.e., total length, embryo mass [including yolk], embryo mass [excluding yolk], yolk volume and deformities) were measured following each oxygen uptake trial. Embryo mass (including yolk) was measured to the nearest 0.1g using an analytical balance and total length and yolk diameter (used to estimate volume) were calculated, to the nearest mm, from scaled photos using FIJI software (Schindelin *et al.*, 2012). Embryo mass (excluding yolk) was estimated by calculating the yolk volume (via photos) and extrapolating the yolk mass based on measurements taken from embryos which did not reach term. Furthermore, gestation duration (days post fertilizations and size (total length and mass) at hatching were analysed between treatments and populations. Developmental abnormalities were recorded, as observed and a subset of hatchlings (two displaying deformities and two control) were X-rayed following natural mortality.

Analysis

Linear mixed effect models (LMM) and linear models (LM) were used to examine all differences in $\dot{M}O_{2Dev}$ and morphometrics between rearing treatments and populations. Individual variation between sharks was controlled for by incorporating individual shark identity as a random factor over development. The fixed effects; development stage and temperature treatment, were used in the models to investigate differences in $\dot{M}O_{2Dev}$, total length, embryo mass [including yolk], embryo mass [excluding yolk], and yolk volume over development.

As time to hatching is significantly influenced by treatment temperature (Pistevos *et al.*, 2015), the time interval between developmental stages may not be similar. Therefore, development stage was treated as a discrete factor to measure changes in oxygen uptake patterns, and growth patterns across development (i.e., compared energetic costs across key developmental stages), thus controlling for developmental rate.

The total oxygen uptake required (i.e., developmental cost) was calculated by extrapolating $\dot{M}O_{2Dev}$ values (mg O_2 kg^{-1} h^{-1}) as the average daily (g O_2 kg^{-1} day^{-1}). Using LoggerPro (v 3.8.7) the total

cost of development ($\dot{M}O_{2total}$ [g O_2]) was measured as the integral of the observed $\dot{M}O_{2Dev}$ values over the duration of development—assuming a linear relationship in uptake rate between developmental stages. To ensure all treatments were time-matched for appropriate comparisons, values encompassed developmental stage 12 through stage 15 (hatching). A generalized mixed model was used to compare $\dot{M}O_{2total}$ across treatments (fixed factor), using development time frame (days) as a random factor.

Generalized mixed models were used to compare time to hatching (days post fertilization [dpf]) and size at hatching within and between populations. Fisher's exact test was used to compare the differences in the occurrence of mortality and developmental deformities between ambient temperatures and EOC temperatures. All analyses were performed in R (Version 3.4.1, R Core Development Team 2013), all assumptions were checked, and data were log- or square-root transformed where necessary to meet assumptions. Tukey post-hoc tests were used for all analyses. All values reported as mean± standard error measure (SEM).

Results

Oxygen uptake rates and embryo growth

a. Across development

Regardless of temperature treatment and population, the embryo's oxygen uptake rate depended on developmental stage (supplementary materials, Table S1). Embryonic oxygen uptake rates were highest prior to stage 12.5 regardless of treatment or population (Fig. 2;). Additionally, as embryos continuously grow and develop, morphometrics variables were also strongly correlated with developmental stage across all variables (supplementary materials, Table S2, S3, S4, S5).

b. Within populations response to elevated rearing temperatures

Developing under EOC temperatures (+3°C above ambient) did not significantly influence oxygen uptake rates of Port Jackson shark embryos within either population, as oxygen uptake rates increased (or decreased) in response to developmental stage, embryos displayed a similar response, both in pattern and magnitude of change (Fig 2., Jervis Bay, LMM, z=2.79, p=0.06; Adelaide; z=-2.54, p=0.11).

Similarly within each population, total length, total mass and embryo mass (excluding yolk) changed at a similar rate between embryos reared under EOC temperature and those reared under ambient treatments (Fig. 3, 4, 5; Jervis Bay: total length, z=0.99, p=0.92; total mass, z=-1.26, p=0.81; embryo mass, z=0.71, p=0.98; Adelaide: total length, z=0.97, p=0.0.93; total mass, z=1.60, p=0.60; embryo mass, z=2.14, p=0.27). However, yolk consumption across development, differed between populations (Fig. 6). Yolk consumption by Adelaide embryos was similar regardless of rearing

temperature (z=-0.10, p=1.00), but within the Jervis Bay population, embryos reared at EOC temperatures displayed smaller yolk sizes across development than ambient conspecifics (yolk volume; z= -4.64, p<0.001).

a. Between populations

Embryos reared under their respective ambient conditions (i.e., Jervis bay reared at 20.6°C and Adelaide embryos reared at 17.6°C) displayed similar oxygen uptake responses across development (z=2.81, p=0.06). However, growth patterns were significantly different between populations (total length, z=3.21, p<0.05; total mass, z=6.30, p<0.001; yolk volume, z= 6.78, p<0.001; embryo mass, 5.44, p<0.001), with Jervis Bay embryos displaying a greater change in size between developmental stages (Fig. 3, 4, 5, 6).

Despite similar oxygen uptake rates between populations reared under their ambient conditions, the response of embryos to predicted end-of-century conditions was dissimilar between populations. Embryos reared at EOC temperatures (23.6°C for Jervis Bay embryos and 20.6°C for Adelaide embryos) displayed dissimilar rates of oxygen uptake or growth (z= 4.42, p<0.001, total length, z=3.13, p<0.05, total mass, z= 4.94, p<0.001; embryo mass, z= 4.04, p<0.001). However, when reared at predicted end-of-century conditions both populations exhibited similar patterns in yolk volume absorption (z= 1.79, p=0.47)

Jervis Bay embryos reared under Adelaide temperatures (17.6°C) displayed different patterns of oxygen uptake across development relative to that of Adelaide conspecifics (z= -4.35, p<0.001). While, the general trend was similar, the magnitude of change across development was not (Fig. 2). Furthermore, correcting for time, Jervis Bay embryos grew larger and absorbed a larger yolk source (total length, z= 4.03, p<0.001, total mass, z= 8.63, p<0.001; yolk volume, z=5.39, p<0.001; embryo mass, z=7.38, p<0.001). Similarly, Adelaide embryos reared at Jervis Bay conditions (20.6°) also showed different trends to that of Jervis Bay conspecifics with Jervis Bay embryos initially taking up oxygen at a slower rate and growing faster than Adelaide embryos (Fig. 3, 4, 5, 6; oxygen uptake rates, z= 4.96, p <0.001; total length, z=3.30, p<0.001; total mass, z= 7.64, p<0.001; yolk volume, 6.18, p<0.001, embryo mass, z=3.75, p<0.01).

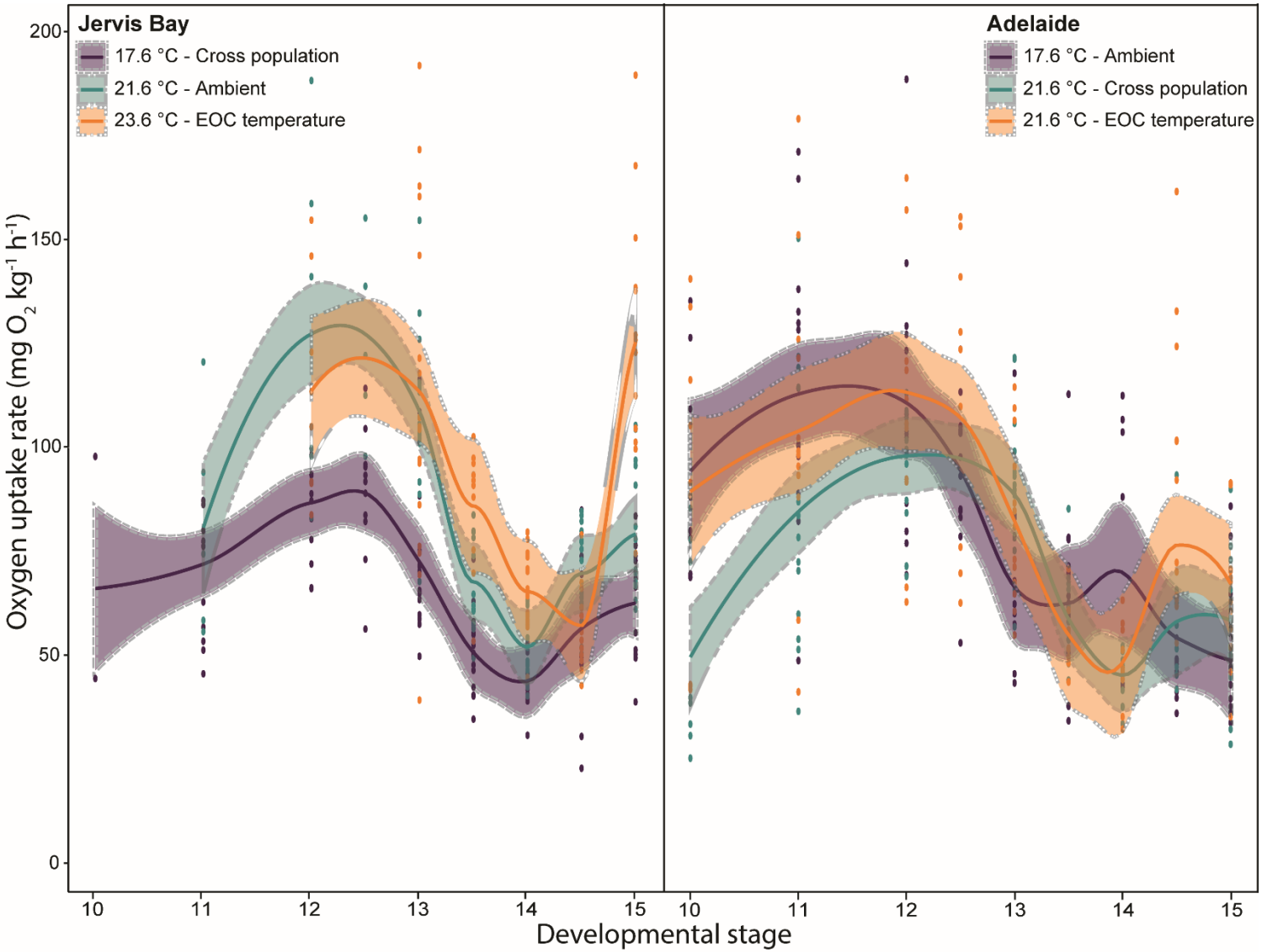

Figure 2: Oxygen uptake rates (mg O_2 kg^{-1} h^{-1}) of embryonic Port Jackson sharks across development from Jervis Bay (left panel) and Adelaide (right panel). Trace represents a line of best fit with a Loess smoothing function.

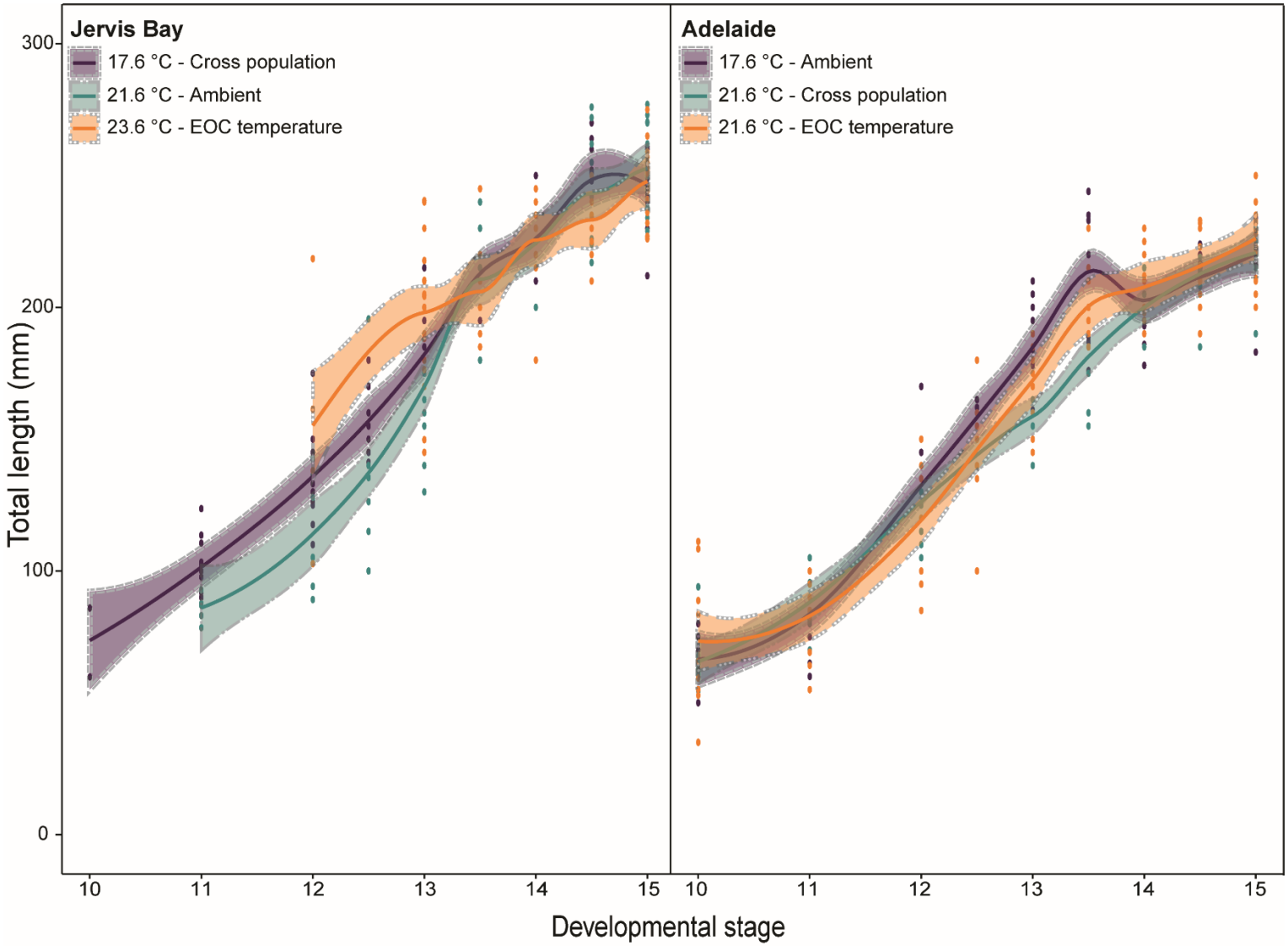

Figure 3: Total length (mm) of embryonic Port Jackson sharks across development from Jervis Bay (left panel) and Adelaide (right panel). Trace represents a line of best fit with a Loess smoothing function.

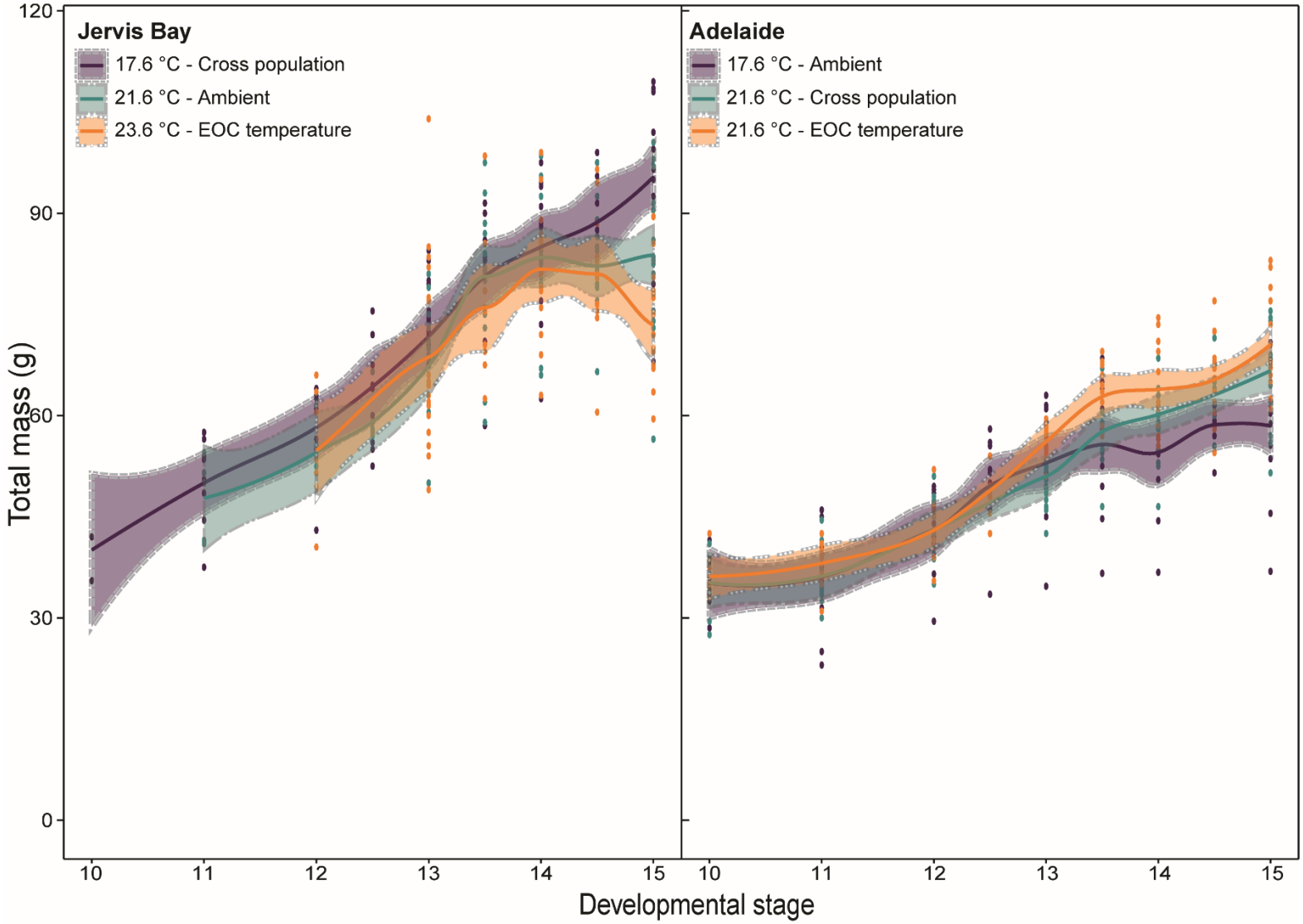

Figure 4: Total mass (g) of embryonic Port Jackson sharks across development from Jervis Bay (left panel) and Adelaide (right panel). Trace represents a line of best fit with a Loess smoothing function.

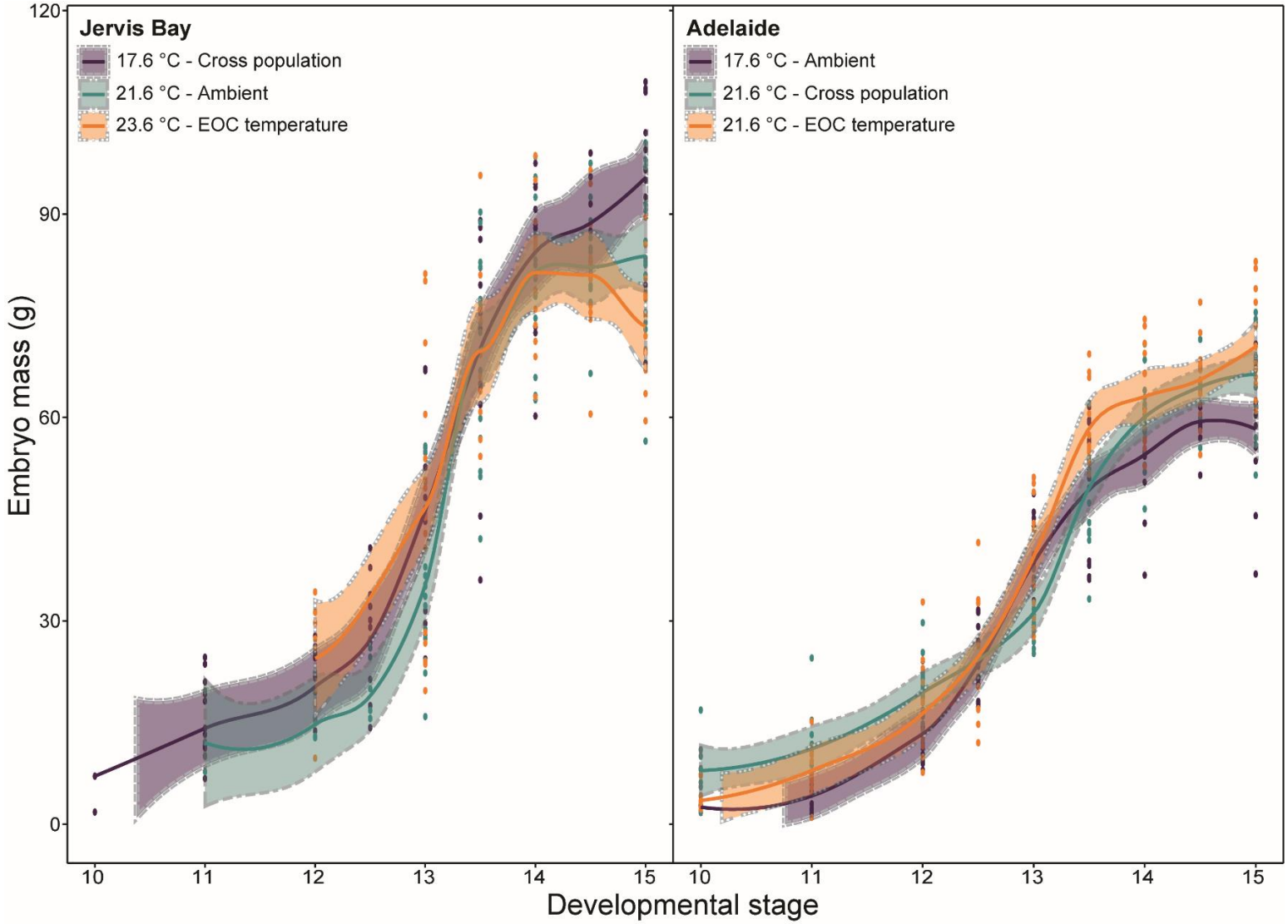

Figure 5: Embryo mass (g) of embryonic Port Jackson sharks across development from Jervis Bay (left panel) and Adelaide (right panel). Trace represents a line of best fit with a Loess smoothing function.

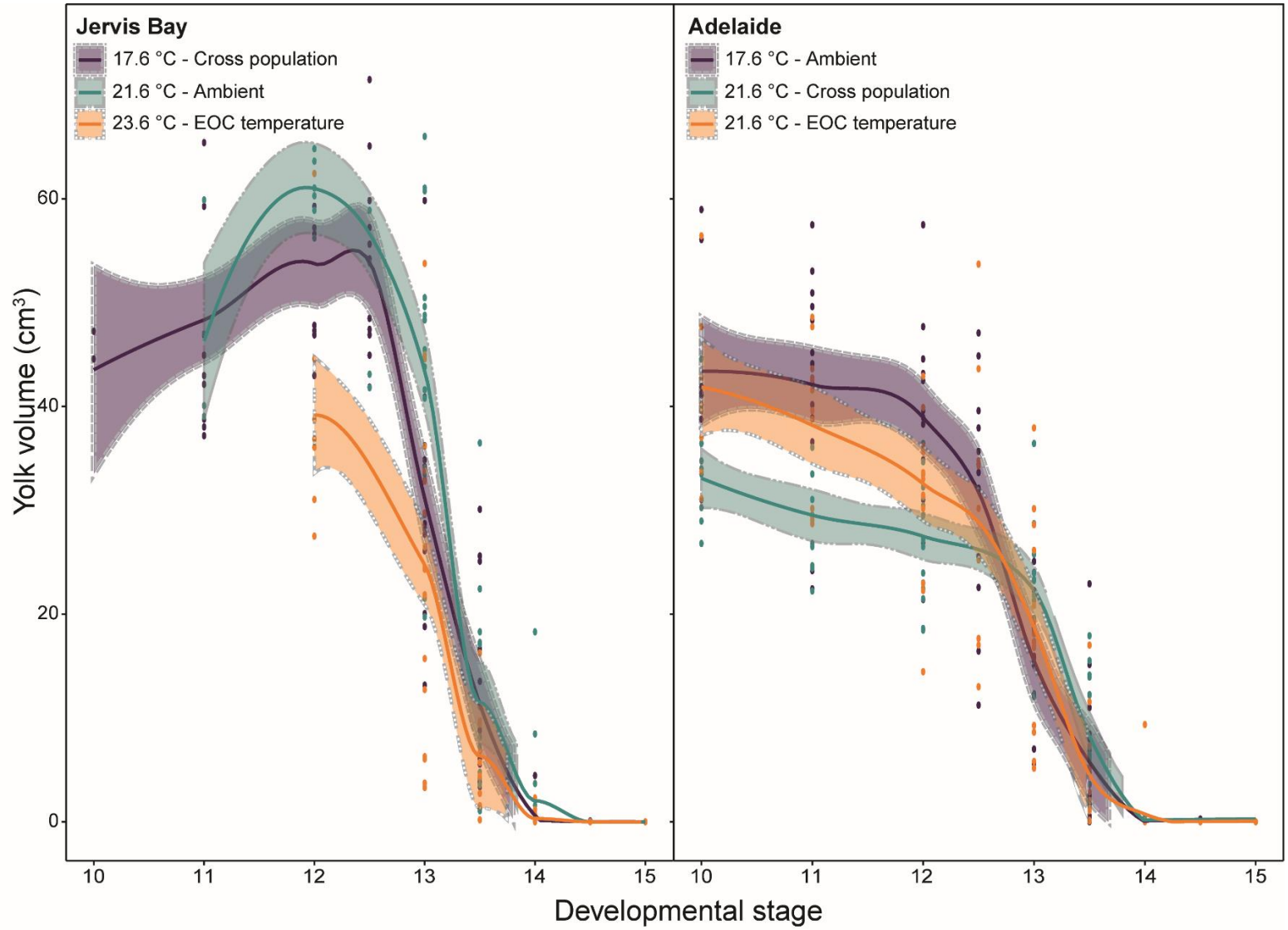

Figure 6: Yolk volume (cm^3) of embryonic Port Jackson sharks across development from Jervis Bay (left panel) and Adelaide (right panel). Trace represents a line of best fit with a Loess smoothing function.

Total oxygen uptake

a. Within populations response to elevated rearing temperatures

Embryos from Jervis Bay displayed no significant increase in total oxygen uptake when reared under elevated temperatures (Fig. 7, GLMM, z= 0.22, p= 0.99), embryos required similar oxygen values (15.37-15.65 g O_2). Similarly, Adelaide embryos displayed no difference in oxygen uptake rates between ambient temperatures and those reared under EOC conditions, taking up 11.82-13.19 g O_2 from developmental stage 12 to 15 (GLMM, z= -0.55., p= 0.99).

b. Between populations

Between Jervis Bay and Adelaide, embryos reared under their respective ambient temperature displayed no difference in total oxygen uptake required for hatching (Fig. 7, GLMM, z= 0.56, p= 0.99). Similarly, there was no difference in total oxygen uptake between embryos reared under their respective EOC conditions (GLMM, z= 1.48, p= 0.64).

Embryos from Jervis Bay reared under 21.6°C temperatures took up a greater amount of oxygen across development 15.37±0.72 g O_2) than Adelaide embryos reared under the same conditions (9.53±0.61 g O_2) (GLMM, z= -7.03, p< 0.001). Conversely, embryos from Adelaide reared under 17.6°C displayed similar oxygen requirements (13.19±1.19 g O_2) to Jervis Bay embryos reared under similar temperature (12.33±0.69 g O_2) (GLMM, z= -0.20, p= 0.99).

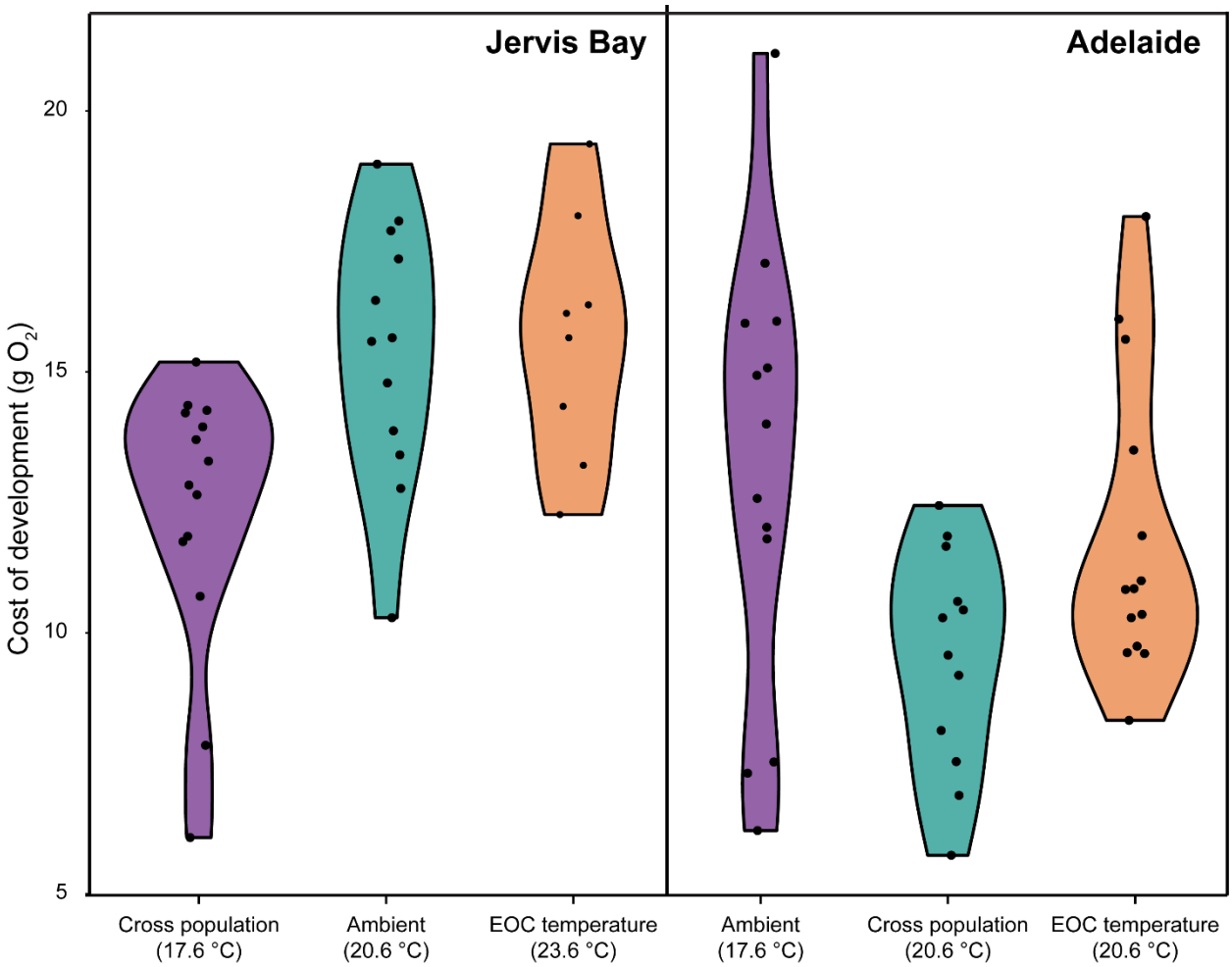

Figure 7: Violin plots depicting the cost of development (g O_2) for each temperature treatment, (ambient conditions, cross populations conditions, and EOC [end-of-century] conditions), from developmental stage 12 until stage 15 (hatching).

Developmental abnormalities

While not significant (Fisher's exact test, p = 0.17), 19.4% of the sharks (6/31) from Adelaide reared at EOC temperatures (20.6°C) displayed either skeletal (see example Fig. 8) or behavioural abnormalities. Two embryos developed cranial lumps, two developed spinal abnormalities and two were unable to swim correctly upon hatching. Additionally, one embryo from Jervis Bay reared under EOC temperatures (23.6°C) developed behavioural abnormalities affecting swimming behaviour, but this was not different from ambient embryos (Fisher's exact test, p = 1.00).

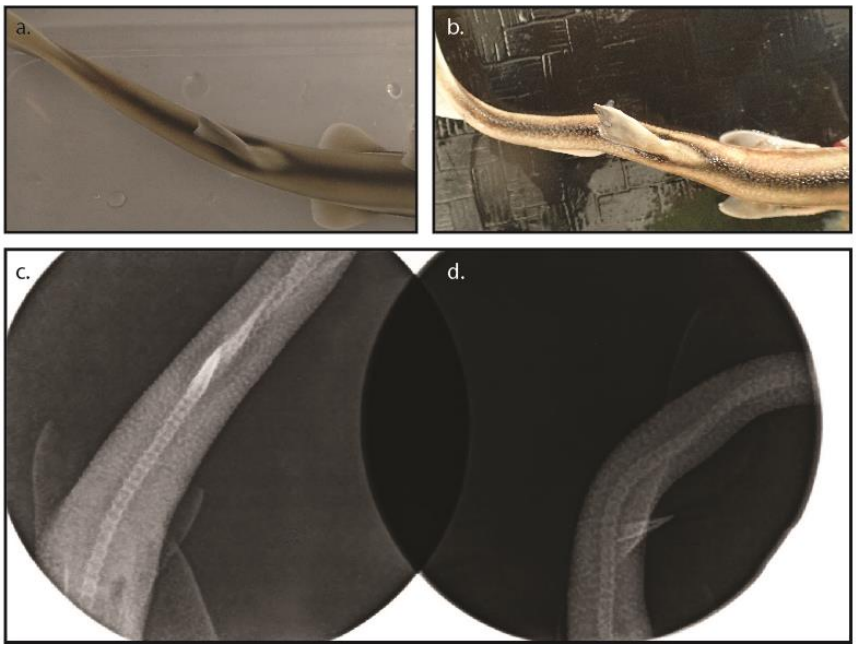

Figure 8: Normal spinal development a) photo and compared to a hatchling which developed a spinal abnormality b). Bottom panel displays X-rays of c) normal and d) abnormal regions.

Gestation duration and size at hatching

The time required for embryos to develop (days post fertilization) was shorter in treatments reared under warmer conditions (Fig. 9). While warmer incubation temperature decreased gestation time in both populations. Embryos from Adelaide were disproportionally influenced by temperature with gestation time 3.2-3.7 months shorter in embryos reared under EOC and Jervis Bay temperatures (20.6°C) compared with those reared under ambient conditions (17.6°C) ($z=-16.84$, $p<0.001$). While not significant, Jervis Bay embryos, hatched earlier as well when reared under warmer temperatures, with embryos reared under EOC conditions (23.6°C) hatching 12.5 days earlier than those reared under ambient conditions (20.6°C) (dpf: EOC, 229.6±3.7 days; ambient, 242.1±3.8 day; $z=-2.66$, $p=0.08$). However, Jervis bay sharks reared under EOC and ambient temperatures did hatch significantly earlier than embryos reared under Adelaide temperatures (17.6°C) (17.6°C vs. 20.6°C; $z= 8.181$, $p<0.001$; 17.6°C vs. 23.6°C; $z= -10.59$, $p<0.001$).

Size (total length and total mass) at hatching was similar within populations, despite rearing temperature (Jervis Bay: total length, $F_{2,43}= 1.02$, $p= 0.37$; total mass, $F_{2,43}=0.59$, $p=0.56$; Adelaide: total length, $F_{2,40}= 0.05$, $p=0.95$; total mass, $F_{2,40}= 1.53$, $p= 0.23$). However, hatchlings were not similar between populations (ANOVA, total length, $F_{5,83}= 13.88$, $p<0.001$, total mass, $F_{5,82}= 16.82$, $p<0.001$). Hatchlings from Jervis Bay, regardless of rearing temperature (total length, 238.16mm±3.82; total mass

82.64g±3.45) were 11% longer and 38.6% heavier than all hatchlings from Adelaide (total length 213.42mm±3.76; total mass, 60.39g±2.15).

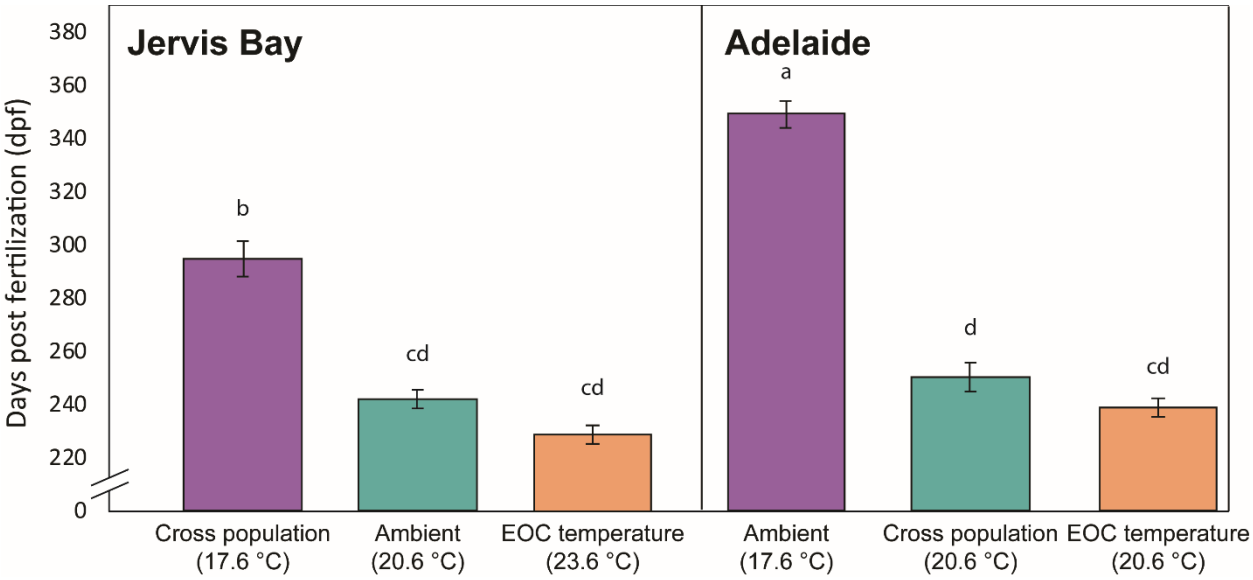

Figure 9: Developmental time (dpf) across rearing temperatures (ambient conditions, cross populations conditions, and EOC [end-of-century] conditions) and populations. Letters demarcate differences between treatments. Mean±SEM.

Discussion

Elevated temperature often imposes significant physiological pressure on traits such as growth and oxygen uptake (Angilletta, 2009). Here, temperature significantly influenced the growth rates of embryonic Port Jackson sharks and to some extent the total oxygen uptake of embryos. While, warmer rearing temperatures did increase the rate of development across all populations, the total cost of development between ambient conditions and end of century (EOC) conditions were similar. However, the development stage (time independent) and parental population, were overall, stronger drivers of oxygen uptake rates and growth patterns. This suggests that while temperature does influence some traits, populations may have local adaptations to local conditions and/or have the capacity for developmental acclimation as rearing conditions change. Unsurprisingly, the speed of development was decreased in embryos reared under elevated temperatures— as in seen in most other ectotherms; however, there are inherent differences in population metrics. Despite this increased development, the size of this species across developmental stages is similar, indicating that unlike many ectotherms, absolute growth is not influenced by temperature (temperature-size rule; see Angilletta and Dunham 2003), but instead may be predetermined by provenance. Unlike many ectotherms, this species

displayed concurrent-gradient patterns between size and temperature, that is, warm water population (Jervis Bay) had inherently larger embryos than the cool water population (Adelaide). Adelaide sharks were also disproportionately influenced by EOC temperatures displaying a 31% decrease in development time compared to, 5% in Jervis Bay embryos, and furthermore, Adelaide hatchlings reared under EOC exhibited more physical and/or behavioural abnormalities (~20%) than those reared under ambient conditions. While not significant, increased abnormality risk coupled with markedly reduced development times and decreased cost of development could indicate that elevated rearing temperatures may influence Adelaide populations more so than Jervis Bay population. Similarly, the capacity for acclimation of total developmental costs, to different thermal regimes was population specific. While Adelaide embryos reared under 20.6°C displayed a 28.6% decrease in total developmental costs (relative to ambient temperatures), Jervis Bay embryos display a 24.5% increase in total costs over this same temperature range. This can suggest that populations display distinct differences despite exposure to similar rearing conditions, likely as a result of adaptation to local conditions. However, the rate of development and thus growth rate, displayed a greater acclimation capacity to changes in rearing temperature. Furthermore, oxygen uptake rates may ultimately reflect the energetic requirements of different processes at specific developmental stages as they arise during ontogeny or they could be a reflection of the allometric scaling relationship between mass and metabolic rate.

Many species exhibit differences in growth metrics within populations and/or between populations. Some teleost fish display phenotypic plasticity in body size with changes in temperature. Within the same population, teleost fish reared under different temperatures may be larger, or smaller at time of hatching (Ojanguren and Braña, 2003). Here, while temperature ultimately decreased the overall development time of Port Jackson sharks, which is consistent with other species (Di Santo, 2015; Rosa *et al.*, 2014), the growth pattern across development and the embryo size at hatching within populations were not different despite the rearing temperature. Similar to many taxa, both endothermic and ectothermic (Stillwell, 2010), elasmobranchs exhibit differences in performance, growth, reproductive cycles, as well as overall size between different populations, which can be attributed to environmental differences (Bethea *et al.*, 2007; Di Santo, 2015; Parsons, 1993; Yamaguchi *et al.*, 2000). Bonnethead sharks (*S. tiburo*) and star spotted dogfish, *Mustelus manazo* from warmer waters are smaller than their conspecifics living in cooler waters (Parsons, 1993; Yamaguchi *et al.*, 2000). Port Jackson sharks from Jervis Bay are much larger than those from Adelaide. While, the drivers influencing the demographics of these populations are not known, this contrasts the patterns predicted by temperature-size rule (Atkinson 1996), in which body mass declines with increasing temperature (Atkinson 1996; Stillwell 2010). As such, the size of hatchlings is likely dependant on maternal input (i.e., yolk resources proportional to maternal mass) rather than rearing conditions, as observed across most

ectotherms (Atkinson 1994, 1995; Stillwell 2010). While the mechanisms behind this species may not be fully understood, species that contract typical temperature-size theory are often influenced by other factors such as varied predation risk, seasonal constraints, and oxygen limitations (Atkinson 1995). Here one possible driver of the differences between populations could be related to different migratory needs. Longer migrations correlate with a larger body mass (Hein *et al.*, 2012). Jervis Bay sharks are known to make annual migrations of at least 1000km (roundtrip) (Bass *et al.*, 2017), however little is known about the migratory patterns of Adelaide sharks, but they could be making shorter migrations. Regardless of the mechanisms driving these size differences, there is little to no plasticity in regard to size at hatching; despite an increase of between 5-31% in development rate, indicating that such size differences are likely innate, reflecting population specific adaptations.

As embryonic Port Jackson sharks developed, oxygen uptake rates and growth rates peak at a particular stage and then decline over the rest of development. Similarly, within populations, Port Jackson shark oxygen uptake rates (relative to embryo size) were strongly correlated with developmental stage. The energetic requirements of an embryo are thought to change over time as the development of new structures (e.g., muscle), the rate of different processes change (e.g., somatic growth), and/or the efficiency of different structures improve (e.g., internal gills) (Leonard *et al.*, 1999; Pelster and Bemis, 1992; Tomita *et al.*, 2014). During stages 11-12, Port Jackson shark embryos displayed an increase in oxygen uptake rates and growth rates. Prior to stage 11, embryos rely on external gill filaments for respiratory functions; however, around this stage, the internal gills become functional (i.e., allowing buccal pumping) and the external gills begin to be reabsorbed (Rodda and Seymour, 2008). As the internal gills become the primary respiratory structure, tail movements, a metabolically expensive movement required to circulate oxygenated water, begin to decrease (Leonard *et al.*, 1999; Tomita *et al.*, 2014). Furthermore, growth rates also peak during this period of development (Rodda and Seymour, 2008). Despite the relative inefficiency of external gill filaments and their associated movements, without this activity embryos may be unable to ensure adequate oxygenation within the eggs (Leonard et al. 1999; Pelster and Beamis 1992). Therefore, the utilization of both internal and external gill structures may be necessary to meet increase oxygen uptake rates needed for rapid somatic growth (Rombough, 2011). As the growth rate begins to decrease and the external gill filaments are reabsorbed, oxygen uptake rates begin to rapidly decline in all treatments and populations. This could be a result of decreased tail movement and increased oxygen uptake efficiency (Leonard *et al.*, 1999; Pelster and Bemis, 1992; Tomita *et al.*, 2014); however, this could also reflect the non-linear allometric relationship between mass and metabolic rate (Bruno *et al.*, 2015; Sims 2000). That is, larger individuals often require less energy per unit of mass relative to smaller individuals. Therefore, over development and thus increasing mass, energy efficiency likely increases resulting in declining oxygen uptake rates (Bruno *et al.,* 2015). While temperature did not influence the overall

pattern of oxygen uptake rates across development, the relationship between temperature and location suggests local adaptation and/or acclimation processes.

Despite the influence elevated temperatures can have on the metabolism of marine organisms, Port Jackson sharks appear to show some resiliency to changes in temperature and perhaps local adaptation or thermal acclimation to these conditions. Embryos reared under their respective ambient conditions, display a similar pattern of oxygen uptake rates across development. Similarly, tropical reef fish found along the Great Barrier Reef show variable metabolic responses between northern and southern populations (Gardiner *et al.*, 2010). In two species, *Ostorhinchus doederleini* and *Pomacentrus moluccensis*, the aerobic scope of each population at their respective ambient temperatures were the same, suggesting that these species had adapted to thermal conditions at the local scale. Furthermore, Donelson *et al.* (2011) suggests that developmental acclimation can occur during the early stages of development. That is, fish reared under altered temperature conditions during early ontogeny may be able to compensate for the increased energetic demand through physiological or behavioural changes, here through reduced activity. However, acclimation can come at a cost, as energy is reallocated away from processes (i.e., growth, skeletal development, laterality) in order to sustain basic maintenance and developmental costs (Donelson *et al.*, 2011; Gervais *et al.*, 2018). In this study, embryos within each population appear to utilize acclimation as a means to mitigate the influence of temperature on their metabolic needs, but there appears to be a strong genetic influence on growth patterns. Increased temperatures and increased development speed may also affect other processes especially if energy is allocated away from developmental processes in favour of maintaining essential processes. While not significant, 19.4% of the sharks from Adelaide reared at elevated temperatures displayed visible developmental abnormalities. Fish and elasmobranchs reared under stressful conditions appear to have an increased risk of developing abnormalities (Gervais *et al.*, 2016; Hubbs, 1959; Pimentel *et al.*, 2014; Pimentel *et al.*, 2016). *Sparus aurata,* reared under elevated temperatures, were 2.57 times more likely to develop skeletal abnormalities (i.e., body malformations, and vertebral curvatures [scoliosis, lordosis, and kyphosis]) than those under control conditions, which was further compounded with increased pCO_2 levels (Pimentel *et al.*, 2016). While not outright lethal, abnormalities will most likely have knock-on effects throughout a sharks' life which in some cases may lead to decreased survival, for example through higher rates of predation or decreased foraging capacity (Afonso et al. 2016).

In summary, despite rearing conditions, Port Jackson embryos may have the capacity to mitigate the influence of temperature on their overall development and may exhibit a greater tolerance to developmental temperatures than expected. As temperatures continue to rise, marine organisms will experience progressively warmer conditions. While some species such as those that inhabit thermally stable conditions (i.e., tropical coral reef fish) are often negatively impacted by rapid environmental

change, species that are regularly exposed to a wide range of temperatures (e.g., coastal temperate species) may be less sensitive to environmental change (Pörtner and Farrell, 2008; Tewksbury *et al.*, 2008; Vinagre *et al.*, 2016). Despite the inherent vulnerabilities of development, embryonic Port Jackson sharks display some robust resilience to changes in developmental temperatures, particularly in terms of energetic costs. Species with the capacity to acclimate and mitigate the effect of warming temperatures will be far more likely to persist.

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Compliance with Ethical Standards Conflict of Interest:

No conflict of interests exists. All animal care and experimental protocols used in this study were approved by Macquarie University Animal Ethics Committee under ARA 2014-027 and conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

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Supplementary materials

Table S1: Mean values (± SEM) for developmental oxygen uptake rates ($\dot{M}O_{2Dev}$ [mg O_2 $kg^{-0.86}$ h^{-1}]) for each developmental stage are in shaded cells. Tukey post-hoc analyses between each stage are provided in non-shaded cells.

Stage	10	11	12	12.5	13	13.5	14	14.5	15
10	76.39±6.62								
11	p< 0.05 z= 3.17	92.21±4.74							
12	p< 0.001 z=4.87	p< 0.05 z=2.03	104.99±3.52						
12.5	p< 0.001 z= 4.22	p= 0.84 z= 1.53	p= 1.00 z= -0.25	101.85±4.19					
13	p= 0.69 z= 1.77	p= 0.54 z= -1.99	p< 0.001 z= -4.43	p< 0.05 z= -3.44	89.93±3.26				
13.5	p< 0.01 z= -3.58	p< 0.01 z= -8.70	p< 0.001 z= -11.46	p< 0.001 z= -9.44	p< 0.001 z= -7.81	61.96±1.93			
14	p< 0.001 z= -6.41	p< 0.001 z= -12.30	p< 0.001 z= -15.42	p< 0.001 z= -12.65	p< 0.001 z= -12.09	p< 0.01 z= -4.00	52.67±1.72		
14.5	p< 0.05 z= -3.51	p< 0.001 z= -8.37	p< 0.001 z= -11.01	p< 0.001 z= -9.24	p< 0.001 z= -7.48	p= 1.00 z= -0.01	p< 0.01 z= 3.83	63.78±2.83	
15	p= 0.93 z= -1.31	p< 0.001 z= -5.81	p< 0.001 z= -8.48	p< 0.001 z= -6.89	p< 0.001 z= -4.56	p< 0.05 z= 3.25	p< 0.001 z= 7.38	p< 0.05 z= 3.13	74.71±3.49

Table S2: Mean values (± SEM) for total length (mm) at each developmental stage are in shaded cells (Jervis Bay values on top; Adelaide values below). Tukey post-hoc analyses between each stage are provided in non-shaded cells.

Stage	10	11	12	12.5	13	13.5	14	14.5	15
10	JB: 72.89±13.08 AD: 70.57±3.01								
11	p< 0.001 z= 7.65	JB: 95.79±3.86 AD: 91.12±2.67							
12	p< 0.001 z= 19.52	p< 0.001 z= 14.17	JB: 132.99±6.44 AD: 131.59±2.83						
12.5	p< 0.001 z= 23.96	p< 0.001 z= 19.85	p< 0.001 z= 7.44	JB: 151.49±6.72 AD: 163.77±3.87					
13	p< 0.001 z= 34.19	p< 0.001 z= 32.47	p< 0.001 z= 18.09	p< 0.001 z= 7.64	JB: 187.98±3.68 AD: 180.23±2.59				
13.5	p< 0.001 z= 40.15	p< 0.001 z= 39.71	p< 0.001 z= 25.91	p< 0.001 z= 14.67	p< 0.001 z= 9.13	JB: 218.74±2.99 AD: 201.77±2.61			
14	p< 0.001 z= 44.41	p< 0.001 z= 44.99	p< 0.001 z= 31.19	p< 0.001 z= 19.06	p< 0.001 z= 14.84	p< 0.001 z= 5.35	JB: 239.04±2.32 AD: 214.49±2.14		
14.5	p< 0.001 z= 44.86	p< 0.001 z= 45.11	p< 0.001 z= 32.36	p< 0.001 z= 21.04	p< 0.001 z= 17.21	p< 0.001 z= 8.33	p< 0.05 z= 3.43	JB: 255.73±1.87 AD: 223.7±2.32	
15	p< 0.001 z= 48.31	p< 0.001 z= 50.09	p< 0.001 z= 36.55	p< 0.001 z= 23.75	p< 0.001 z= 20.74	p< 0.001 z= 3.25	p< 0.001 z= 5.66	p= 0.68 z= 1.79	JB: 227.29±2.58 AD: 261.89±2.5

Table S3: Mean values (± SEM) for total mass (g) (yolk inclusive) at each developmental stage are in shaded cells (Jervis Bay values on top; Adelaide values below). Tukey post-hoc analyses between each stage are provided in non-shaded cells.

Stage	10	11	12	12.5	13	13.5	14	14.5	15
10	JB: 35.48±0.81 AD: 38.75±3.25								
11	p= 0.68 z= 1.79	JB: 49.97±1.59 AD: 36.82±0.76							
12	p< 0.001 z= 8.33	p< 0.001 z= 7.95	JB: 56.23±1.34 AD: 43.07±0.73						
12.5	p< 0.001 z= 11.51	p< 0.001 z= 11.68	p< 0.001 z= 4.88	JB: 62.59±1.70 AD: 49.05±1.61					
13	p< 0.001 z= 20.01	p< 0.001 z= 22.99	p< 0.001 z= 15.51	p< 0.001 z= 7.76	JB: 69.13±1.62 AD: 53.28±0.84				
13.5	p< 0.001 z= 27.27	p< 0.001 z= 31.84	p< 0.001 z= 24.88	p< 0.001 z= 16.07	p< 0.001 z= 10.97	JB: 79.45±1.58 AD: 57.92±1.18			
14	p< 0.001 z= 30.87	p< 0.001 z= 36.04	p< 0.001 z= 29.70	p< 0.001 z= 19.84	p< 0.001 z= 16.15	p< 0.001 z= 4.81	JB: 83.24±1.32 AD: 59.34±1.21		
14.5	p< 0.001 z= 29.92	p< 0.001 z= 34.81	p< 0.001 z=28.50	p< 0.001 z= 19.49	p< 0.001 z= 15.37	p< 0.001 z= 4.55	p= 1.00 z= -0.09	JB: 83.27±1.37 AD: 62.86±1.08	
15	p< 0.001 z= 32.97	p< 0.001 z= 39.11	p< 0.001 z= 32.75	p< 0.001 z= 22.48	p< 0.001 z= 19.22	p< 0.001 z= 7.61	p= 0.13 z= 2.74	p= 0.14 z= 2.73	JB: 83.93±1.99 AD: 65.41±1.61

Table S4: Mean values (± SEM) for embryo mass (g) (yolk exclusive) at each developmental stage are in shaded cells (Jervis Bay values on top; Adelaide values below). Tukey post-hoc analyses between each stage are provided in non-shaded cells.

Stage	10	11	12	12.5	13	13.5	14	14.5	15
10	JB: 4.43±2.65 AD: 4.81±0.69								
11	p< 0.001 z= 5.27	JB: 14.88±1.48 AD: 7.76±0.72							
12	p< 0.001 z= 13.10	p< 0.001 z= 9.40	JB: 21.19±1.36 AD: 16.77±0.94						
12.5	p< 0.001 z= 15.51	p< 0.001 z= 12.56	p< 0.001 z= 4.51	JB: 25.27±1.96 AD: 23.69±1.54					
13	p< 0.001 z= 27.03	p< 0.001 z= 26.99	p< 0.001 z= 18.15	p< 0.001 z= 10.39	JB: 42.04±2.27 AD: 36.26±1.11				
13.5	p< 0.001 z= 37.89	p< 0.001 z= 40.50	p< 0.001 z= 32.52	p< 0.001 z= 22.87	p< 0.001 z= 16.40	JB: 82.27±1.46 AD: 51.71±1.35			
14	p< 0.001 z= 42.50	p< 0.001 z= 45.90	p< 0.001 z= 38.64	p< 0.001 z= 27.74	p< 0.001 z= 23.09	p< 0.001 z= 6.32	JB: 83.24±1.32 AD: 59.16±1.22		
14.5	p< 0.001 z= 42.14	p< 0.001 z= 45.44	p< 0.001 z=38.26	p< 0.001 z= 28.21	p< 0.001 z= 23.22	p< 0.001 z= 7.12	p= 0.98 z= 1.04	JB: 83.27±1.37 AD: 62.85±1.08	
15	p< 0.001 z= 44.14	p< 0.001 z= 48.45	p< 0.001 z= 41.08	p< 0.001 z= 29.90	p< 0.001 z= 25.45	p< 0.001 z= 8.30	p= 0.63 z= 1.86	p= 0.99 z= 0.73	JB: 83.40±2.02 AD: 65.41±1.61

Chapter 4: Phenotypic plasticity or local adaptation: Thermal and spatial effects on the physiology and behaviour of an oviparous elasmobranch, *Heterodontus portusjacksoni*

Phenotypic plasticity or local adaptation: Thermal and spatial effects on the physiology and behaviour of an oviparous elasmobranch, *Heterodontus portusjacksoni*

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Abstract

The rapid rate of increasing sea surface temperatures is likely driving many species towards physiological or behavioural strategies to mitigate the effects of elevated temperatures. Given the large distributions that some species display, exposure to different thermal regimes over time may give rise to population-specific responses to climate change, either through adaptation, acclimation processes, or changes in behaviour. This may be particularly important across early developmental stages, during which small nursery areas can be used for prolonged periods of time, resulting in limited capacity to move and thermoregulate. Here, we reared juvenile Port Jackson sharks, *Heterodontus portusjacksonii*, from two distant Australian populations (Jervis Bay, New South Wales and Adelaide, South Australia) under different rearing temperatures (present day, predicted end-of-century, and a reciprocal cross) to investigate population responses to temperature and the adaptation or acclimation capacity of each population across different physiological and behavioural traits, i.e. oxygen uptake rates, upper thermal limits and swimming activity of juveniles. Rearing temperatures influenced each population differently, but shark upper thermal limits were 10–14°C above their treatment temperature across all temperature and population treatments. Elevated temperatures did not significantly influence the resting oxygen uptake of Jervis Bay sharks; however, sharks reared under elevated temperatures displayed reduced swimming velocity and swimming distance. In contrast, Adelaide sharks displayed a two-fold increase in oxygen uptake rates when exposed to elevated temperatures yet maintained swimming performance. When sharks from different populations were reared under similar temperatures (both at 17.6°C and 20.6°C) sharks displayed similar responses to temperature, indicating that acclimation processes may be responsible rather than local adaptation. Here, thermal exposure to different temperatures promoted acclimation processes. However, the thermal exposure these populations currently experience may incur different energetic costs and populations may therefore display different trade-offs between energetic costs and phenotypic traits. Broadly distributed species are likely to be exposed to varied environmental conditions, including temperatures. Understanding the physiological and behavioural costs associated with warm conditions may elucidate the strategies that potentially sensitive populations (or life-history stages) may use to cope with future climate change.

Keywords: Climate change, Population variability, Respirometry, Thermal limits, Swimming, Acclimation

Introduction

Temperature is one of the most ubiquitous drivers of ectothermic organism's behaviour and physiology (Angilletta, 2009). Most species try to reside within a preferred thermal regime and exposure to conditions outside of their normal range (both warmer and cooler conditions) can negatively impact behavioural and physiological traits such as activity patterns, metabolic rate, and somatic growth (Payne *et al.*, 2016). Global temperatures are increasing and as such, species are continually exposed to progressively warmer temperatures (Collins *et al.*, 2013). Global sea surface temperatures are predicted to increase by 2.6–4 .8°C by the end of the century (Collins *et al.*, 2013), with some warming hot spots, such as waters off the south-east coast of Australia, predicted to warm up to four times faster than global averages (Hobday and Pecl, 2014). This may be particularly detrimental to marine ectotherms, which are thought to be at greatest risk because their body temperatures reflect the external environmental conditions (Angilletta, 2009). As species reach the limits of their thermal niche and the performance of many physiological and behavioural decline, species will have to respond in some way to either maintain their distribution, redistribute, or perish (Habary *et al.*, 2017).

Traits such as metabolic rate, aerobic scope, and activity are regulated by temperature, with performance of each trait peaking at an optimal temperature(s) (T_{opt}) (Pörtner and Farrell, 2008) and declining on either side of this point. Performance declines until critical points are reached such that an organism loses equilibrium or response to external stimuli (critical upper/lower thermal limits), beyond which the organism perishes (lethal temperature). This response curve is referred to as a species' thermal reaction norm (Pörtner and Farrell, 2008) and is generally skewed to the left. The relative width of a species thermal window is reflective of its thermal evolutionary history (Tewksbury *et al.*, 2008). For example, tropical species have evolved a very narrow window as their ambient conditions (daily and seasonally) are relatively stable, while temperate and intertidal species are regularly exposed to a wider range of conditions—often brought about by tidal or seasonal change—have a much wider thermal window (Angilletta, 2009; Pörtner and Farrell, 2008; Tewksbury *et al.*, 2008). Furthermore, as temperatures change away from an organisms thermal optimum, organisms can increase their activity and swimming speed proportionally to the change in their metabolic rate, in an effort to return to more optimal conditions (Bryan *et al.*, 1990). Even within species, populations may respond differently if regularly exposed to different conditions. For example, coho salmon fry (*Onocorhynchus kisutch*) from cooler streams have lower upper thermal limit than populations from warmer streams (Konecki *et al.*, 1995).

Overall, species rely on adaptation, acclimation, and the use behavioural strategies to reduce the costs of living under elevated temperatures. On a long-time scale, species will adapt over many generations to new conditions as species (or population) traits are slowly selected for as a result of

abiotic or biotic pressures driving selection (Angilletta, 2009). Many long-lived species or species with relatively long generation times may not be able to adapt fast enough to match changing climate (Angilletta, 2009; Crozier and Hutchings, 2014; Pörtner and Farrell, 2008). Therefore, many species may need to use acclimation processes as conditions change. These processes are typically non-genetic changes (e.g., utilization of different enzyme classes) that occur over shorter time scales (i.e., hours, days, weeks), but which can also exhibit across generations (Angilletta, 2009; Donelson *et al.*, 2011b; Woods and Harrison, 2002). For example, the upper thermal limits of many fish are highly plastic and reflect a species' environmental conditions; increasing when fish are acclimated to warmer conditions and decreasing when acclimated to cooler temperatures (Angilletta, 2009). Similarly, developmental acclimation plays an important role in the physiological performance of adults in some fish species with rearing conditions during early ontogeny driving how individuals respond to environmental change throughout their life (Donelson *et al.*, 2011a; Donelson *et al.*, 2011b). However, acclimation processes are thought to incur an energetic cost, that is, while these strategies may allow individuals to reside in sub-optimal conditions, there may be trade-offs at the expense of other performance traits (e.g., growth)(Gervais *et al.*, 2018). Therefore, many species, especially fish and sharks, are thought to use behavioural strategies (specifically movement), to mitigate any impacts imposed by temperature (Habary *et al.*, 2017; Nay *et al.*, 2015). Understanding the impacts of temperature on species and the use of these strategies is important in predicting the future health, distributions, and coping mechanisms of species under future climate change scenarios.

While thermal tolerance, specifically a species' thermal limits, is widely studied in a wide range of fish species, it has not been commonly explored in elasmobranchs due to logistical challenges of working with large animals and the long generation time of many species of this group. Port Jackson sharks (*Heterodontus portusjacksoni*), however, are relatively small sharks that can easily be handled and kept in captivity. The species is widely distributed throughout temperate Australia from Western Australia to New South Wales and is oviparous, laying eggs which can take up to 11 months to hatch (Rodda and Seymour, 2008). These eggs experience a broad range of temperatures between seasons and across the species distribution. During embryonic development, embryos are unable to change locations or actively regulate their body temperatures. While juvenile Port Jackson sharks may be able to behaviourally thermoregulate, they remain within nursery areas close to their oviposition sites (McLaughlin and O'Gower, 1971; Powter and Gladstone, 2009) during which they are exposed to summer and winter conditions and thus may still require a wide thermal window to withstand seasonal changes. This species is therefore an ideal model species to investigate the impacts between temperature and interspecific population differences.

As the climate changes, species will likely need to rely on physiological and behavioural strategies to persist under warmer temperatures. These strategies and the energetic trade-off which occur may differ from one population to the next, likely a reflection of local adaptation and acclimation processes (Deutsch *et al.*, 2008; Tewksbury *et al.*, 2008). Here, we were interested in differences between two populations of Port Jackson sharks originating from oviposition sites which experience different thermal regimes (Jervis Bay, New South Wales [NSW] and Adelaide, South Australia [SA]). Specifically, we determine the effects of different temperature regimes on the performance, upper thermal tolerance, and activity patterns of juvenile Port Jackson sharks. Firstly, we established how each population responds to elevated temperatures, specifically how they respond to predicted end-of-century temperatures. Secondly, we investigated the potential for localized adaptations or population plasticity. That is, through a common garden experiment, populations were reared under similar temperatures to investigate inherent differences and trait plasticity through acclimation. Upon hatching, three traits were measured, oxygen uptake rate (physiological trait), upper thermal limit (physiological trait), and swimming activity with acute temperature exposure (behavioural trait). Intermittent flow respirometry was used to measure resting and maximum oxygen uptake rates to provide proxies of metabolic rate as well as the relative scope available for activity. The upper thermal limits of a species readily acclimate based on thermal exposure and can provide ecologically relevant thresholds influencing species behaviour (temperatures which instigate activity) and physiology (loss of equilibrium). Furthermore, swimming is essential for juvenile sharks given they are small and prone to increased predation. However, swimming performance can be limited and may decline in populations reared under warmer conditions as energy is re-allocated towards maintaining basic function. Therefore, swimming performance metrics, such as maximum swimming speed and swimming distance, were measured exposing juveniles to progressively warmer temperatures, which is thought to induce increased activity as individuals seek out more optimal condition to mitigate increasing metabolic rates (Bryan *et al.*, 1990).

Methods

Animal collection and husbandry

Port Jackson shark eggs were collected on snorkel from rocky reefs along the southern side of Jervis Bay, NSW (-35.068°S, 150.684°E) (n=48, 157.36mm ± 1.42; average egg length ± S.E.M) in October and November 2016 and from Christies reef, Adelaide, SA (35.141°S, 138.465°E) (n=29, 135.57mm ± 1.31; average egg length ± S.E.M) in November 2016. Based on egg colour and pliability, eggs were estimated to be no older than six weeks post-oviposition at the time of collection (Rodda and Seymour, 2008). Eggs were transported to the aquarium facilities at Macquarie University in plastic bags filled with seawater and the void filled with pure oxygen. No mortality occurred during transit and all eggs were in

transit no longer than five hours. Eggs were held in 40 l aquaria (up to nine per aquaria) containing aerated, naturally filtered seawater at collection temperatures for one week before slowly transitioning eggs to treatment temperatures at a rate of ±0.5°C day^{-1}. Eggs from each location were randomly assigned to one of three developmental temperatures.

Eggs collected from Jervis Bay (n=16) were reared under 20.6°C (Bureau of Meteorology, 2016), while those from Adelaide (n=14) were reared at 17.6°C (Gaylard, 2004). Eggs reared under elevated temperatures were held under temperatures +3.0°C from collection site's average ambient conditions to simulate predicted end-of-century conditions. Jervis Bay eggs (n=17) were reared under 23.6°C and Adelaide eggs (n= 16) were reared under 20.6°C. Finally, to control for temperature effects between populations, eggs from both populations were exposed to ambient conditions of the alternate collection site. This treatment allowed direct comparison between populations, to observe if temperature drives differences between sharks or if there are innate differences between populations. Eggs from Jervis Bay (n=15) were reared under ambient Adelaide conditions, 17.6°C. As Adelaide eggs reared under predicted end-of-century conditions (20.6°C) mimicked that of ambient Jervis Bay temperatures, this treatment was utilized for cross-population analysis.

Holding-tank temperatures were controlled using an automated mixing system (Simex multicon, CMC99) which mixed water from a warm-water and cold-water sump to achieve treatment temperatures prior to entering the aquaria. Embryos were removed from their egg capsule (developmental stage 10; Rodda and Seymour, 2008) and placed into individual 160 mL containers to continuously monitor embryo development until hatching. Hatching was identified by the loss of mucous coating around the shark and active movement. Upon hatching, sharks were then distributed among four aquaria (40 l each) per treatment temperature (no more than five sharks per aquaria) for remainder of experimental trials.

Respirometry and upper critical thermal experiments began within two weeks of hatching (Jervis Bay: 23.6°C n= 11, 20.6°C n= 16, 17.6°C n=15; Adelaide: 20.6°C n=16, 17.6°C n=14) and were given no less than one-week recovery prior to each experiment. Following upper critical thermal experiments, sharks (n=4, Jervis bay sharks reared at 23.6°C, and n=8, all other treatments) were transferred to SIMS (Sydney Institute of Marine Science) for activity experiments. The different sample size for Jervis Bay sharks reared at 23.6°C was due to mortality events. At SIMS, sharks were held in flow-through aquaria, supplied with seawater heated or cooled to treatment temperatures using a 2000 W titanium stick heater [Full Gauge TIC-17RGT thermostat] or a chiller [TECO, TK3000]) for at least 12 h before introduction to experimental setup. Juveniles were fasted for 48 h prior to all experimentation to ensure a post-absorptive state (Heinrich *et al.*, 2014; Niimi and Beamish, 1974).

Eggs from Jervis Bay, NSW were collected under NSW fisheries permit P08/0010-4.2. No permits were required for collection in South Australia. Animal husbandry and experimental protocols were approved by the Macquarie University Animal Ethics Committee (ARA 2016-027).

Oxygen uptake rate

As a proxy for metabolic rate, oxygen uptake rate ($\dot{M}O_2$) was measured using intermediate-flow respirometry (Svendsen *et al.*, 2016). The system comprised individual 1.95 l chambers placed in a temperature-controlled bath (66 l) and connected to a recirculating pump and a flush pump (Svendsen *et al.*, 2016). The recirculating pump ensured that there was continuous water flow within the chamber and to maintain homogeneous oxygen levels. The flush pump was used to ensure a complete water exchange in the system and that oxygen concentration in the chamber did not fall below 80% air saturation during measurement periods (i.e., period when the flush pump is off) (Clark *et al.*, 2013; Nay *et al.*, 2018; Svendsen *et al.*, 2016). A digital relay timer controlled the flush pump so that every five minutes the chambers were flushed with aerated, filtered seawater for five minutes, then was turned off for five minutes to allow for the recording of oxygen concentrations. In-line with the recirculating pump, temperature-compensated oxygen concentration was measured every 2 s using contactless spots (2mm) with O_2-sensitive REDFLASH dye. The spots were linked, via 2 m fibre-optic cables, to a Firesting Optical Oxygen Meter (Pyro Science e.K., Aachen, Germany).

Prior to placing sharks into individual chambers, sharks were chased for 3-min, followed by a 1-min air exposure to determine maximum oxygen uptake rates ($\dot{M}O_{2Max}$) (Clark *et al.*, 2012; Clark *et al.*, 2013; Roche *et al.*, 2013; Rummer *et al.*, 2016). Within 10 seconds following air exposure, sharks were placed into respirometry chambers to begin the measurement period. Sharks were maintained in chambers for 18 hr, after which they were removed from the chamber. Background oxygen uptake rates were measured before and after all trials and were assumed to have a linear accumulation (Clark *et al.*, 2013). Background oxygen uptake rates were subtracted from each measurement period. Chambers were rinsed between trials with a 10% bleach solution, rinsed with freshwater and sundried to minimize microbial background oxygen uptake.

Oxygen uptake rates were calculated using linear least square regression of oxygen concentration over time in Labchart v8.1.9 (AD Instruments, Colorado Springs, CO, USA). Maximum oxygen uptake rates ($\dot{M}O_{2Max}$) were calculated as the highest rate of change over 30 s during the measurement periods immediately following the placement of the individual into the chamber. This method was deemed appropriate as R^2 values during these periods were above 0.95 (Svendsen *et al.*, 2016). Resting oxygen uptake rates ($\dot{M}O_{2Rest}$) were calculated using the 'mean of the leftmost normal

distribution' method (MLND) (Chabot *et al.*, 2016). Aerobic scope (AS), or the physiological scope for aerobic performance, was determined as the difference between $\dot{M}O_{2Max}$ and $\dot{M}O_{2Rest}$.

Critical thermal limits

Upper critical thermal limits (CT_{max}) were examined following established, non-lethal protocols (Becker and Genoway, 1979; Gervais *et al.*, 2018; Paladino *et al.*, 1980). The experimental tank (245 l) contained four meshed experimental chambers (25 l) and a 2000 W heater (Full Gauge TIC-17RGT thermostat). An air stone and aquarium pump (2500 l h^{-1}) were used to ensure that temperatures throughout the tanks were homogeneous and that the tanks were fully oxygenated. Each experimental chamber was comprised of a rigid, dense mesh, which allowed continuous water flow and eliminated visual cues between experimental chambers. A Firesting Optical Oxygen Meter continuously recorded temperature throughout each trial.

Prior to each trial, the experimental tank was filled with aerated, filtered seawater and set to treatment temperature. Individual sharks were placed into a chamber and allowed to habituate (i.e., cease swimming movements) for 30 minutes. Following this period, the temperature of the system was set to increase at a constant rate of 0.13±0.001°C min^{-1} (calculated using Stevens and Sutterlin, 1976) to ensure that body temperature was allowed time to match that of the environment. Throughout the trial, activity pattern (i.e., swimming or resting) was recorded to establish the temperature at which a shark first became active ($T_{Activity}$). Once swimming activity ceased, sharks were checked for a loss of righting reflex (LRR) by rotating the shark 180° along the longitudinal axis. The temperature at which juveniles exhibited LRR was defined as the upper critical thermal maximum (CT_{max}). Immediately following LRR, the shark was removed from the experimental chamber, placed into an aerated recovery tank—filled with an intermediate temperature (made using equal parts rearing temperature and temperature CT_{max}) temperatures, and allowed to fully recover their righting reflex before transitioning back to treatment temperature.

Activity with step-wise increases in temperature

The experimental arena consisted of a 1.2 m wide, circular tank filled with 25 cm of water (283 l). In the centre of the arena, a 0.25 m wide central PVC cylinder (perforated with holes to allow homogeneous water mixing) housed all heating and water flow components. A recirculating pump (1300 l h^{-1}) was placed inside the central cylinder to ensure homogeneous temperatures and an air stone. The central cylinder also housed a 2000 W heater and an aquarium pump (2500 l h^{-1}). The aquarium pump was placed at the base of the central cylinder with a PVC tube (Ø 25 cm) placed on the outflow to direct water movement to the top of the arena. The heater was placed inside the outflow PVC to allow for temperature manipulation and to ensure homogeneous temperature changes. Power cables and airline

tubing were concealed within a grey cable cover flush to the ground to reduce shark interaction. Strip lighting was fixed around the perimeter of the tank which illuminated the arena evenly. A USB webcam (Microsoft LifeCam HD-3000) was fixed 2 m directly above the tank to record videos (via iSpy connect, www.ispyconnect.com).

The experimental tank was filled with 13°C water, which are within normal winter temperatures of both regions. Sharks were allowed to habituate to the experimental tank and temperature for two hours. Following habituation, sharks were recorded for 10 min following which, the temperature of the tank was increased 2°C over 20 mins until the next temperature was reached. Following another 10 min recording, the temperature was again increased continuing in a step-wise fashion until 31°C (prior to established limits for LRR) was reached.

Videos were imported into LoggerPro (v 3.8.7) for analysis where the x-y coordinate of each shark was recorded every second over the duration of each 10 min step. At each temperature, the total distance travelled (body lengths [BL]) and average swimming speed (BL s^{-1}) were measured to identify the maximum distances swam, maximum swimming velocity, and temperature(s) at which the sharks moved the furthest (T_{MaxD}) or fastest (T_{MaxV}) prior to performance declining. Movement metrics were measured as BL to standardize for size between treatments. Trials were conducted, at most, twice a day, during daylight hours, and the last trial ended no later than two hours before dusk to control for any diel activity patterns.

Analysis

We used a single factor linear models (LM) were used to assess the effect of treatment on nine dependant variables: $\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$, aerobic scope (AS), $T_{activity}$, CT_{Max}, T_{MaxD}, T_{MaxV}, distance travelled, and maximum swimming velocity. All analyses were performed in R (Version 3.4.1, R Core Development Team 2013), all data meet assumptions, and tukey post-hoc tests were used for all analyses. All values reported as mean ± standard error measure (SEM).

Results

Oxygen uptake rate

Adelaide sharks reared at elevated temperatures displayed more than double the $\dot{M}O_{2Rest}$ (82.75±5.22 mg O_2 kg^{-1} h^{-1}) values than Adelaide sharks reared at ambient temperatures (39.98±2.87 mg O_2 kg^{-1} h^{-1}) (Fig. 1; supplementary materials, Table S1, LM, t= 6.09, p< 0.001). Conversely sharks from Jervis Bay exhibited similar $\dot{M}O_{2Rest}$ values regardless of rearing temperature (ambient 71.60±4.75mg O_2 kg h^{-1}; elevated temperature 81.76±6.20mg O_2 kg^{-1} h^{-1}) (LM, t= 1.36, p= 0.66). $\dot{M}O_{2Max}$ and aerobic scope were similar between rearing temperatures in Adelaide sharks (supplementary materials, Table S2, S3);

however, Jervis Bay sharks reared under elevated temperatures displayed greater $\dot{M}O_{2Max}$ values and aerobic scope than sharks reared under ambient conditions ($\dot{M}O_{2Max}$, LM, t= 4.38, p<0.001; AS, t= 1.21, p<0.01).

Jervis Bay sharks reared under Adelaide ambient temperatures [17.6°C] exhibited similar $\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$, and aerobic scope values to that of Adelaide sharks under similar temperatures ($\dot{M}O_{2Rest}$, LM, t= 2.66, p= 0.07; $\dot{M}O_{2Max}$, LM, t= -0.95, p= 0.87; AS, t= -1.99, p= 0.28). Adelaide sharks and Jervis Bay sharks reared under Jervis Bay temperatures (20.6°C) displayed similar $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$ and aerobic scope values ($\dot{M}O_{2Rest}$, LM, t= -1.69, p= 0.45; $\dot{M}O_{2Max}$, LM, t= -0.45, p= 0.99; AS, LM, t= 0.21, p= 0.99).

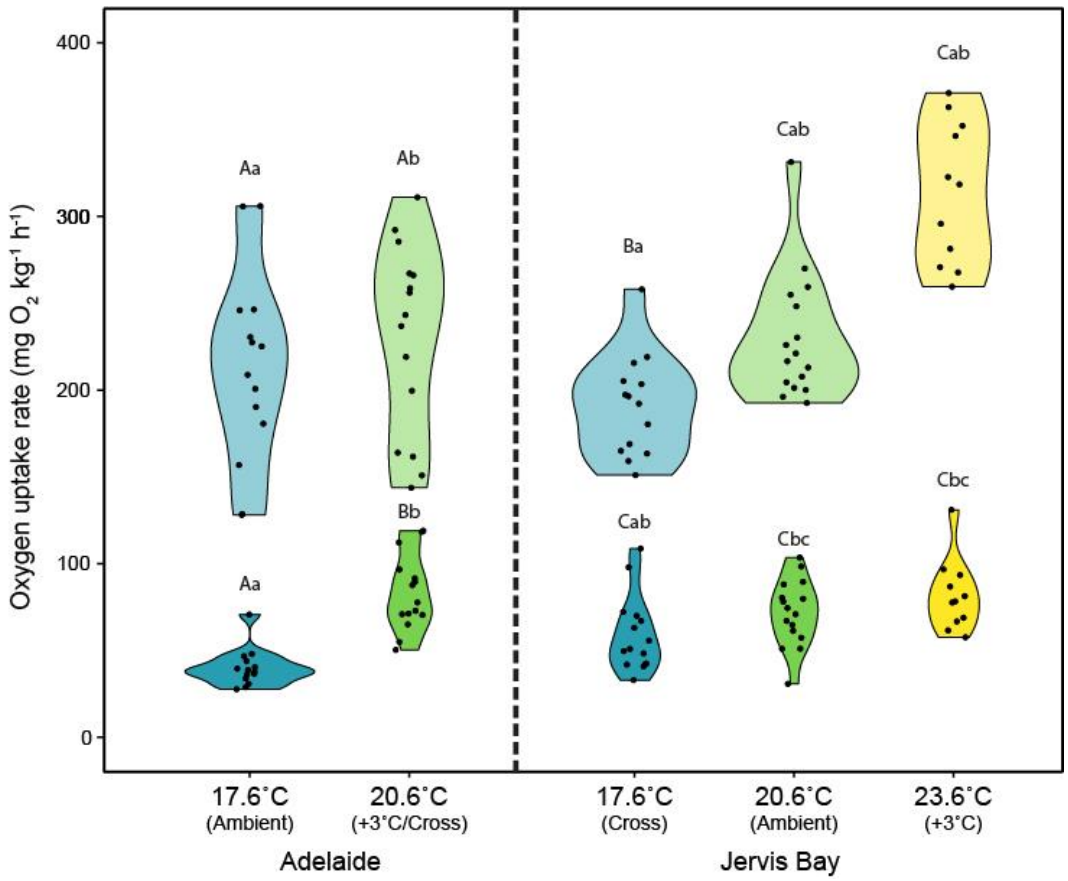

Figure 1: Violin plots depicts the maximum oxygen uptake rate (mg O_2 kg^{-1} h^{-1}) (top-shaded plots) and resting oxygen uptake rate (mg O_2 kg^{-1} h^{-1}) (solid-bottom plots) of juvenile Port Jackson sharks. Adelaide shark treatments (ambient [17.6°C; n=14] and temperatures +3°C above ambient [20.6°C; n= 15]) are displayed to the left of dashed line and Jervis Bay shark treatments (Adelaide ambient temperatures [17.6°C; n= 15], ambient [21.6°C; n=16], and temperatures +3°C above ambient [23.6°C; n= 11]) are to right of dashed lines). Significant differences within populations are denoted by a capital letters while between population differences are denoted by lower case letters.

Critical thermal limits

When reared at elevated temperatures, Adelaide sharks became active at warmer temperatures (23.96±0.61°C) than those reared at ambient conditions (21.10±0.89°C) (Fig. 2; supplementary materials, Table S4, LM, t= 2.83, p< 0.05). However, sharks from Jervis Bay became active at similar temperatures (24.69–25.01°C) regardless of rearing condition (LM, t= 0.31, p=0.99). Within populations, the upper critical thermal limit (CT_{Max}) of juvenile sharks reared at elevated temperatures (Jervis Bay, 34.97±0.09°C; Adelaide, 32.68±0.11°C) was higher than those reared under their respective ambient conditions (Jervis Bay, 33.39±0.11°C; Adelaide, 31.57±0.12°C) (Fig. 2; supplementary materials, Table S5, LM, Jervis Bay, t= 8.10, p< 0.001; Adelaide, t= 5.79, p< 0.001).

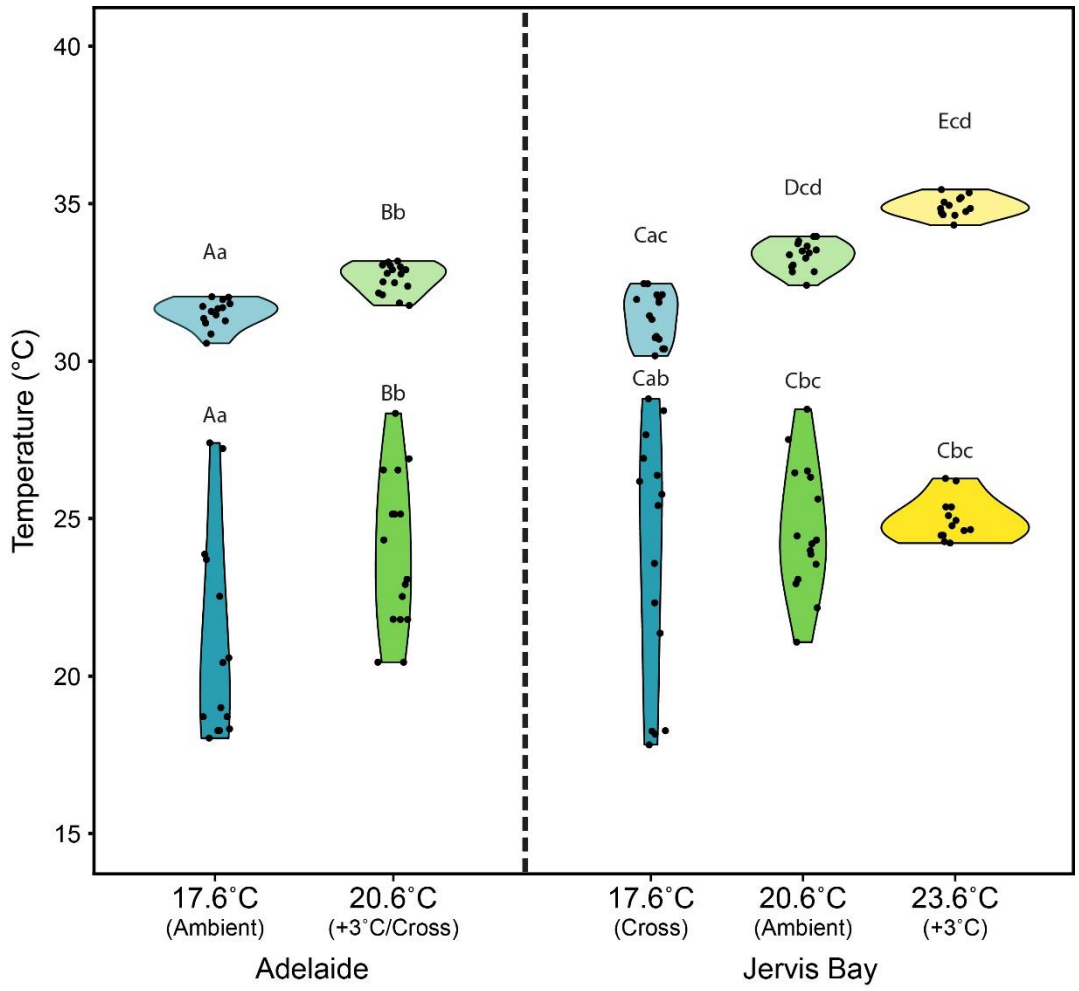

Figure 2: Violin plots depicting the upper critical thermal limits (top-shaded plots) and temperature at which sharks began to swim (solid-bottom plots) of juvenile Port Jackson sharks. Adelaide shark treatments (ambient [17.6°C; n=14] and temperatures +3°C above ambient [20.6°C; n= 15]) are displayed to the left of dashed line and Jervis Bay shark treatments (Adelaide ambient temperatures [17.6°C; n= 15], ambient [20.6°C; n=16], and temperatures +3°C above ambient [23.6°C; n= 17]) are to right of dashed lines). Significant differences within populations are denoted by a capital letters while between population differences are denoted by lower case letters.

Jervis Bay sharks reared under Adelaide conditions became active at similar temperatures to Adelaide sharks under the same temperatures (LM, t= 2.55, p= 0.09), and similarly, Adelaide sharks reared under Jervis Bay conditions became active at similar temperatures to Jervis Bay sharks under the same conditions (LM, t= 0.75, p= 0.94). Sharks from Jervis Bay reared under Adelaide ambient conditions (17.6°C) had similar a CT_{Max} (31.45±0.21°C) to Adelaide sharks reared under ambient conditions (31.57±0.12°C) (Fig. 2, LM, t= -0.65, p= 0.97), but the CT_{Max} of Adelaide sharks reared under Jervis Bay ambient temperatures (32.68±0.11°C) was lower than that of Jervis Bay sharks reared under similar temperatures (33.39±0.11°C) (LM, t= 3.83, p< 0.01).

Activity with step-wise increases in temperature

The greatest velocity of sharks from Jervis Bay reared under elevated temperatures was slower (0.51±0.11 BL s^{-1}) than those under ambient conditions (0.85±0.04 BL s^{-1}) (Fig. 3, LM, t= -3.23, p< 0.05); however, the temperature at which they swam fastest was between 28.25–31.00°C and was similar between rearing temperatures (Fig. 4, LM, t= 1.97, p= 0.30). Furthermore, Jervis Bay sharks reared at elevated temperatures swam less in 10 min (227.04±57.44 BL) than those reared under ambient conditions (489.61±25.11 BL) (Fig. 3, LM, t= -3.56, p< 0.01), but the temperature at which they swam the furthest was similar (27.75-30.50°C; t= 2.45, p= 0.13). Within Adelaide sharks, their fastest average swimming velocity and maximum distance swam were similar between rearing temperatures (LM, velocity, t= 1.78, p= 0.40; maximum distance, t= 0.58, p= 0.97), but those reared at elevated temperatures were fastest at a greater temperature (30±0.65°C) and moved further at a greater temperature (29.5±0.63°C) than those reared under ambient conditions (Fig. 4, T_{MaxV}, 26±0.65°C, LM, t= 4.41, p<0.01;T_{MaxD}, t= 3.55, p< 0.05).

Similarly, there we no differences in T_{MaxD}, T_{MaxV}, total distance swam, or maximum velocity between populations reared under Adelaide ambient temperature as well as between populations reared under Jervis Bay ambient temperatures (Fig. 4; supplementary materials, Table S6, S7, S8, S9)

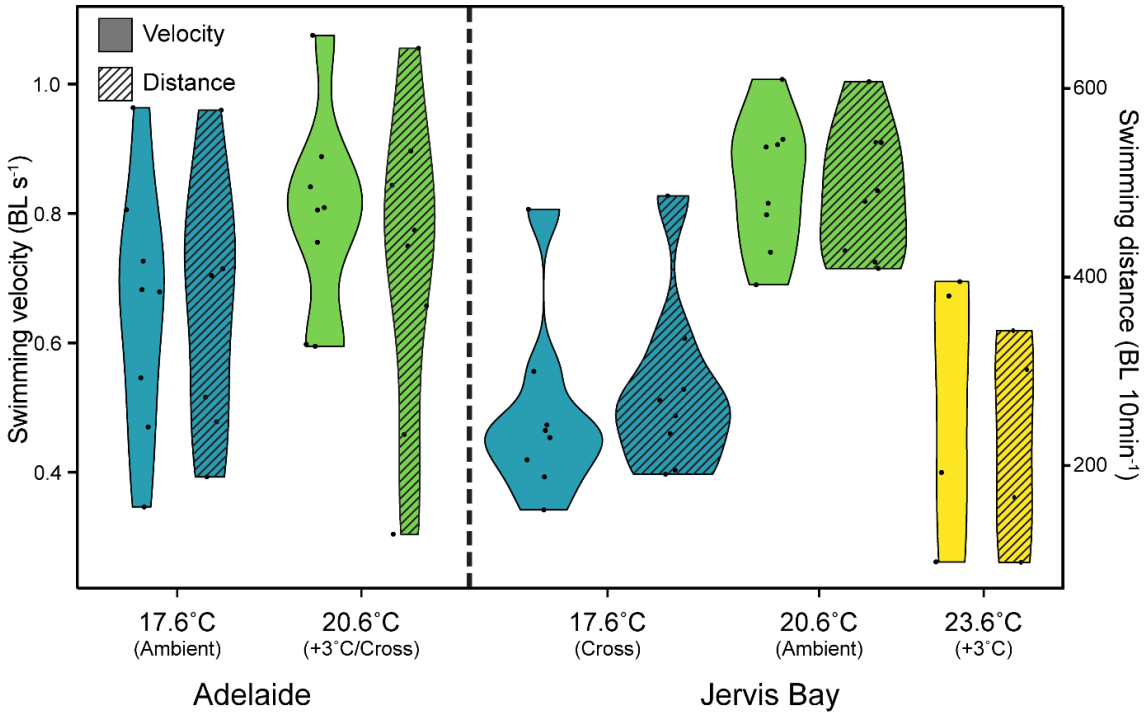

Figure 3: Maximum velocity (BL s^{-1}) (left y-axis, solid violin plots) and maximum distance (BL 10 min^{-1}) (right y-axis, striped violin plots) that Jervis Bay and Adelaide sharks reared under different temperatures, swam during exposure to acute temperature changes. Adelaide sharks are displayed to the left of the dashed line, while those collected from Jervis Bay are to the right.

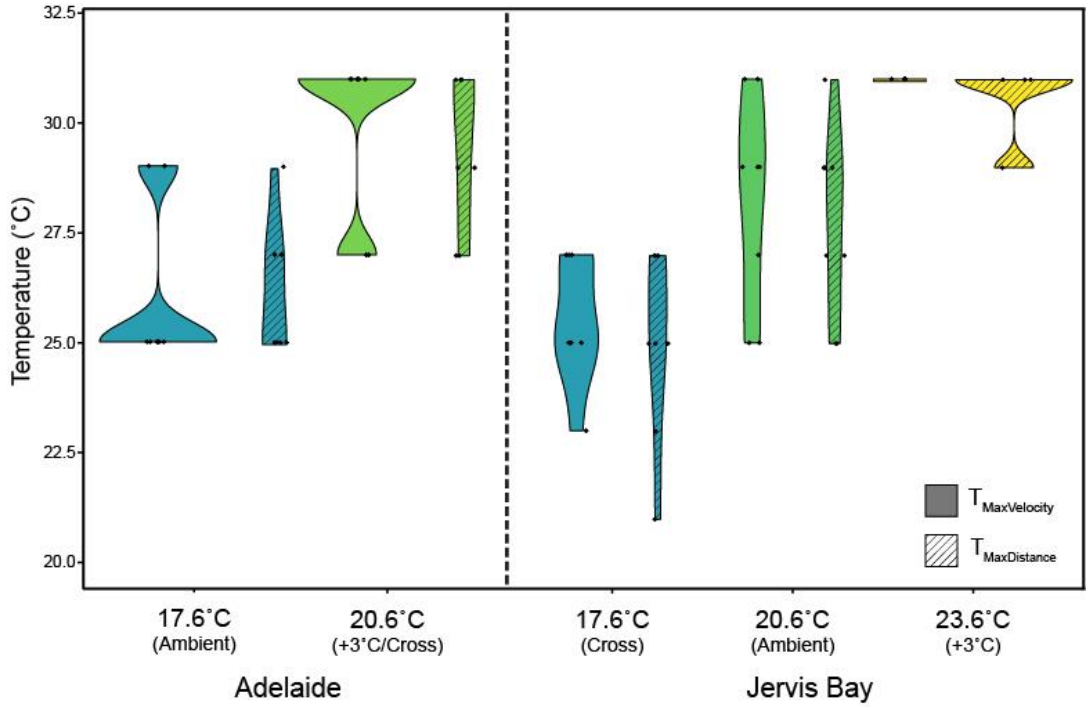

Figure 4: The temperatures at which sharks reared under different temperatures displayed the fastest velocity (solid violin plots) and furthest distance swam (striped violin plots). Adelaide sharks are displayed to the left of the dashed line, while those collected from Jervis Bay are to the right.

Discussion

Rearing temperatures significantly impacted the oxygen uptake rates, upper thermal limits, and swimming activity of juvenile sharks and elicited varied responses between the populations. Jervis Bay juveniles displayed greater maximum oxygen uptake rates and aerobic scope if reared under elevated temperatures and swam 40% slower and swam 53% less than those reared under ambient temperatures. However, Adelaide sharks displayed a different response to elevated temperatures. The resting oxygen uptake rates more than doubled if Adelaide sharks were reared under elevated temperatures while swimming performance was unchanged; juveniles displayed maximum swimming velocity and swimming distance around 4°C higher than those reared under ambient temperatures. As with many other studies, the upper thermal limits of this species are highly plastic and reflect rearing conditions, with elevated temperature correlating with greater upper thermal limits (Jervis Bay, 34.97°C; Adelaide, 32.68°C) relative to those reared under ambient conditions (Jervis Bay, 33.39°C; Adelaide, 31.57°C). However, if controlling for temperature effect, juveniles reared under similar conditions (at either 17.6°C or 20.6°C), readily displayed acclimation potential across most traits. That is, when reared at the same temperature, there were no differences in oxygen uptake, upper thermal limits, or swimming activity. However, while the potential to acclimate may be present, the current environmental conditions these populations are exposed to differ and thus populations at lower latitudes may be limited in their capacity to acclimate some traits (e.g., oxygen uptake rates) and thus may display declines in other traits, such as swimming performance. Temperature can drive many physiological and behavioural processes in ectothermic species, especially during early development, here Port Jackson shark populations readily display acclimation potential across several physiological and behavioural traits.

Temperate species, which experience large variations in thermal exposure on a daily and seasonal basis, tend to reside well below their thermal limits and have a relatively large thermal window before experiencing loss of equilibrium (Vinagre *et al.*, 2016). However, exposure to suboptimal, but not lethal, conditions may incite increased living costs and prompt activity (Bryan et al., 1990). While species can survive exposure to warmer water until loss of equilibrium (LOE), exposure to warming waters increases energetic costs. Therefore, species are thought to increase activity in an attempt to alleviate these costs by moving away from deleterious conditions and towards more forgiving temperatures (Bryan *et al.*, 1990). Here, Port Jackson shark became active only a few degrees above rearing temperature. With progressive temperature exposure, velocity and swimming distance increase until a point at which swimming performance declines, as the cost of activity exceeded the cost of basic maintenance. This could be a thermoregulatory response, as individuals increase activity to move away from areas which incur high metabolic costs (i.e., elevated temperatures or extremely cold

temperatures). Conversely, rather than mitigate these elevated costs, some species increase activity to try and ensure they encounter sufficient prey to meet demand. Populations did not display the capacity to acclimate across all traits, indicating that there may still be population-specific energetic trade-offs between elevated temperatures and physiological and behavioural traits.

Given the plasticity of this species in response to rearing temperatures, the energetic trade-offs between behavioural and physiological traits, will likely be reflective of their current thermal exposure. Jervis Bay sharks reside closer to their upper thermal range limit than Adelaide sharks. While elevated temperatures do not incur an increase in oxygen uptake rates in Jervis Bay sharks, sharks reared under elevated temperatures (or under cooler Adelaide temperature) displayed reduced swimming performance as sharks displayed reduced swimming speeds and swam less than those reared under ambient temperatures. Similar to other species, there may display a trade-off between maintaining baseline energetic costs and performance of non-essential processes. Other species, such as coral trout (*Plectropomus leopardus*), display similar trends of reduced swimming performance under elevated temperatures (Johansen and Jones, 2011; Johansen *et al.*, 2013). While increased activity should prompt movement away from deleterious conditions, decreased activity could also be used as an energy conservation strategy to mitigate energetic costs similar to that observed in cool acclimated fish (Speers-Roesch *et al.*, 2018). Conversely, Adelaide sharks may have a greater flexibility in the strategies they use, given their distance (temperature-wise) from lower latitude range limit. And while the cost of living at relatively warmer conditions was more than double ambient costs for this population, they were able to maintain swimming capacity. Populations closer to their thermal maximum (or minimum to a lesser extent) will incur greater energetic costs which will in turn influence organismal performance traits such as swimming performance, growth, and reproduction capacity. While several traits were examined in this study, there are a plethora of other physiological and behavioural traits which can change with exposure to elevated temperatures (Angilletta, 2009), causing knock-on effects to non-essential processes such as growth, digestion, and reproduction as energy is allocated towards more essential traits (Donelson *et al.*, 2010; Gervais *et al.*, 2018; Munday *et al.*, 2008; Rosa *et al.*, 2016).

Local environmental conditions can have a strong influence on species physiological and behavioural traits and the strategies used by local populations can offer insight into their response to future change (Di Santo, 2015). Adjacent populations will respond differently if conditions are dissimilar enough (Di Santo, 2015). However, some species may display significant capacity to acclimate traits to a range of rearing temperatures, regardless of population. Port Jackson sharks display population specific trade-offs in their response to predicted end of century temperatures, but when reared under similar temperatures sharks displayed no differences in measured traits between populations. There was no difference in the oxygen uptake rates between populations which may suggest the cost of living under these conditions is similar among populations, despite overall increasing costs associated with warmer

temperatures. However, there may be variation in the magnitude at which populations are affected. Adelaide sharks display a 107% increase in their $\dot{M}O_{2Rest}$ values between 17.6°C and 20.6°C, while Jervis Bay sharks only displayed a 23% increase over the same temperature range. Northern and Southern populations of Atlantic cod, (*Gadus morhua* L.) seem to display a similar response. Northern populations (cooler water population) appear to be more sensitive to changes in temperature than those from warmer populations (Grabowski *et al.*, 2009). This is thought to potentially reflect thermal variability between habitats. However, this pattern is atypical across ectotherms as most species display increasing metabolic costs with decreasing latitude and thus lower latitude populations (warmer populations) are often observed to be at greater risk from warming temperatures than high latitude populations (Angiletta, 2009).

In conclusion, many marine organisms have broad distributions, but studies often overlook the physiological differences which may exist across the species range (Grabowski *et al.*, 2009). If changes in environmental factors significantly alters any stage in life, populations may suffer as traits such as growth, swimming performance (and thus predation risk and foraging opportunity), and reproduction can be compromised (Donelson *et al.*, 2010; Johansen *et al.*, 2013). Despite the robust nature of Port Jackson sharks and their capacity to potentially acclimate regardless of populations, we have identified population specific trade-offs in response to predicted end-of-century temperatures. Populations residing closer to their thermal range experienced declines in potential swimming capacity, but the energetic requirements of those exposed to naturally cooler water were more impacted. The physiological and behavioural differences species display between populations revealed in this study can help us predict the susceptibility and response of different populations to changing environmental conditions. While Port Jackson sharks are not a vulnerable species, this study highlights that different populations of the same species can display different responses, both in magnitude and direction, to predicted future temperature conditions. Many of the impacts observed here can have knock-on effects such as decreased growth, limited reproductive output, and decreased ability to evade predators or find food. Long-lived species such as elasmobranchs will likely rely on a range of different strategies to respond to elevated temperature. Mobile species will simply move away from suboptimal temperatures, however those with limited capacity to move, such as juveniles and benthic species, will likely need to rely on species adaptations or acclimation processes to mitigate thermal influence.

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Compliance with Ethical Standards Conflict of Interest:

No conflict of interests exists. All animal care and experimental protocols used in this study were approved by Macquarie University Animal Ethics Committee under ARA 2016-027 and conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

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Supplementary materials

Table S1: Mean values (± SEM) for resting Oxygen uptake rates ($\dot{M}O_{2Rest}$ [mg O_2 kg^{-1} hr^{-1}]) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide Temperature (17.6°C)
Adelaide	Ambient (17.6°C)	39.98 ±2.87 N=14				
	Elevated Temperature (20.6°C)	p< 0.001 * t= 6.089.	82.75 ±5.22 N=16			
Jervis Bay	Ambient (20.6°C)	p< 0.001 * t= 4.520	p= 0.445 t= -1.693	71.60 ±4.75 N=16		
	Elevated Temperature (23.6°C)	p< 0.001 * t= 5.424	p= 0.999 t= -0.195	p= 0.657 t= 1.356	81.76 ±6.20 N=11	
	Adelaide temperature (17.6°C)	p= 0.072 t= 2.659	p< 0.01 * t= -3.491	p= 0.352 t= 1.854	p< 0.05 * t= 3.016	58.87 ±5.56 N=15

Table S2: Mean values (± SEM) for maximum uptake rates ($\dot{M}O_{2Max}$ [mg O_2 kg^{-1} hr^{-1}]) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	212.89±14.71 N=14				
	Elevated Temperature (20.6°C)	p= 0.659 t= 1.352	236.31±14.47 N=16			
Jervis Bay	Ambient (20.6°C)	p= 0.885 t= 0.927	p= 0.991 t= -0.454	229.48±9.06 N=16		
	Elevated Temperature (23.6°C)	p< 0.001 * t= 5.103	p< 0.01 * t= 3.914	p< 0.001 * t= 4.384	313.46±12.29 N=11	
	Adelaide temperature (17.6°C)	p= 0.874 t= -0.953	p= 0.143 t= -2.346	p= 0.312 t= 1.929	p< 0.001* t= 6.072	184.76±9.52 N=15

Table S3: Mean values (± SEM) for aerobic scope (AS [mg O_2 kg^{-1} hr^{-1}]) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	172.91 ±14.32 N=14				
	Elevated Temperature (20.6°C)	p= 0.841 t= -1.029	153.56 ±14.20 N=16			
Jervis Bay	Ambient (20.6°C)	p= 0.917 t= -0.847	p= 0.999 t= 0.208	157.87 ±9.44 N=16		
	Elevated Temperature (23.6°C)	p< 0.05 * t= 2.985	p< 0.01 * t= 3.994	p< 0.01 * t= 1.206	231.69 ±12.91 N=11	
	Adelaide temperature (17.6°C)	p= 0.279 t= -1.994	p= 0.862 t= -0.982	p= 0.748 t= 1.206	p< 0.01 * t= 4.897	125.89 ±11.56 N=15

Table S4: Mean values (± SEM) for temperature (°C) at which sharks instigated movement for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	21.10 ±0.89°C N=14				
	Elevated Temperature (20.6°C)	p< 0.05 * t= 2.831	23.96 ±0.61°C N=16			
Jervis Bay	Ambient (20.6°C)	p< 0.01 * t= 3.552	p= 0.944 t= 0.747	24.69 ±0.50°C N=16		
	Elevated Temperature (23.6°C)	p< 0.01 * t= 3.679	p= 0.845 t= 1.021	p= 0.998 t= 0.314	25.01 ±0.19°C N=11	
	Adelaide temperature (17.6°C)	p= 0.091 t= 2.552	p= 0.999 t= -0.244	p= 0.864 t= 0.979	p= 0.729 t= 1.237	23.72 ±1.04°C N=15

Table S5: Mean values (± SEM) for upper thermal limits (°C) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	31.57 ±0.12°C N=14				
	Elevated Temperature (20.6°C)	p< 0.001 * t= 5.791	32.68 ±0.11°C N=16			
Jervis Bay	Ambient (20.6°C)	p< 0.001 * t= 9.491	p< 0.01 * t= 3.830	33.39 ±0.11°C N=16		
	Elevated Temperature (23.6°C)	p< 0.001 * t= 16.873	p< 0.001 * t= 11.729	p< 0.001 * t= 8.102	34.97 ±0.09°C N=11	
	Adelaide temperature (17.6°C)	p= 0.967 t= -0.646	p< 0.001 * t= -6.565	p< 0.001 * t= 10.333	p< 0.001 * t= 17.784	31.45 ±0.21°C N=15

Table S6: Mean values (± SEM) for the temperature (°C) at which sharks travelled furthest for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	26.25 ±0.53°C N= 8				
	Elevated Temperature (20.6°C)	p< 0.05 * t= 3.549	29.5 ±0.63°C N= 8			
Jervis Bay	Ambient (20.6°C)	p= 0.483 t= 1.638	p= 0.330 t= -1.911	27.75 ±0.75°C N= 8		
	Elevated Temperature (23.6°C)	p< 0.01 * t= 3.789	p= 0.897 t= 0.892	p= 0.127 t= 2.452	30.5 ±0.5°C N= 4	
	Adelaide temperature (17.6°C)	p= 0.651 t= -1.365	P< 0.001 * t= -4.914	p< 0.05 * t= 3.003	p< 0.001 * t= 4.904	25 ±0.65°C N= 8

Table S7: Mean values (± SEM) for the temperature (°C) at which sharks displayed maximum velocity for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	26 ±0.65°C N=8				
	Elevated Temperature (20.6°C)	p< 0.01 * t= 4.406	30 ±0.65°C N=8			
Jervis Bay	Ambient (20.6°C)	p= 0.072 t= 2.729	p= 0.471 t= -1.657	28.25 ±0.84°C N=8		
	Elevated Temperature (23.6°C)	p< 0.01 * t= 4.187	p= 0.962 t= 0.666	p= 0.303 t= 1.967	31 ±0°C N=4	
	Adelaide temperature (17.6°C)	p= 0.977 t= -0.573	P< 0.001 * t= -4.976	p< 0.05 * t= 3.300	P< 0.001 * t= 4.644	25.5 ±0.5°C N=8

Table S8: Mean values (± SEM) for the maximum distance travelled (BL 10min^{-1}) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	376.03±46.09 BL N= 8				
	Elevated Temperature (20.6°C)	p= 0.977 t= 0.576	410.73±58.58 BL N=8			
Jervis Bay	Ambient (20.6°C)	p= 0.343 t= 1.886	p= 0.247 t= 2.092	489.61±25.11 BL N=8		
	Elevated Temperature (23.6°C)	p= 0.278 t= -2.020	p= 0.214 t= -2.175	p< 0.01 * t= -3.561	227.04±57.44 BL N=4	
	Adelaide temperature (17.6°C)	p= 0.512 t= -1.588	p= 0.218 t= -2.165	p< 0.05 * t= 3.475	p= 0.949 t= -0.724	280.40±33.59 BL N=8

Table S9: Mean values (± SEM) for maximum velocity (BL s^{-1}) for each treatment are in shaded cells. Statistical differences between treatments are denoted by (*) following statistical outputs from Tukey post-hoc tests.

		Adelaide		Jervis Bay		
		Ambient (17.6°C)	Elevated Temperature (20.6°C)	Ambient (20.6°C)	Elevated Temperature (23.6°C)	Adelaide temperature (17.6°C)
Adelaide	Ambient (17.6°C)	0.65±0.07 BL s^{-1} N=8				
	Elevated Temperature (20.6°C)	p= 0.399 t= 1.780	0.80±0.06 BL s^{-1} N=8			
Jervis Bay	Ambient (20.6°C)	p= 0.149 t= 2.371	p= 0.976 t= 0.583	0.85±0.04 BL s^{-1} N=8		
	Elevated Temperature (23.6°C)	p= 0.637 t= -1.388	p= 0.055 t= -2.850	p< 0.05 * t= -3.231	0.51±0.21 BL s^{-1} N=4	
	Adelaide temperature (17.6°C)	p= 0.277 t= -2.022	p< 0.005 * t= -3.795	p< 0.01 * t= 4.385	p= 0.999 t= 0.226	0.49±0.06 BL s^{-1} N=8

General discussions and conclusions

During early development, individuals are thought to be inherently vulnerable to a suite of unavoidable impacts such as predation and environmental conditions. However, despite the vulnerable, defenceless picture we paint in regard to embryonic development and other early life stages, Port Jackson sharks, *Heterodontus portusjacksoni*, may be relatively robust with respect to their response predation events and changes in rearing temperature. Responding to such events can be energetically costly, but not necessarily unmanageable. Here, I investigated the impact of predation and temperature across embryonic development and post-hatching life stages of Port Jackson sharks. Overall, this species displays strategies which can aid in minimizing predation risk early in development and while rearing temperature can induce elevated energetic costs in juveniles, embryos may be relatively robust (physiologically) to changes in their thermal environment.

Bottom of the food chain- dangers to early development

The earliest of life stages are incredibly important in promoting healthy populations, but they are at their most vulnerable to predation given their size. Anti-predator strategies are essential to utilize to enhance survival probability but come at an energetic cost which can implicate juvenile growth and energy needs. This balance between costs and benefits should prompt the capacity for individuals, especially during early development, to detect, differentiate, and ultimately refine their anti-predatory response to suit the perceived level of risk. Perhaps, unsurprisingly, embryonic and juvenile Port Jackson sharks were able to detect predator cues and elicit known anti-predatory strategies. Importantly, however they also showed the capacity to differentiate between cues. This can promote young shark fitness as over time their determination of predators may become more efficient and reduce the number of false responses. Given that embryos and juveniles primarily displayed crypsis-like responses, the costs associated with these strategies comes in the form of missed opportunities, especially foraging. While embryos may display minimal costs associate with lost time, frequent use of these anti-predator strategies could delay gestation; while conversely, juvenile growth rates would likely be depressed. Despite the potentially strong and ubiquitous nature of predation across the early life history of elasmobranchs, we know very little in comparison to other vertebrates. The costs associated with consumption are lethal, but even the non-consumptive effects can linger, impacting population growth and health. However, as this thesis illustrates, young elasmobranchs are capable of mitigating this risk and are potentially able to learn, using prior experience to inform future decisions.

The cognitive mechanisms behind predator-prey interactions are well studied in teleost fish (Brown *et al.*, 2011a) and a range of other taxa (Creel, 2018; Hawlena and Schmitz, 2010); however, our understanding in elasmobranchs is greatly lacking. Despite recent interest in elasmobranch cognition

(Guttridge and Brown, 2014; Schluessel *et al.*, 2015; Vila Pouca and Brown, 2018), there are still massive literature gaps in this field. This thesis (chapter 1) is among the first to suggest the capacity for embryonic elasmobranchs to alter their responses based on prior experiences, thus suggesting that embryonic sharks may have the capacity to learn. This capacity is already known from teleosts and amphibians (Atherton and McCormick, 2015; Brown *et al.*, 2011b; Ferrari and Chivers, 2009) and thus is somewhat unsurprising that it might also exists in sharks. These mechanisms, especially during very early life stages ultimately aid in the survival of young not just during predator interactions. The capacity to discriminate odours is not limited to more efficient anti-predator responses, but odour discrimination also aids in prey detection. Despite the importance of elasmobranchs across marine ecosystems, and their ecological role as both 'predators' and 'prey', the mechanistic pathways and behavioural/physiological responses that elasmobranchs utilize as 'prey' are still poorly understood. It is also clear from this thesis that a greater understanding of the development of the sensory system during early ontogeny is required and this may be in part elucidated by examining behavioural and physiological responses to a range of sensory stimuli.

Changing climate

Sea surface temperatures are largely impacting the physiology and behaviour of many marine species (Beever *et al.*, 2017; Crozier and Hutchings, 2014). Here ontogeny played a significant role in the relative vulnerability of this species to predicted end of the century changes in water temperature. Contrary to what is assumed in teleost fish (Pörtner and Farrell, 2008), embryonic sharks displayed no impact of temperature on their metabolic or growth rates across rearing temperature(chapter 3), but upon hatching juveniles were relatively more vulnerable, showing elevated metabolic rates and declines in swimming activity with elevated temperature (depending on provenance)(chapter 4). While embryos may be able to maintain survival and development under warming temperatures, juveniles may begin to display deleterious consequences (i.e., metabolic rate, depressed swimming activity) particularly as they have to contend with stressors beyond development. Despite conventional thoughts, the vulnerability of this species may not be greatest during embryonic development, but instead upon hatching. Further work is required to examine the long-term implications for early exposure to high temperature. However, the magnitude and strength of these impacts are likely linked to a population's proximity to thermal distribution limits. The impacts of temperature observed herein highlighted the importance of ontogeny in assessing a population's vulnerability to climate change, and furthermore, the stages most vulnerable to thermal changes may not always the same for all populations. Changes in morphology, behaviour, and habitat may all contribute to the relative tolerance of an age class to shifting environmental temperatures. While younger life stages in this case may be constrained by an egg capsule or by the safety of nursery areas, Port Jackson shark adults make relatively long migrations (Bass

et al., 2017) and thus have a greater capacity to thermoregulate. If adults are thermoregulating, their thermal window may be relatively narrower than that of younger life stages which are largely restricted in their available thermal habitats. As conditions change, it would be expected that populations may begin shifting to cooler waters (higher latitudes or deeper waters), and already adult Port Jackson may already be feeling thermal pressure. Their abundance along their high latitude distribution is increasing, suggesting that adults may be slowly redistributing (Last *et al.*, 2010). Nonetheless, there is some expectation that this process may be hampered by their high fidelity to breeding reefs (Bass *et al.*, 2017).

Ontogenetic shifts coincide with a plethora of changes, and a change in thermal environment may influence the relative tolerance of different life stages to thermal extremes. Given that eggs must develop over a 10-12 month period in variable seasonal temperatures, Port Jackson sharks, and most likely other temperate oviparous species, are relatively robust during development. One future direction would be to explore the impact of temperatures across the adult stages of sister species (e.g., adult Port Jackson sharks and the crested horn shark, *Heterodontus galeatus*). While, these species inhabit the same habitats, develop under similar conditions, and likely consume similar prey, as adults one is highly migratory, while the other is not. It might be expected that Port Jackson sharks lose the capacity to cope with warmer temperatures as adults and that by residing in relatively colder water they are more sensitive to thermal change.

As the climate continues to warm, sharks will be impacted by a suite of other factors which will all interact with each other. Temperature does not occur in isolation in nature, and is just one, albeit one of the most influential factors associated with climate change. While in this thesis two different stimuli were measured independently, this is not indicative of multifaceted stressful conditions experienced in the wild. As thermal extremes become more prevalent, species will be experiencing a suite of different abiotic and abiotic stressors, some of which may work antagonistically. For example, increases in water temperature co-occurs with declines in oxygen concentration as well as reductions in pH due to ocean acidification. Individuals have to deal with these abiotic stressors whilst simultaneously coping with biotic stressors such as predator avoidance or foraging competition. As the cost of living increases with elevated temperatures, the trade-offs between behaviour and physiology will likely impact continued survival, especially within lower-latitude populations (Laubenstein *et al.*, 2019). Here, I illustrated that there are likely also population specific responses to warming water. Swimming performance, in Jervis Bay sharks, was hampered under future conditions. Such declines in swimming performance can ultimately depress the efficiency of movement-based activities such as anti-predator responses, migration and foraging. Additionally, ocean acidification is already known to impair hunting capacity in elasmobranchs (Dixson *et al.*, 2015). Similarly, teleost fish are known to misidentify potential

predators and show impaired decision making under these conditions (Dixson *et al.*, 2010; Munday *et al.*, 2012; Munday *et al.*, 2014; Munday *et al.*, 2009a; Munday *et al.*, 2009b), but again, in the context of elasmobranch as prey, this topic has largely been ignored.

I have identified a large knowledge gap in our understanding of elasmobranchs, and while manipulation in laboratory systems is limited, it is not impossible. As the climate changes, species will be exposed to increasingly suboptimal conditions, across a variety of stressors (e.g., increasing temperature, progressive ocean acidification, and increasing hypoxia zones). Future studies need to target the interactions between these variables and how multiple stressors will impact elasmobranch populations, not only during short-term acclimation studies, but also across generations. Transitioning from short-term acclimation to transgenerational studies can elucidate the potential for additional strategies (e.g., transgenerational acclimation, parental transference, adaptation potential) that species may be able to use to cope with changing conditions. While elasmobranch studies in regards to climate change are increasing (Chin *et al.*, 2010; Di Santo, 2015; Gervais *et al.*, 2018; Johnson *et al.*, 2016; Rosa *et al.*, 2014), to date, nearly all experimental work is focused on benthic, oviparous species. And while these small species are well suited for study, there are a range of other species which exhibit totally different characteristics which remain completely unstudied in this field (e.g., ram ventilators). We need a greater understanding of the physiology, behaviour, and cognitive capacities, across a range of elasmobranchs to highlight the patterns and traits that may be most impacted to better protect those species (or populations) that may be most vulnerable to change.

Conclusion

Despite the varied life-history characteristics that elasmobranchs display, most are vital in the maintenance of a healthy marine ecosystem and/or are important to humans from a socioeconomic perspective (Hammerschlag *et al.*, 2019). However, as a whole, elasmobranchs populations are rapidly declining worldwide with nearly 25% of species threatened or endangered and around 46% classified as data deficient (Dulvy *et al.*, 2014). As water temperatures continue to change, the relationships between temperature and the traits which promote survival will be important in predicting patterns of distributions and species persistence in the future. Will they adapt, will they move, or will they perish? With the unprecedented rapid rate of climate change, adaptation in elasmobranchs is thought to be unlikely given their k-selected life-history characteristics; however, given the potential for large movements, some elasmobranch populations may be more prone to redistributing as a mitigation strategy. Large mobile species such as tiger sharks, *Galeocerdo cuvier*, may already be following their thermal preferences and may become more prominent in higher latitude locations (Payne *et al.*, 2018). While movements may aid in the continued survival of these mobile species, shifting distributions is not a long-term solution for all species and for those that do move, there will be impacts on the novel

environments and food webs they are shifting into. This is particularly the case for coastal dependent species whose distributions rely on coastal ecosystems at higher latitudes for their continued existence. In the case of Port Jackson sharks, they could shift their distribution to encompass Tasmania, but there is simply nothing beyond that landmass for them to colonise.

The evolutionary pressure that predation and environmental stimuli exert, likely shaped the robust nature of these young sharks. By being able to respond to these risks, embryo fitness and survival is enhanced. Here, Port Jackson shark embryos displayed the capacity to endure different stressors and mitigate the energetic costs associated with them. Given the declines in elasmobranch populations worldwide, population recovery may be hampered by factors such as climate change. Yet we largely do not know to what extent most species are influence by temperature or predation nor the synergies between them. Species generally regarded as being tolerant of extreme conditions (Heinrich *et al.*, 2014; Wise *et al.*, 1998) are now known to experience significant mortality when reared under prolonged elevated temperatures (Gervais *et al.*, 2018). Even for those which are relatively tolerant of changing conditions, there may be costs and associated declines in performance which can hamper the demographics and/or recovery of populations. Certainly, there are few studies examining the long-term implications of exposure to high temperature and this remains a serious challenge. As the climate changes we need to quickly improve our understanding of the behaviour, movement, and physiology across ontogeny, especially targeting a wider range of species and incorporating multiple stressors over longer, generational time-frames. Identifying resilient species and/or population can aid in the early development and implementation of targeted management techniques to better protect at risk elasmobranchs now and in the future. Without elasmobranchs we lose many vital ecosystem functions potentially leading to tropic cascade, loss of climate change mitigation processes, and potentially ecosystem collapses/ phase shifts (Hammerschlag *et al.*, 2019).

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Appendix I- Research communications

I. **Gervais C.**, Fuchert T., Rummer J., Huveneers C., Brown C. (2019) Sharks International, Joao Pessoa, Brazil

II. **Gervais, C.**, Rummer J., Brown C. (2018) How hot is too hot? Temperature's role on the development and physiology of a benthic shark. Macquarie University HDR conference

III. **Gervais C.** (2018) Baby sharks: the cute side of sharks. Reef HQ Aquarium, Shark night, public talk

IV. **Gervais, C.**, Rummer J., Brown C. (2017) Fight, flight, or freeze? Physiological and behavioural responses of juvenile Port Jackson sharks (*Heterodontus portusjacksoni*) to conspecific death cues. Macquarie University HDR conference.

V. **Gervais C.**, Nay, T., Johansen J.,Renshaw G., Steffensen J., Rummer J., (2016) Too hot to handle? The use of movement by a benthic elasmobrach species, *Hemiscyllium oceallatum*, to alleviate effects of temperature. 4th ASFB/OCS conference, Hobart, Australia

Appendix II- Additional research conducted during candidature

I. **Gervais CR**, Nay TJ, Renshaw G, Johansen JL, Steffensen JF, Rummer JL (2018) Too hot to handle? Using movement to alleviate effects of elevated temperatures in a benthic elasmobranch, Hemiscyllium ocellatum. Marine Biology 165: 162

II. Nay TJ, **Gervais CR**, Hoey AS, Johansen JL, Steffensen JF, Rummer JL (2018) The emergence emergency: A mudskipper's response to temperatures. Journal of Thermal Biology 78: 65-72

III. Vila Pouca C, **Gervais C**, Reed J, Brown C (2018) Incubation under Climate Warming Affects Behavioural Lateralisation in Port Jackson Sharks. Symmetry 10: 184

IV. Vila Pouca C, **Gervais CR**, Reed J, Michard J, Brown C (2019) Global warming enhances quality discrimination in sharks. Behavioural Ecology and Sociobiology 73: 93

V. Hernandez AV, **Gervais CR**, Rummer JL, Porter ME (*In Review*) Born to walk: swimming and aquatic walking kinematics of epaulette sharks. *Journal of Experimental Biology*

VI. Kadar JP, Vila Pouca C, Perryman R, Pini-Fitzsimmons J, Chambers S, **Gervais C**, Brown C (*In Review*) Behaviour. In Mehods for FIsh Biology II

VII. Luongo SM, Ruth A, **Gervais CR**, Johansen JL, Domenici P, Korsmeyer KE, Steffensen JF. Bidirectional flow regimes increase energetic costs for a labriform fish: Cymatogaster aggregate. (In prep)

VIII. Nay TJ, Longbottom RJ, **Gervais CR**, Johansen JL, Rummer JL, Steffensen JF, Hoey AS. Microhabitat use and temperature utilization in a coral reef flat specialist, the epaulette shark Hemiscyllium ocellatum. (In prep)

Appendix III- Funding sources

I. 2019 Fisheries Society of the British Isles- Travel award, AUD $1800

II. 2018 Society of Experimental Biology- Travel grant, AUD $850

III. 2018 Department of Biological Sciences- Grant matching scheme, AUD $1850

IV. 2017 Wainright Fellowship $2,000

V. 2017 Friday Harbour Laboratories Adopt-a-student endowment, AUD $2500

VI. 2016 ASFB John Glover Travel Fund AUD $400

VII. 2015-2019 Department of Biological Sciences AUD $3,000 (per annum)

Appendix IV- Animal ethics and fisheries approvals