Ecological energetics of climate change for tropical sharks

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General abstract

Climate change is predicted to affect the survival and reproductive success, or fitness, of marine ectotherms through a combination of physiological and behavioural effects. Sharks and rays, which are among the most threatened vertebrate taxa, experience reduced physiological performance and behavioural impairments when tested under simulated ocean warming (i.e., temperature) and acidification (i.e., CO_2) conditions. Research effort has predominantly focused on sharks occupying a narrow ecological niche (i.e., benthic, temperate, oviparous species in low trophic levels), such that predicted responses to climate change conditions are not representative of most other groups. Here, this thesis addresses the knowledge gap concerning climate change effects on active, tropical species in high trophic levels, for which no data exist. I test the hypothesis that climate change will have affect the fitness of reef shark populations through reductions in physiological performance, using the blacktip reef shark (*Carcharhinus melanopterus*) and sicklefin lemon shark (*Negaprion acutidens*) as experimental models.

In **Chapter 2** of this thesis, I characterised *in situ* thermal dependence of growth and metabolic rate in relation to nursery area use in populations of neonatal *C. melanopterus* and *N. acutidens* around the island of Moorea, French Polynesia. *Carcharhinus melanopterus* did not exhibit strong thermal dependence of growth and metabolic rate and did not use nursery areas. Conversely, *N. acutidens* used a nursery area; yet, this species also did not exhibit strong thermal dependence of growth or metabolic rate. Together, **Chapter 2** supports the hypothesis that sharks in nursery area systems exhibit reduced thermal dependence of performance and suggests that climate change can affect shark performance in nursery areas to act on the fitness of reef shark populations.

In **Chapter 3**, I reviewed the available literature to identify physiological performance traits that can be applied to predict climate change effects on fitness. Oxygen uptake rates are proxies for aerobic metabolic rates that have been applied to predict changes in fitness, spatial ecology, and bioenergetics of fishes in response to climate change. This review also highlighted a lack of understanding of the effects of environmental change on aerobic scope in sharks, which represents an organism's capacity to supply oxygen to multiple, simultaneous, oxygen-demanding processes. **Chapter 3** set the foundation for **Chapters 4-6**, which tested oxygen uptake metrics to understand their applicability to predicting fitness consequences in sharks under global climate change conditions.

In **Chapter 4**, I characterised *in situ* thermal dependence of stress physiological status, oxygen uptake rates, and recovery from exercise in *C. melanopterus* and *N. acutidens*. Of the measured physiological status markers, only blood glucose concentration in *C. melanopterus* and haemoglobin concentration in *N. acutidens* increase with temperature; all other metrics (blood pH, lactate concentration, haematocrit, mean

corpuscular haemoglobin concentration) were unaffected over a range of 28-31 °C. Oxygen uptake rates relating to exercise performance (i.e., maximum oxygen uptake, post-exercise oxygen uptake and recovery) could only be measured in *C. melanopterus* and were unaffected by temperature over a range of 28-32 °C. Thus, **Chapter 4** suggests that physiological performance in *C. melanopterus* and *N. acutidens* under stress does not exhibit thermal dependence *in situ* and that *N. acutidens* are more sensitive to stress than *C. melanopterus*.

In **Chapter 5**, I characterised physiological and behavioural responses of *C. melanopterus* to temperature change. First, I tested thermal dependence of oxygen uptake rates, growth, and environmental tolerance traits in laboratory acclimated sharks using an ecologically relevant range of acclimation temperatures (28 and 31 °C). Second, I tested the effects of temperature (25, 30, and 35 °C) and pH on haemoglobin-oxygen (Hb-O_2) affinity *in vitro*. Third, I observed thermal preference behaviours *in situ* using temperature data-loggers deployed on wild sharks and in the environment. Oxygen uptake rates (aerobic scope, post-exercise oxygen consumption, and recovery) and growth (rate, body condition, and food conversion efficiency) were unaffected by acclimation temperature. Conversely, thermal tolerance and hypoxia tolerance were increased following acclimation to ocean warming conditions and were associated, suggesting a common mechanism. Haemoglobin-oxygen affinity decreased with increasing temperature and pH-sensitivity of Hb-$_{O2}$ binding was strong at 30 °C, but absent at 25 and 35 °C. There was evidence of avoidance of temperatures 31 °C water *in situ*. Taken together, **Chapter 5** suggests that *C. melanopterus* exhibit a suite of physiological and behavioural capacities to tolerate temperature change.

In **Chapter 6**, I tested for physiological and behavioural responses of *C. melanopterus* to simulated ocean warming and acidification conditions. Sharks were acclimated to ambient (28 °C, 650 μatm), elevated CO_2 (28 °C, 1050 μatm), elevated temperature (31 °C, 650 μatm), and elevated temperature and CO_2 conditions (31 °C, 1050 μatm). Oxygen uptake rates (aerobic scope, maximum oxygen uptake rate, post-exercise oxygen uptake and recovery), hypoxia tolerance, haematological (haematocrit and haemoglobin concentration) and physiological status (blood pH and lactate concentration), and behavioural traits (lateralisation and activity level) were unaffected by temperature and CO_2. However, high temperature and CO_2 conditions interacted synergistically to increase minimum oxygen uptake rates, meaning that the energetic costs associated with the maintenance of homeostasis would be predicted to increase under climate change conditions. Thus, **Chapter 6** suggests that *C. melanopterus* do not respond behaviourally to multiple climate change stressors but exhibit variable physiological responses.

This thesis demonstrates differential responses of reef shark neonate populations to global change stressors, highlighting the potential for deleterious outcomes. Ocean warming and acidification are predicted to increase energetic costs in *C. melanopterus*. In addition, this thesis advances nursery area theory for sharks

by suggesting that thermal dependence of physiological performance is associated with nursery areas, and that these physiological traits can be acted on by climate change to affect fitness in tropical shark populations. These data are applicable to conservation planning in for reef shark populations throughout French Polynesia and offer an important new avenue for targeted research effort to understand the magnitude of the climate change threat to the world's shark populations.

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Chapter 1: General introduction

Organismal performance is inexorably linked with Darwinian fitness, meaning that the ability of organisms to perform physiological functions and behaviours ultimately affects their survival and reproductive success. Metabolic rate is a fundamentally important physiological trait that represents an organism's rate of energy uptake and is thought to set the rates of ecological processes (Brown *et al.*, 2004). Fry, a fish biologist, coined the terms 'scope for metabolic activity' and 'aerobic scope' to represent the capacity of an organism to supply oxygen to match multiple, simultaneous metabolic demands (Fry, 1947). Aerobic scope, which is calculated in ectotherms as the difference between standard metabolic rate (SMR, the metabolic rate of fasted, ectothermic organism at a stable temperature) and maximum metabolic rate (MMR, the highest achievable metabolic rate), is, therefore, considered as a proxy of the total aerobic capacity of an organism to perform physiological functions and behaviours that relate to Darwinian fitness (Farrell, 2016). Fry went on to characterise the effects of environmental factors on metabolic rate in ectothermic fishes, noting that environmental factors can be lethal, controlling (i.e., rates of chemical reactions are influenced), limiting (i.e., supply of energetic substrates or removal of metabolic wastes is influenced), masking (i.e., invoke regulation or compensation), or directive (i.e., invoke signal transduction) (Fry, 1971). Expanding upon Fry's paradigm, the effects of environmental factors on aerobic scope, among other traits, have received much attention to test so-called unifying theories to predict the effects of global climate change on the distribution, fitness, and survival of ectothermic organisms (Pörtner *et al.*, 2017), with considerable research effort focused on understanding the fate of fishes (Lefevre, 2016).

1.1 The ecophysiology of climate change in fishes

Climate change is among the greatest modern threats to marine organisms (Pörtner and Farrell, 2008; Hoegh-Guldberg and Bruno, 2010). Since the Industrial Revolution, anthropogenic gas emissions (i.e., greenhouse gases including carbon dioxide, CO_2) have contributed to warming of the earth's atmosphere (IPCC, 2014). The world's oceans act as a tremendous heat sink, resulting in a steady increase in the heat content and, therefore, surface temperatures of the oceans that is referred to as 'ocean warming' (Hansen *et al.*, 2006). The most pessimistic projection, or 'Representative Concentrations Pathway' (RCP), RCP 8.5 (i.e., an unabated greenhouse gas emission scenario), predicts increases in sea surface temperatures (SST) of 1-5 °C by the year 2100, depending on climatic region (IPCC, 2014). In addition to absorbing heat, the oceans absorb CO_2, which acidifies water and reduces the saturation state of carbonate minerals (e.g., aragonite, calcite) in a process referred to as 'ocean acidification' (Meinshausen *et al.*, 2011). Present day partial pressures of CO_2 (pCO_2) in the open ocean average 400 μatm and are expected to increase to approximately 1000 μatm by the year 2100 according to RCP 8.5 (IPCC, 2014). The increase in heat content of the oceans also reduces the solubility of gases in water, including oxygen, thereby contributing to a third global climate change phenomenon, referred

to as ‘ocean deoxygenation’ (Breitburg *et al.*, 2018). Knowledge of the effects of changes in temperature, CO_2, and oxygen on the physiology of marine ectotherms can shed light on possible responses of organisms to these global change phenomena (i.e., ocean warming, acidification, and deoxygenation). Fortunately, decades of research effort have been dedicated to understanding the effect of these fundamental environmental factors on the physiology of marine ectotherms, including fishes (Fry, 1971; Randall and Brauner, 1991; Claireaux and Lefrançois, 2007). In the following subsections of section 1.1, I briefly summarise the effects of temperature, CO_2, and oxygen on relevant aspects of the physiology of fishes and conclude with their application to a proposed unifying hypothesis of the effects of climate change on marine ectotherms.

Effects of temperature on the physiology of fishes

Temperature is the most well-studied and influential controlling environmental factor of the physiology of fishes (Fry, 1971; Schulte, 2015). Thus, temperature is used to explain trends in the abundance and distribution of fishes in relation to populations’ thermal tolerance limits and thermal dependence of physiological performance (Pörtner and Knust, 2007; Sunday *et al.*, 2012; Payne *et al.*, 2016). Effects of temperature occur at all levels of biological organisation (Schulte *et al.*, 2011). Changes in temperature invoke molecular responses (e.g., gene expression; Fangue *et al.*, 2006; Houde *et al.*, 2019; Bernal *et al.*, 2020), biochemical responses (e.g., enzyme activity and substrate interactions; Hochochka and Somero, 2002; Strobel *et al.*, 2012; Ekström *et al.*, 2017), subcellular and cellular responses (e.g., membrane effects on mitochondrial respiration; Martinez *et al.*, 2016; Chung *et al.*, 2017; Pichaud *et al.*, 2019), organ system responses (e.g., cardiovascular function; Sandblom *et al.*, 2016; Keen *et al.*, 2017; Gilbert and Tierney, 2018), and whole-organism responses (e.g., metabolic rate, growth; Killen *et al.*, 2010; Eliason *et al.*, 2011; Audzijonyte *et al.*, 2020). Most biological rates increase exponentially with temperature. For example, SMR typically doubles or triples (i.e., a temperature quotient, Q_{10}, of 2-3) with a 10 °C increase in temperature (Clarke *et al.*, 1999; Sandblom *et al.*, 2014; Seebacher *et al.*, 2015). However, the sensitivity of biological rates to temperature change is dependent on the temperature range (Gilbert *et al.*, 2020), rate of temperature change (Allen *et al.*, 2016), and duration of temperature exposure (Nyboer and Chapman, 2017). Further, fishes exhibit a remarkable capacity for developmental (Spinks *et al.*, 2019), reversible (da Silva *et al.*, 2019), and transgenerational acclimation to temperature change (Donelson *et al.*, 2012). Biological rates can be measured to estimate performance metrics (e.g., aerobic scope) that can be modelled with ‘thermal performance curves’ or ‘reaction norms’ that are left-skewed, exhibit an optimal temperature where the biological rate is maximised, and predict a critical upper temperature limit where the biological rate is zero (Payne and Smith, 2017). As such, there is much interest in quantifying thermal dependence of performance, thermal limits, and acclimation capacity in fishes to predict populations’ responses to ocean warming.

Effects of CO_2 on the physiology of fishes

Carbon dioxide is a fundamentally important environmental factor affecting the physiology of fishes (Fry, 1971). Fishes experience elevated pCO_2 in their environment at CO_2 seeps (Munday *et al.*, 2014), low-oxygen environments (Gobler and Baumann, 2016), in aquaculture (R. P. Ellis *et al.*, 2017), and *via* ocean acidification (Baumann, 2019). Fishes are highly sensitive to increases in environmental pCO_2 because they have low arterial blood pCO_2 (relative to air breathers) owing to the high ventilation volume of gills, solubility of CO_2 in water, and counter-current nature of gas exchange across the gills (Evans *et al.*, 2005; Bayley *et al.*, 2019). In addition, fishes experience internal increases in pCO_2 in their blood as a metabolic waste product of aerobic cellular respiration (Hillman *et al.*, 2013). The hydration of CO_2 in the blood produces carbonic acid that results in an acid-base disturbance. Fishes can modulate ventilation volume (e.g., *via* swimming speed, ventilation rate) to reduce their blood pCO_2 (Esbaugh, 2018), but CO_2 efflux is physically limited by structural constraints of the respiratory system (Evans *et al.*, 2005; Hillman *et al.*, 2013). Instead, fishes are highly competent at acid-base regulation, primarily through the exchange of protons (H^+) and bicarbonate (HCO_3^-) with the environment (Brauner *et al.*, 2019). Thus, a typical (and simplified) response to an increase in environmental pCO_2 is an increase in blood pCO_2, a decrease in pH, and then a net increase in [HCO_3^-] that recovers pH (Wood, 1991). The consequences of acid-base disturbance and regulation affect multiple physiological systems, including aerobic metabolism (e.g., cost of acid-base regulation reflected in SMR; Heuer and Grosell, 2016; Lefevre, 2016), calcification processes (e.g., otolith formation; Bignami *et al.*, 2013, 2014), and neurological function (e.g., hyperpolarisation of inhibitory neurotransmitters, reduced sensitivity of olfactory epithelia; Nilsson *et al.*, 2012; Heuer *et al.*, 2016; Porteus *et al.*, 2018). Further, fishes demonstrate developmental, reversible, and transgenerational acclimation responses to elevated pCO_2 (Miller *et al.*, 2012; Schunter *et al.*, 2016, 2018). Whilst fishes can experience and compensate for environmental pCO_2 that exceeds projected values for ocean acidification (Ishimatsu *et al.*, 2008), there is mounting evidence to suggest that exposure to ~ 900-1000 μatm pCO_2 (i.e., RCP 8.5) has the potential for physiological and behavioural consequences (Heuer and Grosell, 2014; Tresguerres and Hamilton, 2017). Therefore, studies aiming to test the effects of simulated ocean acidification conditions in fishes measure both physiological and behavioural endpoints.

Effects of oxygen on the physiology of fishes

Oxygen is a limiting factor of aerobic metabolism in fishes (Fry, 1971). Fishes have evolved a remarkable capacity to uptake oxygen from water, which is a tremendously viscous, dense fluid relative to air (Rummer *et al.*, 2013a; Randall *et al.*, 2014). Life is supported aerobically in fishes, and life without oxygen (i.e., anaerobic) is time limited. Because oxygen is the final electron acceptor in the electron transport chain, oxygen partial pressures (pO_2) set the rates of aerobic cellular respiration (McClelland, 2011). Aerobic metabolic rates are dependent and independent of environmental pO_2

along a continuum; species that regulate metabolic rate independent of environmental pO_2 are referred to as 'oxyregulators' and species whose metabolic rates conform to environmental pO_2 are referred to as 'oxyconformers' (Alexander and McMahon, 2004; Mueller and Seymour, 2011). Most fishes are believed to be oxyregulators (Burggren *et al.*, 2019; Svendsen *et al.*, 2019); however, MMR in fishes (and ectotherms) is reduced by decreasing environmental pO_2 (Claireaux and Lefrançois, 2007), but does not increase with environmental pO_2 above saturation (Lefrançois and Claireaux, 2003). Instead, SMR is regulated until pO_2 is too low and fishes transition from oxyregulating to oxyconforming (Ultsch and Regan, 2019). Reductions in environmental pO_2 can lead to hypoxia that is often defined as a reduction in dissolved oxygen concentration below 2 mg O_2 L^{-1} in marine systems (Breitburg *et al.*, 2018). Fishes can experience hypoxia in habitats with high rates of respiration such as estuaries or tidal pools (Richards, 2011), and in stratified waters including oxygen minimum zones (Seibel, 2011). Hypoxia tolerance can be defined using various metrics that relate to multiple physiological systems (Wood, 2018), including the pO_2 that reduces SMR (Negrete and Esbaugh, 2019; Reemeyer and Rees, 2019), the pO_2 that induces loss of equilibrium (Fangue *et al.*, 2001; Snyder *et al.*, 2016), and the pO_2 that reduces retinal sensitivity to light (Robinson *et al.*, 2013; McCormick *et al.*, 2019). Exposure to hypoxia initiates a suite of physiological responses including the expression of hypoxia inducible factor proteins and heat-shock proteins (Borowiec *et al.*, 2018; Williams *et al.*, 2019). Fishes can improve tolerance to low-pO_2 environments *via* developmental and reversible acclimation (Motyka *et al.*, 2017; Wood *et al.*, 2017, 2019; Gilmore *et al.*, 2019). Thus, quantifying hypoxia tolerance in fishes is of interest for defining the effects of ocean deoxygenation.

From Fry's Paradigm to oxygen- and capacity-limited thermal tolerance

Aerobic scope has received much attention for predicting consequences of environmental change in fishes through integration of Fry's Paradigm (Fry, 1971; Claireaux and Lefrançois, 2007; Farrell, 2016). Proposed by Pörtner *et al*., the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis, has been proposed to explain the responses of ectotherms to climate change (Pörtner and Knust, 2007; Pörtner, 2010; Pörtner *et al.*, 2017). This hypothesis posits that thermal tolerance is limited by physiological oxygen supply capacity, as demonstrated by thermal dependence of traits of the oxygen cascade, including aerobic scope (Eliason *et al.*, 2011), cardiac output (Eliason *et al.*, 2013), and arterial blood flow (Pörtner and Knust, 2007). Some species also demonstrate oxygen dependence of thermal tolerance (Giomi *et al.*, 2019) and associations between thermal and hypoxia tolerance (Anttila *et al.*, 2013). Thermal performance curves of traits relating to oxygen supply capacity (e.g., aerobic scope, swimming performance, arterial blood flow) have been used to explain decreasing abundance in fish populations with increasing temperature (Pörtner and Knust, 2007; Farrell *et al.*, 2008). Further, upper critical temperatures that performance curves predict align with warm temperature boundaries of populations' latitudinal distributions (Payne *et al.*, 2016). As such, there is some empirical support for using the OCLTT hypothesis to predict effects of ocean warming on the abundance and distribution of

fishes. Regarding aerobic scope, the OCLTT hypothesis predicts that thermal dependence of aerobic scope follows a conventional thermal performance curve, and that ocean warming will lead populations of fishes to either redistribute or acclimate to maintain aerobic scope and a sufficient safety margin from upper thermal limits (Pörtner, 2002). Ocean acidification is predicted to have similar effects as ocean deoxygenation, where aerobic scope is reduced (i.e., deoxygenation reduces MMR and acidification increases SMR) and upper thermal limits are reduced (Pörtner, 2010), thereby tightening metabolic constraints on fishes (Deutsch *et al.*, 2015). However, the interactive effects of ocean warming, acidification, and deoxygenation are complex and unpredictable and warrant detailed investigation (Lefevre, 2016; Boyd *et al.*, 2018).

A growing number of studies also provide evidence that is inconsistent with assumptions of the OCLTT hypothesis. For instance, aerobic scope increases across an ecologically relevant temperature range without a clear optimum or upper critical temperature in a number of marine ectotherms (Lefevre, 2016). Further, comprehensive tests of the hypothesis have failed to demonstrate associations between aerobic scope and fitness-related metrics, such as growth (Gräns *et al.*, 2014). Others have suggested that considering performance metrics of multiple physiological systems is a better approach than relying on aerobic scope as a "catch-all" metric of climate change vulnerability (Kellermann *et al.*, 2019); this notion forms the basis of the multiple performances multiple optima (MPMO) hypothesis, as proposed by Clark *et al.* (2013). For example, thermal performance of other metrics, including somatic and gonadal growth, and activity, is associated with abundance and warm latitudinal boundaries in some populations of fishes (Gannon *et al.*, 2014; Payne *et al.*, 2016, 2018). Despite debate over whether hypotheses like OCLTT or MPMO are universal or unifying, there are species for which there is strong support for links between aerobic scope and Darwinian fitness, such as the association between spawning migration success and aerobic scope in salmonids (Eliason and Farrell, 2016). Indeed, the mechanistic basis of thermal tolerance as proposed by the OCLTT hypothesis is not universal (Jutfelt *et al.*, 2018), and there are several other 'high-profile' hypotheses, including MPMO, the Metabolic Theory of Ecology (Brown *et al.*, 2004), Gill Oxygen Limitation (Pauly and Cheung, 2017), and Dynamic Energy Budget Theory (Nisbet *et al.*, 2000) that emphasise testing aerobic scope (among other traits, i.e., MPMO) and can be tested to predict populations' responses to climate change. However universal these hypotheses may be, there is much value in investigating mechanisms of vulnerability to climate change in fishes, including among the most threatened and data-deficient fishes.

1.2 Conservation status of sharks and rays

The Chondrichthyan fishes (sharks, rays, and chimaeras) are one of the most threatened and data-deficient aquatic vertebrate taxa (Dulvy *et al.*, 2014). Throughout a 450 million year evolutionary history, these fishes have expanded into every major aquatic ecosystem, from freshwater and estuarine habitats (Grant *et al.*, 2019), to coral reefs (Roff *et al.*, 2016), the poles (Edwards *et al.*, 2019), and the

deep-sea (Simpfendorfer and Kyne, 2009). Further, this taxonomic group includes the oldest living vertebrates (Nielsen *et al.*, 2016), the largest fishes (Lawson *et al.*, 2019), and embody the longest evolutionary history of extant vertebrates (Stein *et al.*, 2018). Throughout their evolutionary history, many shark, ray, and chimaera species experienced selection for life-history traits that are characteristic of *K*-selected organisms (Cortés, 2000), including late maturation and long gestation producing few offspring, and an absence of parental care (Carrier and Pratt, 2004). Together, these life-history traits render many species highly susceptible to population declines (Kindsvater *et al.*, 2016). Targeted and incidental overfishing is the greatest modern threat to Chondrichthyan fishes (Dulvy *et al.*, 2014; Oliver *et al.*, 2015), and Chondrichthyan fishes face a greater conservation challenge and extinction risk relative to most other vertebrate taxa (Dulvy *et al.*, 2017). One quarter of all known species (n = 1192; Stein *et al.*, 2018) are estimated to be threatened with extinction, and of these, large-bodied species that use shallow habitats are at greatest risk (Dulvy *et al.*, 2014). Global climate change is the least understood threat to Chondrichthyan fishes; only one species, the New Caledonia catshark (*Aulohalaelurus kanakorum*), is currently listed as threatened by global climate change (Dulvy *et al.*, 2014).

1.3 Climate change as an emerging threat to sharks and rays

Knowledge of the effects of global climate change phenomena in Chondrichthyan fishes is very recent. Responses of sharks and rays to ocean acidification were not examined until recently (*c.* 2014), because extant Chondrichthyan fishes evolved during periods of much higher atmospheric CO_2 and were thought to be resilient to increases in ambient pCO_2 (Rosa *et al.*, 2017; Rummer and Munday, 2017). The first study to consider the vulnerability of sharks, rays, and chimaeras to climate change was an ecological risk assessment for the Great Barrier Reef (Australia) that concluded that freshwater/estuarine species and coastally distributed species (i.e., sharks and rays) were most at risk; although, this study also concluded that the study species had low relative vulnerability to ocean acidification (Chin *et al.*, 2010). Furthermore, managers and stakeholders in shark sanctuaries (i.e., marine protected areas specifically for sharks that usually span a country's entire jurisdiction) suggested that, for some countries, ocean warming and acidification were threats of least concern to sharks relative to fishing-related threats like targeted fishing, bycatch, or ghost fishing (Ward-Paige and Worm, 2017). Since this initial consideration of the effects of climate change in sharks and rays, studies have predicted changes in distribution patterns in response to ocean warming (Fuentes *et al.*, 2016) and documented physiological and behavioural responses to simulated ocean warming and acidification conditions that suggest consequences for biological fitness (Rosa *et al.*, 2017). Altogether, recently compiled evidence suggests that climate change is a more significant threat to sharks and rays than was previously considered (Chin *et al.*, 2010; Rosa *et al.*, 2017). Together, global change stressors (e.g., ocean warming, acidification, and deoxygenation) appear to have the potential to affect the distribution, physiology, and behaviour of sharks and rays.

Climate change effects on the distribution of sharks

Ocean warming is predicted to affect the distribution of sharks. Both sea surface temperature and chlorophyll *a* (a correlate of primary productivity and biodiversity) have been predicted to affect the distribution of whale sharks (*Rhincodon typus*; Sequeira *et al.*, 2014), blue sharks (*Prionace glauca*), various lamnid species (Hazen *et al.*, 2013), and tiger sharks (*Galeocerdo cuvier*; Payne *et al.*, 2018). These studies suggest that consequences of redistribution include increased interactions of sharks with human bathers (Payne *et al.*, 2018) and with fisheries (Hazen *et al.*, 2013), possibly exacerbating current conservation issues for sharks. For tiger sharks, there is evidence of an association between distribution and thermal dependence of physiological traits, where tiger sharks are most abundant at temperatures that support high activity levels (Payne *et al.*, 2018). Ocean deoxygenation is predicted to affect the distribution of sharks *via* expansion of oxygen minimum zones into shallower habitats (Seibel, 2011; Hazen *et al.*, 2013). Ocean acidification is also predicted to affect the distribution of sharks and rays by exacerbating effects of warming and deoxygenation on physiological performance (Rosa *et al.*, 2017). Beyond sharks, ocean warming, acidification, and deoxygenation conditions were found to reduce aerobic scope and habitat availability in jumbo squid (*Dosidicus gigas*) and yellowfin tuna (*Thunnus albacares*), which suggests a physiological mechanism underlying changes in the distribution and abundance of these oceanic top predators (Rosa and Seibel, 2008; Del Raye and Weng, 2015). Thus, studies that associate physiological performance with abundance will be invaluable for predicting changes in species' distribution in and beyond the open ocean.

Climate change effects on the physiology of sharks and rays

Ocean warming and acidification are predicted to affect sharks and rays across multiple physiological systems. To date (*c.* 2019), few studies ($n < 20$), of fewer species ($n < 10$), have investigated the effects of ocean warming (Gervais *et al.*, 2016, 2018; Crear *et al.*, 2019; Hume, 2019), ocean acidification (Green and Jutfelt, 2014; Heinrich *et al.*, 2014; Johnson *et al.*, 2016; Lopes *et al.*, 2018; Pegado *et al.*, 2018, 2019; Dziergwa *et al.*, 2019), or both global change stressors (Rosa *et al.*, 2014, 2016b, 2016a; Di Santo, 2015, 2016, 2019; Pistevos *et al.*, 2015, 2017, 2019; Schwieterman *et al.*, 2019b) in sharks and rays using climate change relevant experimental conditions. It is important to note, however, that there exists a large body of relevant literature on the ecophysiology of sharks and rays, including the effects of temperature (e.g., Miklos *et al.*, 2003; Dabruzzi *et al.*, 2013; Bernal *et al.*, 2018; Payne *et al.*, 2018), CO_2 (e.g., Randall *et al.*, 1976; Heisler *et al.*, 1988; M. S. Graham *et al.*, 1990; Choe and Evans, 2003; Bouyoucos *et al.*, 2020), and oxygen (e.g., Butler and Taylor, 1975; Carlson and Parsons, 2001; Speers-Roesch *et al.*, 2012), but not necessarily under climate change relevant conditions. Tropical carpet sharks are the most studied group, with ten studies of three species (*Chiloscyllium plagiosum*, *C. punctatum*, and *Hemiscyllium ocellatum*). Temperate skates are the next most studied group, with five studies of four species (*Amblyraja radiata*, *Leucoraja erinacea*, *Raja microocellata*, and *R. eglanteria*).

The temperate Port Jackson shark (*Heterodontus portusjacksoni*) appears in three studies, the temperate small-spotted catshark (*Scyliorhinus canicula*) has been studied twice, and the endemic shyshark (*Haploblepharus edwardsii*) and widely-distributed sandbar shark (*Carcharhinus plumbeus*) have only been studied once. It is noteworthy that the majority of research effort has focused mostly on temperate, oviparous species with benthic, inactive lifestyles (Rosa *et al.*, 2017), except for the viviparous, ram-ventilating sandbar shark. This group may, therefore, possess differential tolerance of climate change conditions than other species and not be representative of all sharks and rays.

Effects of ocean warming

Survival, growth, and metabolic rates exhibit acclimation responses to simulated ocean warming conditions in some sharks and rays (Gervais *et al.*, 2016, 2018; Crear *et al.*, 2019; Hume, 2019). Survival of developing embryos and hatchlings is reduced following acclimation to 3-4 °C of warming in epaulette sharks (Gervais *et al.*, 2016, 2018), brown-banded bamboo sharks (Rosa *et al.*, 2014), Port Jackson sharks (Vila Pouca *et al.*, 2019), and little skates (Di Santo, 2015). Further, growth is reduced in epaulette (Gervais *et al.*, 2018) and Port Jackson sharks (Pistevos *et al.*, 2015), and body condition is reduced in brown-banded bamboo sharks (Rosa *et al.*, 2014). Oxygen uptake rates have been commonly measured as proxies for whole-organism metabolic rates for most species studied. Minimum oxygen uptake rates (i.e., proxies of SMR) predictably increased with warming in sharks (*C. plumbeus* and *C. punctatum*) and rays (*A. radiata*, *L. erinacea*, and *R. eglanteria*) suggesting an increase in energetic costs associated maintenance metabolism (Rosa *et al.*, 2014; Di Santo, 2015, 2016; Crear *et al.*, 2019; Schwieterman *et al.*, 2019b). Conversely, maximum oxygen uptake rates (i.e., proxies of MMR) were not affected by temperature in rays (*A. radiata*, *L. erinacea*, and *R. eglanteria*) or in sandbar sharks at the highest test temperatures (Di Santo, 2016; Crear *et al.*, 2019; Schwieterman *et al.*, 2019b), which suggests that, whilst upper limits to oxygen uptake are less plastic than minimum oxygen uptake rates (Sandblom *et al.*, 2016), maximal aerobic activity is not more energetically costly under elevated temperatures. As a result, aerobic scope had an optimal temperature in little skates, such that warming beyond the optimal temperature reduces performance and survival (Di Santo, 2015, 2016). In sandbar sharks, aerobic scope plateaued at high temperatures and hypoxia tolerance was maintained, but increases in activity could not be supported aerobically, which suggests that aerobic capacity is constrained by oxygen demand from other physiological systems (Crear *et al.*, 2019). Finally, aerobic scope did not exhibit a temperature effect in thorny or clearnose skates, but hypoxia tolerance in these species decreased with warming (Schwieterman *et al.*, 2019b). Overall, little skates are one of the most comprehensively studied species regarding the effects of simulated ocean warming conditions, with available data demonstrating an association between reductions in fitness and performance with warming beyond an optimal temperature. Indeed, little skates partially support the OCLTT and MPMO hypotheses, where thermal performance of multiple physiological systems associated with fitness is used to predict global change effects (Clark *et al.*, 2013; Pörtner *et al.*, 2017). Thus, the fitness effects

of ocean warming appear pronounced in these few existing studies, but further investigation into the mechanisms of vulnerability to ocean warming in sharks and rays are needed to more broadly predict the nature of consequences associated with global climate change in these data-deficient species.

Effects of ocean acidification

Ocean acidification is predicted to affect survival, growth, enzyme activity, and oxygen uptake rates in some shark and ray species. Simulated ocean acidification conditions target 300-600 μatm increases in pCO_2 (Rosa *et al.*, 2017). Notably, the effects of acidification on survival were much less pronounced than ocean warming. For instance, acidification reduced survival in juvenile brown-banded bamboo sharks (Rosa *et al.*, 2014), but not in other species for which warming has been found to reduce survival (e.g., epaulette sharks; Johnson *et al.*, 2016, Port Jackson sharks; Pistevos *et al.*, 2015, little skates; Di Santo, 2015). Growth was reduced in Port Jackson sharks (Pistevos *et al.*, 2015) and white-spotted bamboo sharks (Pegado *et al.*, 2018), but not in small-spotted catsharks (Green and Jutfelt, 2014) or epaulette sharks (Johnson *et al.*, 2016). In brown-banded bamboo and epaulette sharks, and small-spotted catsharks, minimum oxygen uptake rates were unaffected by acidification (Green and Jutfelt, 2014; Heinrich *et al.*, 2014; Rosa *et al.*, 2014). Only one study in small-spotted catsharks tested the effects of acidification on maximum oxygen uptake rates and aerobic scope but found no effect (Green and Jutfelt, 2014). Maintenance of minimum oxygen uptake rates was associated with increases in haemoglobin concentrations and plasma bicarbonate, presumably to buffer the blood acidosis in epaulette sharks (Heinrich *et al.*, 2014), and increases in plasma bicarbonate and sodium in small-spotted catsharks (Green and Jutfelt, 2014). Generally, sharks (e.g., epaulette sharks, puffadder shysharks, and small-spotted catsharks) have demonstrated excellent acid-base buffering capacity when exposed to ocean acidification conditions (Green and Jutfelt, 2014; Heinrich *et al.*, 2014; Dziergwa *et al.*, 2019; Pegado *et al.*, 2019). Epaulette sharks also maintained hypoxia tolerance under acidification conditions (Heinrich *et al.*, 2014). Conversely, brown-banded bamboo sharks experienced reductions in the activity of digestive enzymes and aerobic enzymes in the muscle, and increases in anaerobic enzyme in the muscles that were associated with maintenance of minimum oxygen uptake rates (Rosa *et al.*, 2014, 2016a, 2016b). Oxygen uptake rates of rays (*A. radiata*, *L. erinacea*, and *R. eglanteria*) were differentially sensitive to acidification relative to sharks. Minimum oxygen uptake rates increased in response to acidification in both clearnose and little skates (Di Santo, 2015, 2016; Schwieterman *et al.*, 2019b); there was no effect in thorny skates. Maximum oxygen uptake rates and aerobic scope were unaffected by acidification in clearnose and thorny skates, but acidification reduced hypoxia tolerance in both species (Schwieterman *et al.*, 2019b). In little skates, aerobic scope was unaffected by acidification although maximum oxygen uptake rates increased and recovery from exercise lasted longer in acidified conditions (Di Santo, 2016). Thus, ocean acidification alone has the potential to affect fitness and physiological performance in sharks and rays. There is value in testing the effects of individual global climate change stressors but, as sharks and rays appear to exhibit some resilience to

simulated ocean acidification conditions, studies that test the combined effects of multiple stressors (i.e., ocean warming and acidification) are needed and will be more representative of end-of-century conditions.

Interactive effects of ocean warming and acidification

Together, ocean warming and acidification are predicted to have interacting effects on survival, growth, and oxygen uptake rates in sharks and rays. Only five species have been tested under combinations of temperature and pCO_2 that are representative of end-of-century conditions, and three of these species display a range or interaction effects across multiple physiological systems. Only oxygen uptake rates were tested in clearnose and thorny skates in one study and will not be discussed here (Schwieterman *et al.*, 2019b). In brown-banded bamboo sharks, high temperature and pCO_2 reduced the condition, metabolism, and survival of sharks with a larger effect size than was observed for each stressor in isolation (Rosa *et al.*, 2014). In addition, high temperature and pCO_2 acted antagonistically to reduce digestive enzyme activities in sharks when compared to sharks maintained under current levels (Rosa *et al.*, 2016a), which suggests that sharks will be at a disadvantage in meeting higher energy demands under high temperature and pCO_2 (Rosa *et al.*, 2014). Further, brown-banded bamboo sharks exhibited the highest levels of peroxidative damage in the brain that were associated with reduced body condition and survival at high temperatures and pCO_2; antioxidant enzyme activity in the brain and muscle was also elevated under high temperatures and pCO_2, but the magnitude of this effect was not as extreme as peroxidative brain damage (Rosa *et al.*, 2016b). In little skates, aerobic scope, body mass, and the intensity of exercise performance were reduced under high temperature and pCO_2 in juveniles (Di Santo, 2016), but not embryos (Di Santo, 2015). In another study, body condition of little skates was reduced by high temperature and pCO_2, and temperature and pCO_2 had opposing effects on skeletal mineralisation (Di Santo, 2019). Finally, in Port Jackson sharks the combined effects of high temperature and pCO_2 acted antagonistically to impair foraging behaviour (Pistevos *et al.*, 2017), reduce growth (Pistevos *et al.*, 2015), and affect skeletal mineralisation (Pistevos *et al.*, 2019). Altogether, the few experiments conducted thus far demonstrate that acclimation to representative end-of-century temperature and pCO_2 conditions reduces survival, growth, and aerobic metabolic performance in temperate and tropical sharks and rays. These few studies present compelling evidence that sharks and rays experience physiological responses under combined ocean acidification and warming conditions with the potential for population- or ecosystem-level consequences. Indeed, these studies also suggest inconsistent and variable effects of temperature on pCO_2 conditions on shark and ray physiology. As these two stressors can exhibit seemingly unpredictable, interactive effects, there is a critical need for studies that consider the effects of multiple global change stressors in sharks and rays.

Climate change effects on the behaviour of sharks

Predator-prey behaviours of sharks are predicted to respond to ocean warming and acidification. Behavioural responses to simulated ocean acidification conditions, warming conditions, or both global change stressors have been tested only in sharks and not rays (Rosa *et al.*, 2017). Changes in behaviour are typically investigated as a physiological consequence of ocean acidification conditions because increasing pCO_2 is associated with disrupted $GABA_A$ inhibitory neurotransmitter function (Nilsson *et al.*, 2012; Heuer *et al.*, 2016) and reductions in olfactory system and central brain function (Porteus *et al.*, 2018). Behaviour experiments have been conducted in Port Jackson sharks, epaulette sharks, smooth dogfish (*Mustelus canis*), and small-spotted catsharks with a focus on foraging, activity levels, shelter seeking, and lateralisation. In most cases, sharks' ability to locate food through olfaction was decreased by ocean acidification conditions (Dixson *et al.*, 2015; Pistevos *et al.*, 2015, 2017); however, foraging was unaffected by acidification conditions in epaulette sharks (Heinrich *et al.*, 2016). Activity levels are generally unaffected by acidification conditions (Dixson *et al.*, 2015; Heinrich *et al.*, 2016; Pistevos *et al.*, 2017), but activity patterns were altered in small-spotted catsharks (Green and Jutfelt, 2014). Of other measured behaviours, shelter seeking was unaffected by acidification conditions in epaulette sharks (Heinrich *et al.*, 2016). Turning preference (i.e., relative lateralisation) was unaffected by acidification conditions in small-spotted catsharks; yet, the strength of turning preference (i.e., absolute lateralisation) was increased (Green and Jutfelt, 2014). The combined effects of warming and acidification conditions were tested on the behaviour of Port Jackson sharks and revealed that, while temperature increased activity levels independent of acidification, acidification had an antagonistic effect on foraging behaviour, which suggests that hunting becomes energetically less efficient under a more realistic warming and acidification scenario (Pistevos *et al.*, 2015, 2017). Finally, the effects of ocean warming during embryonic development were tested on behaviours relating to cognitive function in Port Jackson shark hatchlings. Interestingly, these studies suggest that sharks develop a strong turning preference in response to warming (Vila Pouca *et al.*, 2018) and that learning performance was improved with warming despite increased embryo mortality (Vila Pouca *et al.*, 2019). Overall, these data support the claim that acidification and warming conditions affect behaviour in sharks (Rosa *et al.*, 2017), and hunting behaviour in sharks will deviate from optimal foraging as transport costs and time invested in finding prey increase. However, it will be necessary to identify the possible effects of ocean acidification and warming on predator-prey behaviours in high trophic level species with greater potential to affect ecosystem processes than small mesopredator species (Nagelkerken and Munday, 2016; Hammerschlag *et al.*, 2019), which may serve functionally redundant ecosystem roles (Heupel *et al.*, 2014; Frisch *et al.*, 2016; Roff *et al.*, 2016).

1.4 Knowledge gaps concerning global change effects in sharks

Mechanisms of vulnerability to climate change are unclear in sharks and rays. Only two species (*C. punctatum*; Rosa *et al.*, 2014, 2016a, 2016b; *L. erinacea*; Di Santo, 2015, 2016, 2019; Di Santo *et al.*, 2016) have undergone comprehensive laboratory examination of the effects of global change phenomena (ocean warming, acidification, and deoxygenation) on traits like aerobic scope, growth, and survival to predict climate change effects on fitness. In particular, aerobic scope is a key trait that is hypothesised to underpin species' vulnerability to climate change (*via* OCLTT); although, studies of the effects of temperature and pCO_2 on aerobic scope in sharks and rays are very few in number (Green and Jutfelt, 2014; Crear *et al.*, 2019; Schwieterman *et al.*, 2019b), and no study has examined the effects of oxygen on aerobic scope in sharks and rays. As such, the applicability of overarching, mechanistic frameworks like OCLTT or MPMO to predict global climate change effects in sharks and rays is extremely limited (Lefevre, 2016). Yet, many shark and ray species achieve prohibitively large body sizes as sub-adults or adults for measurement of physiological traits like aerobic scope (but techniques are becoming available; Payne *et al.*, 2015; Lawson *et al.*, 2019). Instead, predicting climate change effects on populations of neonates or juveniles is a valuable endeavour because these early life history stages are predicted to have a more constrained aerobic scope than older life history stages, which predicts greater vulnerability to climate change stressors by OCLTT (Pörtner *et al.*, 2017). Further, there is value in studying climate change effects on populations of shark and ray juveniles because high rates of juvenile survival are critical to support low population growth rates that are characteristic of many sharks and rays (Kindsvater *et al.*, 2016). Moving forward, there is a need for studies of sharks and rays that not only generate knowledge of the performance of key physiological and behavioural traits under relevant climate change conditions, but validate findings of laboratory studies against trends observed *in situ* to suggest potential mechanisms underlying the responses of sharks and rays to climate change.

1.5 Studying reef shark neonates as experimental models in a tropical nursery area

In this thesis, I address the knowledge gaps outlined in the previous section by studying reef shark neonates in a tropical nursery area. My experimental models are the blacktip reef shark (*Carcharhinus melanopterus*) and sicklefin lemon shark (*Negaprion acutidens*). Blacktip reef sharks take 4-8 years to reach sexual maturity (Chin *et al.*, 2013), have litters of 3-4 pups after 10-11 months of gestation (Stevens, 1984; Porcher, 2005; Chin *et al.*, 2013), achieve a maximum total length of 1.5 m (Papastamatiou *et al.*, 2009a; Mourier *et al.*, 2013b), and are considered high-level mesopredators as adults (Frisch *et al.*, 2016; Roff *et al.*, 2016). Sicklefin lemon sharks reach sexual maturity at approximately 2.2 m total length, have litters of up to 12 pups following 10-11 months of gestation (Stevens, 1984), achieve a maximum total length of at least 3 m and (Clua *et al.*, 2010; Mourier *et al.*, 2013a), as adults, are considered apex predators (Frisch *et al.*, 2016). As neonates, however, both

species occupy functional roles as mesopredators in their respective ecosystems (Heupel *et al.*, 2014). Further, neonates of these species co-occur across their range (Matich *et al.*, 2017; Oh *et al.*, 2017b; Weideli *et al.*, 2019b), and their tendency to exhibit small home ranges (Papastamatiou *et al.*, 2009a; Oh *et al.*, 2017b; George *et al.*, 2019) and predictable use of habitats as putative nursery areas (Mourier and Planes, 2013; Mourier *et al.*, 2013a) means that both species can reliably be collected for use in laboratory experiments with sufficient replication. The small size of neonates (< 70 cm total length; Mourier *et al.*, 2013b; Weideli *et al.*, 2019), amenability to captivity (Baldwin and Wells, 1990; Wells *et al.*, 1992; Davie *et al.*, 1993; Chin *et al.*, 2015), and ability to actively ventilate *via* buccal pumping (personal observation) facilitate the use of these animals in laboratory experiments, including respirometry that restricts the capacity for swimming for ram ventilation. Finally, there is added value in studying the effects of global change on tropical reef sharks because tropical marine ectotherms are predicted to live at temperatures closer to their upper thermal limits relative to temperate species (Comte and Olden, 2017; Stuart-Smith *et al.*, 2017). Tropical species are adapted to a narrow temperature range relative to temperate species (Payne and Smith, 2017), such that increases of only several degrees could lead to reductions in physiological performance of individuals with the potential for population-level fitness consequences (Rummer *et al.*, 2014). Thus, the amenability of these species to laboratory experiments and reliable access to animals *in situ* makes them ideal for combining laboratory and field approaches to test mechanisms of vulnerability to climate change.

My study site is the island of Moorea in French Polynesia, where blacktip reef shark and sicklefin lemon shark populations have been extensively studied since 2007 (Mourier and Planes, 2013; Mourier *et al.*, 2013a). Both populations are fragmented and have low genetic diversity (Mourier and Planes, 2013; Vignaud *et al.*, 2014); adult populations of blacktip reef sharks and sicklefin lemon sharks around Moorea are made up of approximately 240 and 40 individuals, respectively (Mourier *et al.*, 2012, 2013a). Both species give birth during austral summer in shallow, nearshore habitats that may serve as putative nursery areas to supposedly increase fitness in these populations (Mourier and Planes, 2013; Mourier *et al.*, 2013a). Neonates exhibit high fidelity to parturition sites and maintain very small home ranges (Mourier and Planes, 2013; Mourier *et al.*, 2013a), which suggests that neonates experience dynamic environmental conditions of these shallow, nearshore habitats with little capacity for redistribution. Neonatal reef sharks around Moorea typically occur in water less than a meter deep and within 50 m of shore. The first six months of the neonate phase, in which natural mortality can be highest among neonate shark populations (Heupel and Simpfendorfer, 2002), coincides with austral summer. Average summer water temperature in neonate habitat is approximately 30 °C and can experience diel fluctuations from 26-36 °C (personal observation). Daily pH and pCO_2 oscillations in Moorea's lagoon range from 8.20 and 280 µatm, respectively, during the day to 7.96 and 550 µatm, respectively, at night (Comeau *et al.*, 2014); however, fluctuations in neonate habitat can be more extreme (e.g., pH 7.92-8.47; personal observation). As oxygen and pCO_2 oscillations are commonly

associated in aquatic systems (Gobler and Baumann, 2016), dissolved oxygen concentration fluctuates between supersaturation (i.e., > 100% air saturation or 6 mg O_2 L^{-1}) during the day and hypoxia (i.e., ~ 50% air saturation or 3 mg O_2 L^{-1}) at night (personal observation). Climate change is predicted to have an impact on these abiotic conditions with unknown consequences for resident shark populations (Ward-Paige and Worm, 2017; Andréfouët and Adjeroud, 2019). Relative to other regions, French Polynesia is projected to experience average ocean warming and below-average ocean acidification (Pendleton *et al.*, 2016). Climate change projections for the year 2100 for the tropical Pacific include 2.5-3.0 °C of ocean warming and an increase in pCO_2 from an average in the lagoon of 400 µatm to 1000 µatm (Comeau *et al.*, 2014; Field *et al.*, 2014; Le Moullac *et al.*, 2016). Ocean warming is also predicted to exacerbate deoxygenation (Breitburg *et al.*, 2018). It is interesting to note that targeted shark fishing has been banned throughout French Polynesia since 2006 (Ward-Paige and Worm, 2017). Thus, in the absence of this major threat, Moorea is an ideal system for studying the isolated effects of global climate change on reef shark populations.

1.6 Thesis aims and outline

The purpose of this thesis is to investigate mechanisms of vulnerability to climate change in reef shark neonate populations. Specifically, I aim to combine field observation of thermal dependence of various performance traits with laboratory characterisation of environmental tolerance thresholds and acclimation responses of performance traits to simulated climate change conditions to define vulnerability. Overall, my thesis addresses the hypothesis that climate change conditions reduce performance of key physiological traits (e.g., aerobic scope, growth, environmental stress tolerance) that are associated with fitness in reef shark neonates. To test this overarching hypothesis, my thesis investigates the following hypotheses:

> **Chapter 2**: growth and routine oxygen uptake rates exhibit minimal thermal dependence *in situ*;
>
> **Chapter 4**: stress physiological status, maximum oxygen uptake rates, and recovery exhibit thermal dependence *in situ*;
>
> **Chapter 5**: thermal preference *in situ* is associated with effects of acute temperature change on blood-oxygen binding, and effects of thermal acclimation on environmental tolerance thresholds, oxygen uptake rates, and growth;
>
> **Chapter 6**: acclimation to simulated ocean warming and acidification conditions interactively affect lateralisation, activity, environmental tolerance thresholds, oxygen uptake rates, and physiological status.

I address these hypotheses in four corresponding data chapters and a literature review (**Chapter 3**), and then I discuss my overarching hypothesis in a final, synthesis chapter (**Chapter 7**).

In **Chapter 2**, I test **Hypothesis #1** by pairing long-term temperature records with catch data to define thermal dependence of growth rate, and by exposing sharks in the laboratory to simulated extreme diel temperature fluctuations to define thermal dependence of routine oxygen uptake rates. This approach makes it possible to define the sensitivity of fitness-related performance traits to temperature *in situ*.

In **Chapter 3**, I conduct a systematic literature review to understand how oxygen uptake rates can be used to predict changes in fitness in reef sharks. In so doing, this chapter sets a foundation for **Chapters 4-6** that test these metrics and their applicability to predicting changes in fitness in reef sharks.

In **Chapter 4**, I test **Hypothesis #2** by exposing two reef shark species to an exercise challenge *in situ* and measuring sharks' stress physiological status and oxygen uptake rates, and extrapolating recovery times. I test sharks at a range of seasonal temperatures to define thermal dependence of these physiological traits. This approach makes it possible to define reef sharks' physiological sensitivity to temperature while under stress.

In **Chapter 5**, I test **Hypothesis #3** by 1) defining the effects of acclimation temperature on oxygen uptake rates, environmental tolerance thresholds, and growth *ex situ*, 2) defining the temperature- and pH-sensitivity of blood-oxygen binding *in vitro*, and 3) observing thermal preference of sharks *in situ*. By relating thermal dependence of various physiological traits at different timescales (i.e., acute *vs* acclimation) to thermal preference *in situ*, this approach makes it possible to understand reef sharks' capacity to respond physiologically and behaviourally to temperature change.

In **Chapter 6**, I test **Hypothesis #4** by defining the combined effects of multiple global change stressors in a model reef shark. I test behaviour (i.e., lateralisation, activity) and physiological performance (i.e., environmental tolerance thresholds, oxygen uptakes, stress physiological status) under simulated ocean warming and acidification conditions. As such, this approach makes it possible to understand the resilience of a reef shark to global change stressors.

In **Chapter 7**, I synthesise **Chapters 2-6**. This approach makes it possible to discuss my overarching hypothesis in the context of previous work and the new knowledge generated by this thesis.

This thesis offers a novel evaluation of the potential mechanisms of vulnerability to climate change in marine ectotherms, using a reef shark model. The data presented in this thesis may contribute to further research in the fields of ecophysiology, elasmobranch biology and ecology, global change biology, and conservation physiology. The significance of this thesis is that the data presented herein advance knowledge of the effects of global climate change stressors in marine ectotherms. Further, the broader impact of this thesis will be the contribution of information on environmental tolerance thresholds and consequences of environmental change for future efforts to conserve, manage, and protect shark populations threatened with anthropogenic environmental change. Fishing is the greatest threat to sharks, but climate change is an emerging threat that must be understood to predict whether sharks – a

group of animals that have survived all five previous mass extinctions – will succumb to an Anthropocene extinction.

Chapter 2: Defining temperature dependence of growth and metabolic rate of reef shark neonates in a nursery area system

2.1 Summary

Using habitats as nursery areas is a fitness-enhancing behaviour of marine fishes and invertebrates However, very little is known about the role that temperature, an influential controlling factor of the physiology of fishes, plays in the suitability of habitats as nursery areas. Sharks are known to use shallow, nearshore habitats that experience extreme and dynamic thermal conditions as nursery areas. Therefore, it is hypothesised that fitness related physiological traits should exhibit minimal thermal dependence for sharks to successfully exploit nursery areas. Here, I tested the thermal dependence of growth and metabolic rate of reef shark neonates in a nursery area system to advance our understanding of shark nursery area use. I first determined if ten sites around Moorea, French Polynesia met nursery area criteria for *Carcharhinus melanopterus* and *Negaprion acutidens* neonate populations using four consecutive years of abundance survey data. I defined thermal dependence of growth *in situ* by modelling growth in recaptured individuals in response to thermal exposure. Thermal dependence of metabolic rate was quantified *ex situ* using respirometry. Both species exhibited differential habitat use and sensitivity to temperature. There was no evidence of nursery area use in *C. melanopterus*. However, *N. acutidens* used two sites as a larger nursery area. Thermal exposure was not a better predictor of growth in recaptured sharks than time-at-liberty. Metabolic rate less than doubled with a 10 °C increase in temperature. Together, minimal thermal dependence of these traits supports the hypothesis that reef shark neonates use Moorea as a nursery area system.

Associated publication

There is no publication associated with this chapter.

Data availability

Data presented in this manuscript are available from the Research Data Repository (Tropical Data Hub) at James Cook University: https://doi.org/10.25903/5eb256b915630

2.2 Introduction

Nursery areas are key aspects of the ecology of many marine and estuarine fishes and invertebrates. Beck *et al*. (2001) originally hypothesised that nursery areas for juveniles of a species are habitats that must contribute a higher density of recruits to the adult, reproductive population when compared to other adjacent, non-nursery habitats. As such, using nursery areas is a fitness-maximising behaviour (Beck *et al.*, 2001; Fodrie *et al.*, 2009; Nagelkerken *et al.*, 2015). The nursery area concept has received particular attention among studies examining elasmobranch fishes (Heupel *et al.*, 2007, 2018; Martins *et al.*, 2018). Elasmobranch fishes (sharks and rays) exhibit life-history traits that are characteristic of *K*-selected organisms (Cortés, 2000); long generation times render many species extremely vulnerable to population declines resulting from anthropogenic impacts (Dulvy *et al.*, 2014). As such, there is interest in identifying shark and ray nursery areas for conservation and management of imperilled species because of the supposed fitness benefits associated with these habitats (Heupel *et al.*, 2007; but see also Kinney and Simpfendorfer, 2009).

The nursery area concept proposed by Beck *et al*. (2001) has since been refined to identify shark nursery areas. Three criteria proposed by Heupel *et al.* (2007) are: 1) juvenile sharks are more abundant in nursery areas relative to other habitats, 2) juveniles are resident within nursery areas for extended periods of time, and 3) use of nursery areas by juvenile sharks is stable over time (Heupel *et al.*, 2007). Key assumptions underlying the fitness benefits of shark nursery areas are that nursery areas provide ample prey abundance and refuge from predation to improve survival of juveniles to maturity (Heithaus, 2007; Heupel *et al.*, 2007). Many studies on shark nursery areas only go so far as to test the criteria to identify nursery areas (Heupel *et al.*, 2018), but do not evaluate the associated effects on fitness. Prey and refuge benefits are not qualities of all nursery areas, as some are either characterised by one or the other. Such examples have been published for scalloped hammerhead sharks (*Sphyrna lewini*) in a food-limited nursery area (Bush and Holland, 2002; Lowe, 2002; Duncan and Holland, 2006). It is probable that other unidentified benefits of nursery area use also contribute to fitness.

Temperature is a powerful controlling factor of the physiology of ectotherms (Fry, 1947) and is, therefore, likely to influence the benefits associated with shark nursery areas. This idea was originally proposed as an area for further research by Heithaus (2007). Previous studies have quantified the effects of temperature on metabolic rates in several shark species (Lear *et al.*, 2017); yet, few have directly applied these data within the context of nursery area use. One example includes bioenergetic modelling in juvenile sandbar sharks (*Carcharhinus plumbeus*) in a Chesapeake Bay nursery area (Dowd *et al.*, 2006a, 2006b; Crear *et al.*, 2019). Similarly, bioenergetics modelling has been applied to predict temperature effects on mass loss in freshwater sawfish (*Pristis pristis*) and euryhaline bull sharks (*Carcharhinus leucas*) in nursery area systems (Lear *et al.*, 2020). Another study observed an association between temperature and abundance of juvenile *C. leucas* that revealed a potential shift in

nursery area use (Bangley *et al.*, 2018). Activity levels have also been examined in juvenile sawfishes (*P. pectinata* and *P. pristis*), lemon sharks (*Negaprion brevirostris*), nurse sharks (*Ginglymostoma cirratum*), and *C. leucas* to examine the sensitivity of this performance trait to temperature across various nursery area systems (Lear *et al.*, 2019, 2020). Indeed, understanding the sensitivity of physiological performance traits to temperature (i.e., thermal dependence) can explain habitat use in fishes (Gannon *et al.*, 2014; Payne *et al.*, 2016, 2018).

Sharks in nursery areas should exhibit minimal thermal dependence of physiological performance. Shallow, nearshore habitats where shark nursery areas may occur can be dynamic environments that experience extreme environmental conditions (Knip *et al.*, 2010). Neonate and juvenile sharks in nursery areas can exhibit site fidelity and small home ranges (Chapman *et al.*, 2009), which suggests that these animals can physiologically tolerate extreme abiotic conditions without leaving the safety of their nursery areas. For example, juvenile *P. pristis* in their Fitzroy River nursery area experience temperatures that exceed their optimal temperature for activity for almost 40% of the year (Lear *et al.*, 2019). Further, sharks and rays that are confined to nursery area systems seem to exhibit low thermal sensitivity of activity levels and metabolic rate (Lear *et al.*, 2019, 2020), which is adaptive because high thermal sensitivity in highly variable thermal environments can be energetically costly (da Silva *et al.*, 2019). This also broadly agrees with evidence that juvenile fishes have greater thermal tolerance than other ontogenetic stages (Dahlke *et al.*, 2020). Thus, examining thermal dependence of fitness related traits (e.g., metabolic rate) may contribute to an understanding of how sharks exploit habitats as nursery areas.

The purpose of this study was to understand the effects of temperature on the growth and metabolic rates of neonatal reef sharks in putative shark nursery areas. I hypothesised that these fitness related traits should exhibit minimal thermal dependence if Moorea is a nursery area system. My first objective was to identify shark nursery areas, following criteria proposed by Heupel et al. (2007), for two reef shark species found around Moorea, French Polynesia. My second objective was to characterise thermal dependence of growth *in situ* and routine metabolic rate *ex situ*. Together, combining abundance surveys with physiological data makes it possible to determine whether Moorea is a nursery area system for neonatal reef sharks. These data have significance for advancing nursery area theory so that we may better understand why habitats function as nursery areas, the benefits sharks derive from these habitats, and whether environmental stressors (e.g., marine heatwaves, climate change) may ultimately affect the suitability of habitats as shark nursery areas.

2.3 Materials and methods

Authorisation to collect, posses, and transport sharks and shark tissues in French Polynesia was obtained from the Ministère de la Promotion des Langues, de la Culture, de la Communication, et de

l'Environnement of French Polynesia (Arrêté N° 9524). Experimental protocols were approved by the James Cook University Animal Ethics Committee (A2089 & A2394).

The Centre de Recherches Insulaires et Observatoire de l'Environnement (CRIOBE) has conducted a fisheries-independent survey of neonate and juvenile sharks at 14 unique sites around Moorea since 2007 (Mourier and Planes, 2013). Here, I fished the ten most productive sites over three parturition seasons (October-March) during 2016-2019, and I included available data from the previous fishing season (i.e., 2015-2016). The ten sites – Apaura, Haapiti, Maharepa, Paorea, Papetoai, Pihaena, Tiki, Vaiane, Vaiare, and Valorie – are distributed around the entire perimeter of Moorea (Figure 2.1). Two species of sharks were targeted in these sites: the blacktip reef shark (*Carcharhinus melanopterus*) and the sicklefin lemon shark (*Negaprion acutidens*).

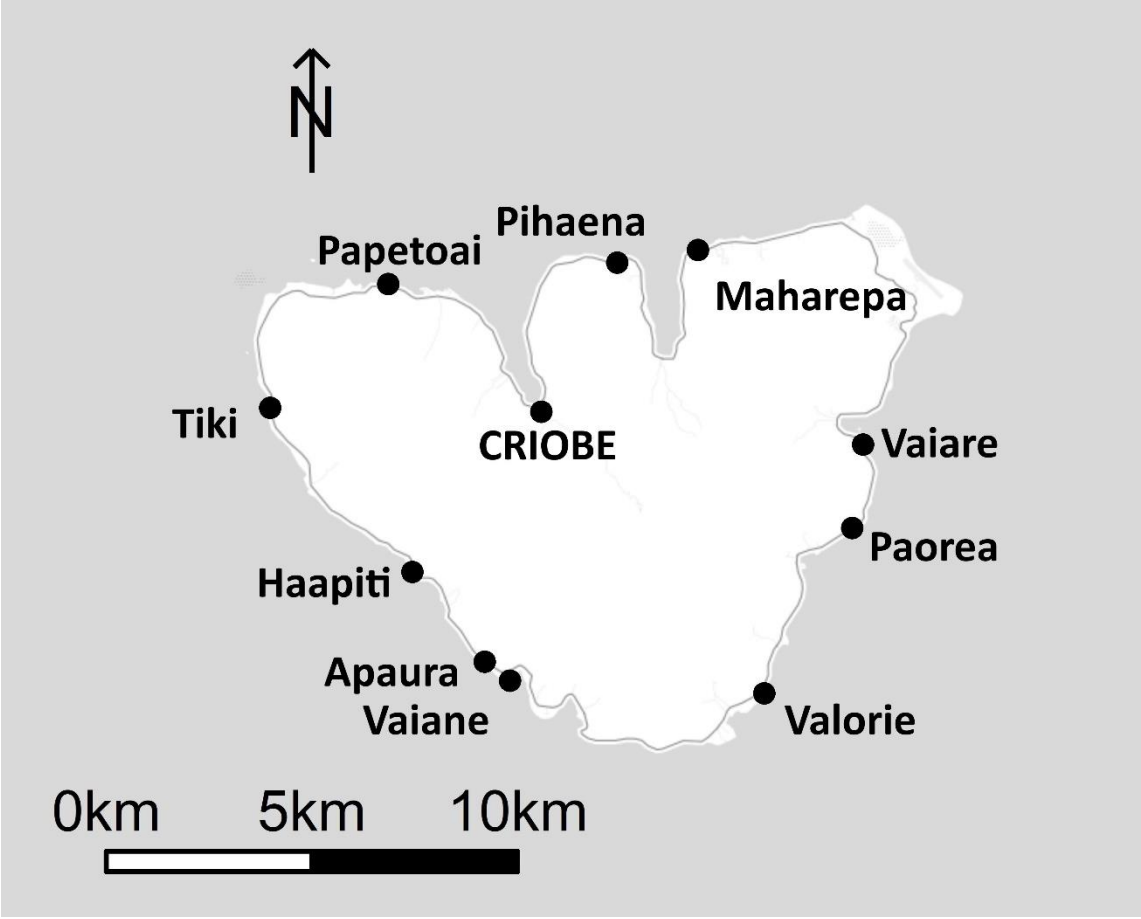

Figure 2.1 Shark capture locations and putative nursery areas around Moorea, French Polynesia (S 17°30'; W 149°50'). Some animals were brought to the Centre de Recherches Insulaires et Observatoire de l'Environnement (CRIOBE) to characterise physiological performance.

Experimental model and subject details

Carcharhinus melanopterus

Blacktip reef sharks were captured at ten sites. All *C. melanopterus* collected for this study were neonates or juveniles aged 0-1 years as confirmed by the presence of open or recently closed umbilical scars (Chin *et al.*, 2015). Although the sex of individuals can be visually identified, sex was not included in analyses or interpretation of the data because *C. melanopterus* do not sexually mature until 4-8 years of age (Chin *et al.*, 2013).

Throughout the study, *C. melanopterus* were captured using monofilament gillnets (50 m long by 1.5 m depth; 5 cm mesh size, 10 cm stretch) set perpendicular from shore that were fished from approximately 1700-2000 throughout the parturition season (Mourier and Planes, 2013). Total fishing effort was 92 sets totalling 242 hours in 2015/2016, 90 sets totalling 247 hours in 2016/2017, 78 sets totalling 206 hours in 2017/2018, and 93 sets totalling 221 hours in 2018/2019, resulting in the capture of 147, 152, 135, and 94 individual sharks, respectively. All *C. melanopterus* that were captured throughout the study were tagged for identification in the event of a recapture with a coloured T-bar (Hallprint, Hindmarsh Valley, SA, Australia) or a passive integrated transponder (Biolog-id SAS, Paris, France), weighed to the nearest 10 g in a soft plastic bag suspended from a digital scale, and measured to estimate body condition (Weideli *et al.*, 2019a). Weights and total lengths (curvilinear, snout to upper caudal lobe tip) of *C. melanopterus* during each season were 0.65-1.36 kg and 510-800 mm, 0.72-1.97 kg and 513-750 mm, 0.67-1.67 kg and 502-658 mm, and 0.54-1.88 kg and 476-684 mm, respectively. Of these sharks, 12, 24, 23, and 15 individuals were recaptured at least once during each season, respectively. Capture durations were typically under five minutes, and processing lasted 10-15 minutes before releasing *C. melanopterus* at their original capture site.

A subset of *C. melanopterus* (n = 10) were transported from two sites, Papetoai and Vaiare to the CRIOBE. Prior to departing, sharks were held in individual vinyl bags (0.2 m diameter and 1.0 m long) with mesh ends to allow water flow for no more than 30 minutes post-capture. Sharks were then transported by car in 200 L insulated coolers in groups of 1-3 sharks per cooler. Water in coolers was continuously aerated, and transport from the farthest site, Vaiare, took approximately 30 minutes whilst transport from the closest site, Papetoai, took approximately 10 minutes. At the CRIOBE, sharks were maintained in groups of five in two 1250 L, circular holding tanks. The wet lab holding facility at the CRIOBE supplied tanks continuously with sand filtered seawater from Opunohu Bay. Holding tanks were covered with 60% shade cloth that exposed animals to a natural photoperiod. After approximately one week of habituation, water temperatures were adjusted in 0.5 °C day^{-1} increments using chillers (TK-1000/2000, TECO S.r.l., Ravenna, Italy) to a target temperature of 28 °C that was maintained for two weeks. Sharks were fed 5% of their body mass in fresh tuna (*Thunnus* spp.) supplied from a local restaurant. However, sharks were fasted for 48 hours prior to experimentation. After 22-32 days in

captivity and following experimentation, sharks were released to their original site of capture using the identical transport methods described previously.

Negaprion acutidens

Sicklefin lemon sharks were collected and treated identically to *C. melanopterus*. Neonatal *N. acutidens* were present at all sites except for Paorea and Vaiare. In addition, *N. acutidens* was observed to move between Apaura and Vaiane; these two sites were, therefore, treated as one ('Apaura-Vaiane') for this species. All *N. acutidens* collected for this study were neonates or juveniles. The age of juveniles could not be determined; however, these sharks were confirmed to be immature because size-at-maturity is approximately 2200 mm (Stevens, 1984). Fishing yielded 30 individual sharks in 2015/2016, 43 in 2016/2017, 26 in 2017/2018, and 77 in 2018/2019. Weights and total lengths of *N. acutidens* were 0.94-3.65 kg and 582-872 mm, 0.92-2.92 kg and 594-850 mm, and 0.88-6.66 kg and 566-1100 mm, respectively for 2016-2019. Sharks were not weighed in 2015/2016; total lengths of *N. acutidens* caught during 2015/2016 were 580-940 mm. Of these sharks, 12, 4, 9, and 30 individuals were recaptured at least once during each season, respectively. In 2018/2019, a subset of *N. acutidens* (n = 8) were transported from two sites, Pihaena and Tiki, to the CRIOBE. Sharks were housed in two groups of four under identical conditions as described for *C. melanopterus* and were released to their original site of capture after 22-32 days in captivity and following experimentation using the identical transport methods described previously.

Identifying shark nursery areas

The first study objective was to determine which shark habitats around Moorea serve an ecologically important role as shark nursery areas. Fishing survey data were used to quantify catch-per-unit-effort (CPUE, sharks h^{-1}) for each species per gillnet set per site and per season (i.e., 2015/2016, 2016/2017, 2017/2018, 2018/2019). These CPUE data were then used to test the three shark nursery area criteria proposed by Heupel and colleagues to identify which of the ten sites function as shark nursery areas and which do not (Heupel *et al.*, 2007, 2018). For a location to be identified as a shark nursery area, neonates and juveniles must be more abundant in the target location relative to other locations (criterion 1), individuals must use the target area for extended periods of time (criterion 2), and abundance must not decline at the target location over time (criterion 3) (Heupel *et al.*, 2007, 2018).

Shark nursery area criteria were assessed quantitatively following Froeschke and colleagues (Froeschke *et al.*, 2010), except for criterion 2. Criteria were tested separately for each species. Bootstrap hypothesis testing was used to test criterion 1. All sites were tested for whether sharks were caught during at least one season. For each site, seasonal mean CPUE was scored '1' if its value was larger than the population mean CPUE or '0' otherwise. Then, seasonal mean CPUE was sampled with replacement (n = 1000), and each iteration was scored as described above. Significance was determined by calculating *P*-values as the number of iterations with values greater than or equal to the measured value (i.e., the sum of

measured seasonal mean CPUE scores) divided by the total number of iterations. Criterion 1 could therefore be quantitatively assessed by determining which sites have significantly higher CPUE than the population mean for the entire study period. A *P*-value of 0.05 was selected to identify sites as candidate 'nursery areas.' All other sites (i.e., $P > 0.05$) that did not satisfy criterion 1 could not be classified as nursery areas. Analyses were conducted in R using the 'stats' and 'nlme' packages (Pinheiro *et al.*, 2018; R Core Team, 2018).

Criterion 2 was tested using a generalised linear model. The mean seasonal recapture rate for each site was fit with site as a nominal variable, assuming log-ratio transformed recapture rate data followed a Gaussian distribution. Sites were only tested if sharks were recaptured during three or more seasons. In a second model, time at liberty (i.e., the elapsed time between recapture events) was fit with site as a nominal variable, assuming time at liberty data followed a Poisson distribution. For both models, confidence intervals of effect size were produced for all sites. Confidence intervals of effect size of fixed effects terms were generated from 1000 posterior simulations using the 'arm' R package (Gelman and Su, 2018). Criterion 2 could therefore be quantitatively assessed by determining whether recapture rates and time at liberty differed between sites that did and did not satisfy criterion 1. Together, these analyses made it possible to determine whether recapture rates and time at liberty differed between sites and over time.

Criterion 3 was tested using a weighted least-squares regression. Mean CPUE was fit with site as a nominal variable, season as a continuous variable, and the interaction of site and season. Sites were only tested where sharks were captured for three or more seasons. Confidence intervals of effect size were produced for all levels of the interaction term so that criterion 3 could be quantitatively assessed by comparing mean effect sizes of the slopes. A positive effect size or an effect with a confidence interval overlapping zero suggests that CPUE was increasing or stable during the study period, respectively. A negative effect size would suggest that CPUE decreased during the study period and would not satisfy criterion 3. This approach made it possible to identify within-site relationships between CPUE and sampling season, focusing on sites that satisfied criterion 1.

Effects of temperature on growth and metabolic rate

The second study objective was to test for thermal dependence of growth and metabolic rate. To accomplish this, I paired environmental temperature data with growth rates measured *in situ* and identified temperature-scaling of metabolic rates in the laboratory.

Growth

Growth was measured using mass data from shark recaptures and temperature data from data loggers at each capture location. At the end of the study, the absolute change in mass was calculated over an individual's longest time at liberty (*C. melanopterus*, mean ± standard deviation = 35.8 ± 40.6 days,

range = 9-300 days; *N. acutidens*, mean ± standard deviation = 65.6 ± 86.4 days, range = 2-395 days). Change in mass was then regressed against growing degree days (GDD, °C days), which is a commonly used temperature-derived metric to model growth performance in fishes (Neuheimer and Taggart, 2007). Environmental temperature data were recorded using one or two temperature loggers (UA-002-64, Onset Computer Corporation, Bourne, MA, USA) that were deployed up to 50 m from shore in representative microhabitats (e.g., in mangrove stands, between coral structures, over sand substrate, etc.) at each site. Loggers recorded temperature continuously at 10-min resolution, producing a four-year time series (2015-2019) at most sites. Where two loggers were deployed, an average was taken of each time-stamped value for subsequent analyses. Temperatures recorded by loggers were assumed to be representative of temperatures experienced by sharks because neonatal sharks around Moorea have very small home ranges; *C. melanopterus* neonates have an average core habitat size of 0.02 km^2 (personal observation).

For each 24-hr period (i.e., 1200-1200), a degree day (DD) was calculated from temperature time series data recorded by temperature loggers deployed at each site using the following equation:

$$DD = \left(\frac{T_{Max} + T_{Min}}{2}\right)$$

where T_{Max} and T_{Min} are the maximum and minimum daily temperature, respectively (Chezik *et al.*, 2014). For each recaptured shark, a GDD value was calculated as the sum of DD during the shark's time at liberty in the relevant site. Owing to variability in recapture rates between sites, change in mass was modelled against GDD for each species across sites following a simple linear relationship (Neuheimer and Taggart, 2007). Change is mass was also regressed against time at liberty for comparison to determine if GDD or time at liberty were better predictors of change in mass.

Metabolic rate

Sharks underwent intermittent-flow respirometry to measure oxygen uptake rates ($\dot{M}O_2$, in mg O_2 kg^{-1} h^{-1}) as a proxy for metabolic rate (Svendsen *et al.*, 2016). Specifically, routine $\dot{M}O_2$ ($\dot{M}O_{2Routine}$) is analogous to an ectotherm's routine metabolic rate (RMR), which is simply any metabolic rate measured from resting to maximum metabolic rate. Temperature-scaling was investigated by exposing sharks to rapid changes in ambient temperature simulating an extreme diel temperature cycle and measuring $\dot{M}O_{2Routine}$ at five temperature steps: 28 °C, 25 °C, 28 °C, 33 °C, and 28 °C (Di Santo and Bennett, 2011). The 8 °C difference is representative of the maximum daily temperature range observed *in situ* (~10 °C; environmental temperature range = 26-36 °C). This experimental design made it possible to test for effects of heating and cooling on $\dot{M}O_2$ by consistently returning to 28 °C.

Sharks were transferred from holding tanks to respirometry chambers and were habituated to 28 °C for six hours. Oxygen uptake rates were then recorded over the final two hours at 28 °C. Water temperature

was then cooled to 25 °C at a rate of 0.88 ± 0.18 (mean ± standard deviation) °C h^{-1}. Oxygen uptake rates were measured for two hours once the system stabilised at 25 °C, after which, water temperature was heated to 28 °C at a rate of 1.15 ± 0.44 °C h^{-1}. Oxygen uptake rates were measured for two hours upon reaching 28 °C, after which, water temperature was further heated to 33 °C at a rate of 1.12 ± 0.27 °C h^{-1}. Oxygen uptake rates were measured for two hours at 33 °C, after which, water temperature was cooled back to 28 °C at a rate 1.35 ± 0.25 °C h^{-1} of for a final two hours of measurement. Overall, heating (1.14 ± 0.34 °C h^{-1}) and cooling rates (1.12 ± 0.32 °C h^{-1}) were similar, but higher than maximum *in situ* rates of temperature change (0.53 ± 0.33 °C h^{-1}). In total, $\dot{M}O_2$ was measured in three two-hour periods at 28 °C, for two hours at 25 °C, and for two hours at 33 °C, for a total trial duration (excluding habituation) of 20.5-25 hours.

Static, intermittent-flow respirometry chambers (32 l volume including recirculating loop tubing; 70 cm long, 24 cm in diameter) were submerged in an aerated water bath (550 l) that was temperature-controlled using an aquarium chiller (TK-1000/2000, TECO S.r.l., Ravenna, Italy). Up to four chambers were tested at a time. Chambers were configured with a 2500 l h^{-1} aquarium pump (EHEIM GmbH & Co KG, Deizisau, Germany) connected to the chamber in a closed, recirculating loop to ensure homogenous mixing of oxygenated water. A second pump was configured to flush water from the external bath into individual chambers and out through overflow tubing above the water's surface. Dissolved oxygen (DO, in mg O_2 l^{-1}) concentrations were measured using robust fibre-optic probes (PyroScience GmbH, Aachen, Germany) that were fed into chambers through the overflow tubing. Probes were connected to a Firesting Optical Oxygen Meter (PyroScience GmbH, Aachen, Germany). A single temperature probe was connected to the oxygen meter and placed into the water bath to allow for temperature compensation of DO measurements; the oxygen meter compensated for barometric pressure with an internal meter and salinity with a manually input value. The flush pump was controlled by a relay device that was automated using custom software on a laptop computer (National Instruments, Austin, TX, USA). The relay turned the flush pumps off for five minutes to yield a measurable decline in DO inside chambers (i.e., the "measurement" phase). Flush pumps were then turned on for 10 minutes to reintroduce oxygenated water from the bath and restore DO inside chambers to saturation (i.e., the "flush" phase). A single flush-measurement phase cycle lasted 15 minutes, and two hours of $\dot{M}O_2$ measurement at each target temperature yielded eight $\dot{M}O_2$ values.

Oxygen uptake rate was calculated following:

$$\dot{M}O_2 = SV_{Resp}M^{-1}$$

where S is the absolute value of the slope of the linear decline in DO during a five-minute measurement phase with a coefficient of determination greater than 0.95 (in mg O_2 l^{-1} s^{-1}), V_{Resp} is the volume of water (in l) in the chamber (i.e., excluding the shark's volume), and M is the shark's mass in kg, measured upon removal from respirometry chambers. Values of S were extracted from raw Firesting meter output

in R using custom code (A. Merciere and T. Norin, unpublished data). At each temperature (i.e., 25, 28, and 33 °C), a single $\dot{M}O_{2Routine}$ value was calculated for each shark as the mean value across all measurements. To account for exogenous sources of respiration inside chambers (i.e., background respiration), $\dot{M}O_2$ measurements were made in empty chambers immediately before and after trials with sharks. Background $\dot{M}O_2$ was accounted for by fitting a line to the two background $\dot{M}O_2$ values that were measured at a known time, predicting the value of background $\dot{M}O_2$ at the time of measurements when sharks were inside chambers, and subtracting background $\dot{M}O_2$ from shark $\dot{M}O_2$ (Rummer *et al.*, 2016).

This approach made it possible to calculate a temperature-scaling quotient (Q_{10}) for $\dot{M}O_{2Routine}$ that characterises the change in $\dot{M}O_{2Routine}$ over a 10 °C change in temperature and is calculated using the following equation:

$$Q_{10} = (K_2 \cdot K_1^{-1})^{(10 \cdot (t_2 - t_1)^{-1})}$$

where K_1 and K_2 are $\dot{M}O_2$ at temperatures t_1 and t_2, respectively. Temperature-scaling quotients were calculated for individuals of each species between each temperature step at 28 to 25 °C, 25 to 28 °C, 28 to 33 °C, and 33 to 28 °C. In addition, overall Q_{10} values were calculated from 25 to 33 °C.

Temperature effects on $\dot{M}O_{2Routine}$ were investigated using linear mixed effects models. For each species, $\dot{M}O_{2Routine}$ was fit with temperature step (i.e., 28 °C [1], 25 °C, 28 °C [2], 33 °C, and 28 °C [3]) as a nominal fixed effect with shark ID as a random effect. Models were validated through visual inspection of plots of model residuals and fitted values (to test homogeneity), model residuals and fixed effects (to test independence), and quantile-quantile plots of model residuals (to test normality). Similarly, Q_{10} was investigated for each species by fitting Q_{10} with temperature range (i.e., 28 to 25 °C , 25 to 28 °C, 23 to 33 °C, and 33 to 28 °C) as a nominal fixed effect with shark ID as a random effect. Significance was determined by generating confidence intervals of effect size for fixed effects terms with posterior simulations (n = 1000).

2.4 Results

Identifying shark nursery areas

Apaura-Vaiane was the only site where CPUE of *N. acutidens* was significantly higher than the population mean and was the only candidate nursery area. As such, this site was considered to have satisfied criterion 1 (Table 2.1; Figure 2.2). All other sites (Haapiti, Maharepa, Paorea, Papetoai, Pihaena, Tiki, Vaiare, and Valorie) were considered non-nursery areas.

Table 2.1 *P*-values from bootstrap hypothesis testing to test shark nursery area criterion 1 (i.e., higher neonate abundance relative to adjacent habitats). Bolded sites were identified as nursery areas ($P < 0.05$).

Carcharhinus melanopterus		*Negaprion acutidens*	
Site	*P*-value	Site	*P*-value
Apaura	1.000	**Apaura-Vaiane**	**0.011**
Haapiti	0.125	Haapiti	1.000
Maharepa	0.811	Maharepa	1.000
Paorea	0.425	Papetoai	1.000
Papetoai	0.108	Pihaena	0.747
Pihaena	0.961	Tiki	0.063
Tiki	0.422	Vaiare	1.000
Vaiane	0.797	-	-
Vaiare	0.447	-	-
Valorie	0.973	-	-

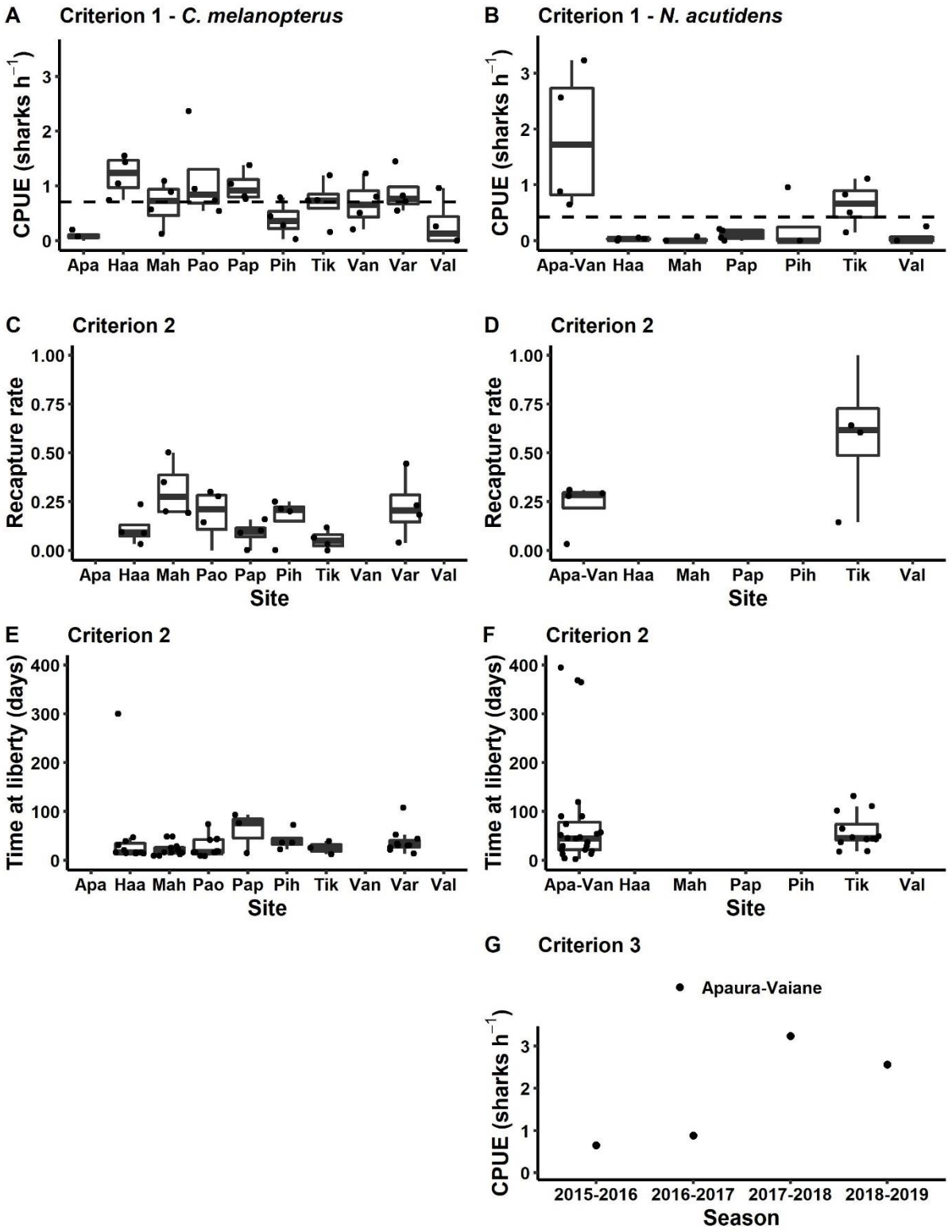

Figure 2.2 Testing shark nursery area criteria for *Carcharhinus melanopterus* (left panels) and *Negaprion acutidens* (right panels) around Moorea, French Polynesia. To qualify as a shark nursery area, habitats must exhibit higher relative neonate abundance (criterion 1), neonates must exhibit residency (criterion 2), and habitat use must be stable through time (criterion 3). Abundance surveys

were conducted over four consecutive parturition seasons from 2015-2019. Individual observations of catch-per-unit-effort (CPUE, panels A, B, and G) represent mean values per season within a site. For recapture rate (panels C and D), individual observations represent the overall recapture rate for each season within a site, whilst individual observations of time at liberty represent values for individual sharks across all four seasons. Criterion 3 is presented only for sites that satisfied criteria 1 and 2. Abbreviations: Apaura, Apa; Apaura-Vaiane, Apa-Van; Haapiti, Haa; Maharepa, Mah; Paorea, Pao; Papetoai, Pap; Pihaena, Pih; Tiki, Tik; Vaiane, Van; Vaiare, Var; Valorie, Val.

Recapture rates did not significantly differ between sites for *C. melanopterus* and *N. acutidens* (Table 2.1; Figure 2.2). However, *C. melanopterus* exhibited significantly different time at liberty between sites; although, these sites did not satisfy criterion 1. Recapture rates did not differ between Apaura-Vaiane and Tiki for *N. acutidens*, but time at liberty was greater at Apaura-Vaiane (Table 2.2; Figure 2.2).

Table 2.2 Generalised linear model output to test shark nursery area criterion 2 (i.e., neonates exhibit residency). Mean effect size and 2.5% and 97.5% confidence interval limits are presented. Bolded sites satisfied criterion 1 (i.e., higher neonate abundance relative to adjacent habitats).

	Carcharhinus melanopterus				*Negaprion acutidens*			
Response	Parameter	Mean	2.5%	97.5%	Parameter	Mean	2.5%	97.5%
Recapture rate	Haapiti	-1.74	-2.58	-0.89	**Apaura-Vaiane**	**-1.08**	**-2.39**	**0.24**
	Maharepa	1.13	-0.07	2.32	Tiki	1.07	-0.94	3.07
	Paorea	0.31	-0.88	1.50	-	-	-	-
	Papetoai	-0.22	-1.41	0.98	-	-	-	-
	Pihaena	0.26	-0.93	1.45	-	-	-	-
	Tiki	-0.51	-1.70	0.68	-	-	-	-
	Vaiare	0.62	-0.58	1.81	-	-	-	-
Time at liberty	Haapiti	3.87	3.78	3.95	**Apaura-Vaiane**	**4.42**	**4.38**	**4.47**
	Maharepa	-0.76	-0.91	-0.61	Tiki	-0.35	-0.44	-0.27
	Paorea	-0.59	-0.73	-0.45	-	-	-	-
	Papetoai	0.24	0.07	0.41	-	-	-	-
	Pihaena	-0.14	-0.32	0.03	-	-	-	-

	Tiki	-0.64	-0.88	-0.40	-	-	-	-
	Vaiare	-0.21	-0.34	-0.08	-	-	-	-

There were significant interactions between site and season for *C. melanopterus* and *N. acutidens*. Catch-per-unit-effort decreased over time for *C. melanopterus* at Maharepa, and at Haapiti and Papetoai for *N. acutidens*. For all other sites, CPUE did not increase or decrease over time (Table 2.3; Figure 2.2). Therefore, Apaura-Vaiane met the criteria of a shark nursery area for *N. acutidens*.

Table 2.3 Weighted least-squares regression output to test shark nursery area criterion 3 (i.e., consistent habitat use over time). Mean effect size and 2.5% and 97.5% confidence interval limits are presented. Bolded sites satisfied criteria 1 (i.e., higher neonate abundance relative to adjacent habitats) and 2 (i.e., neonates exhibit residency).

Carcharhinus melanopterus				*Negaprion acutidens*			
Parameter	Mean	2.5%	97.5%	Parameter	Mean	2.5%	97.5%
Apaura	0.19	0.01	0.38	**Apaura-Vaiane**	**-0.19**	**-2.29**	**1.90**
Haapiti	0.91	-0.19	2.01	Haapiti	0.26	-1.83	2.36
Maharepa	1.18	0.53	1.83	Papetoai	0.27	-1.84	2.39
Paorea	0.45	-1.87	2.78	Tiki	0.20	-2.02	2.42
Papetoai	0.78	-0.06	1.63	Season	0.81	0.04	1.58
Pihaena	0.63	-0.05	1.31	**Season*Apaura-Vaiane**	**0.81**	**-0.87**	**4.41**
Tiki	0.83	-0.33	1.99	Season*Haapiti	-0.83	-1.59	-0.06
Vaiane	0.61	-0.67	1.89	Season*Papetoai	-0.79	-1.57	-0.02
Vaiare	1.34	0.71	1.97	Season*Tiki	-0.55	-1.37	0.26
Season	-0.04	-0.11	0.02	-	-	-	-
Season*Apaura	-0.04	-0.19	0.10	-	-	-	-
Season*Haapiti	0.08	-0.32	0.48	-	-	-	-

Season*Maharepa	-0.24	-0.48	-0.01	-	-	-	-
Season*Paorea	0.24	-0.61	1.09	-	-	-	-
Season*Papetoai	0.05	-0.26	0.36	-	-	-	-
Season*Pihaena	-0.13	-0.38	0.11	-	-	-	-
Season*Tiki	-0.08	-0.51	0.34	-	-	-	-
Season*Vaiane	-0.01	-0.47	0.46	-	-	-	-
Season*Vaiare	-0.22	-0.45	0.01	-	-	-	-

Effects of temperature on growth and metabolic rate

Growth

Growing degree days exhibited strong positive relationships with growth (i.e., increase in mass during time at liberty) in *C. melanopterus* (mean effect size = 0.12, 95% confidence interval = 0.09-0.14) and *N. acutidens* (mean effect size = 0.24, 95% confidence interval = 0.20-0.27; Figure 2.3). Similarly, time at liberty was an equally good predictor of growth (*C. melanopterus*, mean effect size = 3.47, 95% confidence interval = 2.79-4.14; *N. acutidens*, mean effect size = 6.81, 95% confidence interval = 5.90-7.72).

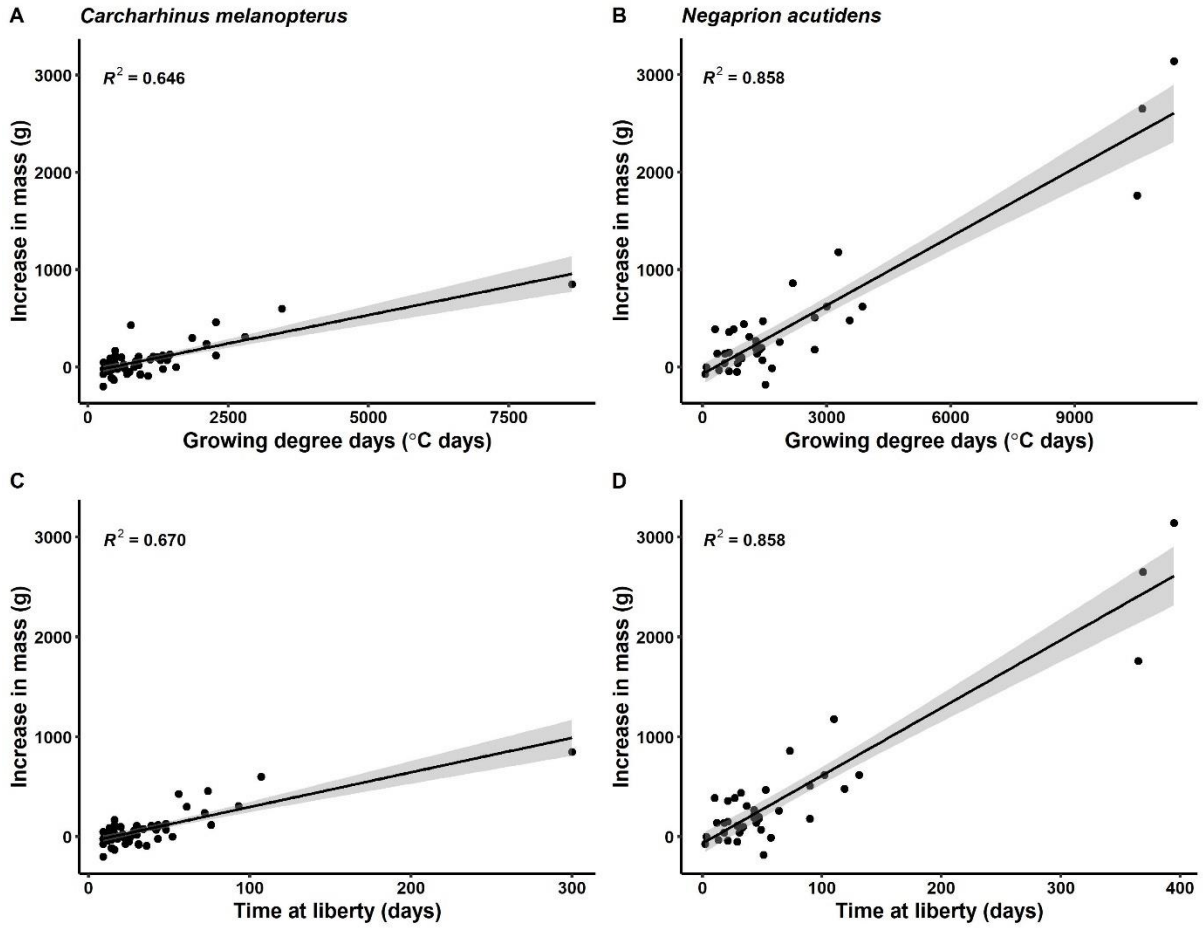

Figure 2.3 Growth (i.e., increase in mass) as a function of growing degree days (A, B) and time at liberty (C, D) in neonatal *Carcharhinus melanopterus* and *Negaprion acutidens*. Growth was measured in free-ranging sharks that were recaptured up to 395 days at liberty. Growing degree days were calculated as the sum of degree days, the average of the daily minimum and maximum environmental temperatures within a shark's habitat, during an individual shark's time at liberty. Individual observations represent growth and growing degree days or time at liberty for individual sharks. Shading around regression lines represent 95% error bars. Coefficients of determination (R^2) are presented in the upper left corner of each panel.

Metabolic rate

Temperature step had significant effects on $\dot{M}O_{2Routine}$ in *C. melanopterus* and *N. acutidens* (Table 2.4; Figure 2.4). In *C. melanopterus*, $\dot{M}O_{2Routine}$ decreased upon initially lowering the temperature to 25 °C, and then increased until 33 °C. In *N. acutidens*, temperature effects were most apparent between the 25 and 33 °C steps (Table 2.4; Figure 2.4). Overall, $\dot{M}O_{2Routine}$ was 112.9 mg O_2 kg^{-1} h^{-1} at 25 °C, 140.7 mg O_2 kg^{-1} h^{-1} at 28 °C, and 167.6 mg O_2 kg^{-1} h^{-1} at 33 °C for *C. melanopterus* and 138.9 mg O_2 kg^{-1} h^{-1} at 25 °C, 170.2 mg O_2 kg^{-1} h^{-1} at 28 °C, and 195.29 mg O_2 kg^{-1} h^{-1} at 33 °C for *N. acutidens*. In general,

Q_{10} did not differ whether heating or cooling was occurring (Table 2.4; Figure 2.4). Instead, Q_{10} for *C. melanopterus* was larger at the cooler range (i.e., 25-28 °C) than at the warmer range (i.e., 28-33 °C). No trend with temperature range was apparent for *N. acutidens*. Temperature quotients (Q_{10}) across the entire 25-33 °C range were 1.72 and 1.61 in *C. melanopterus* and *N. acutidens*, respectively.

Table 2.4 Model outputs of the effects of temperature on oxygen uptake rates of reef sharks. For mixed effects models, mean effect size and 2.5% and 97.5% confidence interval limits are presented. Abbreviations: routine oxygen uptake rate, $\dot{M}O_{2Routine}$; temperature quotient. Q_{10}.

		Carcharhinus melanopterus			*Negaprion acutidens*		
Model	Parameter	Mean	2.5%	97.5%	Mean	2.5%	97.5%
$\dot{M}O_{2Routine}$ ~ Temperature Step + Shark ID (random)	Intercept	148.24	129.32	167.16	194.75	157.91	233.67
	25 °C	-35.34	-46.89	-23.06	-56.18	-72.41	-40.02
	28 °C [2]	-5.02	-16.83	6.64	-46.54	-63.00	-30.28
	33 °C	19.05	6.83	31.67	0.05	-15.24	15.71
	28 °C [3]	-17.51	-26.25	-5.59	-29.46	-45.63	-14.05
Q_{10} ~ Temperature Range + Shark ID (random)	Intercept	2.46	1.90	3.09	1.62	0.86	2.43
	28 to 25 °C	0.46	-0.18	1.16	2.20	1.14	3.25
	28 to 33 °C	-1.02	-1.74	-0.35	0.12	-0.99	1.22
	33 to 28 °C	-0.76	-0.05	-1.49	-0.21	-1.28	0.90

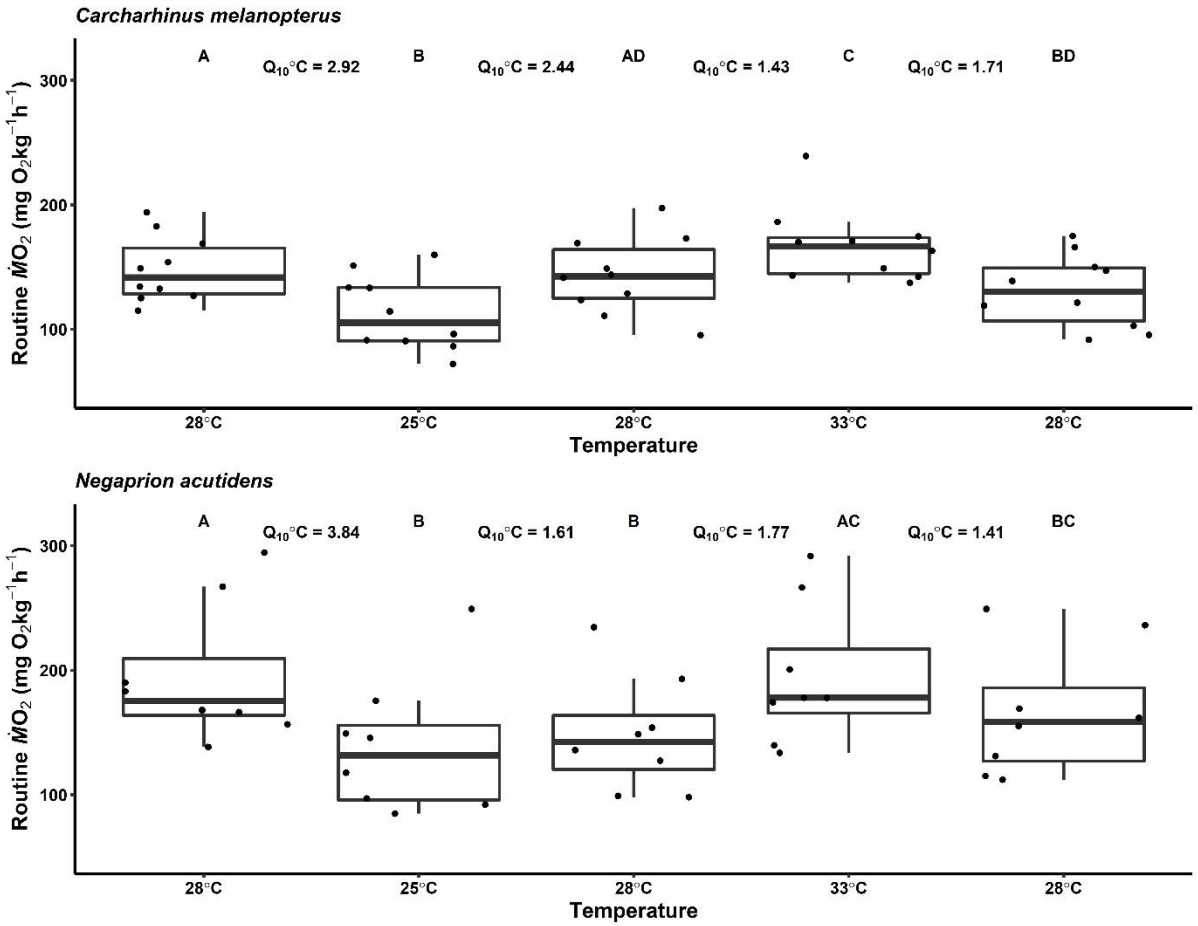

Figure 2.4 Temperature-scaling of oxygen uptake rates ($\dot{M}O_2$) in *Carcharhinus melanopterus* and *Negaprion acutidens*. Temperature quotients (Q_{10}) are presented for each temperature change (i.e., 28-25 °C, 25-28 °C, 28-33 °C, and 33-28 °C). Individual observations represent values for individual sharks, and differing letters denote statistically significant differences in routine $\dot{M}O_2$ between temperatures.

2.5 Discussion

The purpose of this study was to understand temperature's influence on fitness related performance traits of reef shark neonates in a putative nursery area system. This study offers support for the hypothesis that neonates and juveniles that use confined habitats like nursery areas exhibit minimal thermal dependence of performance. Recently, Lear *et al*. (2019, 2020) provided evidence for six species from five nursery area systems that juvenile sharks and rays using confined habitats like nursery areas exhibit reduced thermal sensitivity of activity levels and routine metabolic rates. Indeed, Moorea was confirmed to be a nursery area system. *Negaprion acutidens* appear to use discrete nursery areas, whereas *C. melanopterus* were equally abundant and may use the entire island as a nursery; although, this could not be tested. Further, performance traits measured in reef shark neonates revealed minimal thermal dependence. Growth, as measured *in situ* as changes in body mass, did not vary with thermal exposure. Routine metabolic rate, as estimated from oxygen uptake rates measured *ex situ*, exhibited

below-average (i.e., $Q_{10} < 2$; Clarke *et al.*, 1999) thermal sensitivity. Indeed, this study also provides support for the notion that fully developed, early life history stages in fishes have high thermal tolerance (Dahlke *et al.*, 2020). As such, this study suggests that thermal dependence of performance is a trait worthy of consideration for identifying nursery area use in neonate and juvenile shark populations, where more transient populations should exhibit greater thermal dependence of performance than resident populations (Lear *et al.*, 2019). Temperature may, therefore, be an important driver underlying the three proposed nursery area criteria through its effects on performance and possibly abundance in some species. However, heatwave conditions and ocean warming may have the potential to act on these defining traits of nursery areas. Therefore, this study suggests an additional mechanism by which the fitness of sharks, and possibly other species that rely on nursery areas, is threatened by climate change.

Reef shark neonates exhibited differential patterns of habitat use. A single nursery area was identified for *N. acutidens*. Nursery area use has been suggested for both *C. melanopterus* (Papastamatiou *et al.*, 2009; Mourier and Planes, 2013; Oh *et al.*, 2017b) and *N. acutidens* (Filmalter *et al.*, 2013; Mourier *et al.*, 2013; Hodgkiss *et al.*, 2017; Oh *et al.*, 2017b), but nursery area criteria were not previously tested for either species (Heupel *et al.*, 2018). Refuge from predation is likely not a unique trait of the Apaura-Vaiane nursery area because all habitats where sharks were collected in this study were shallow enough to exclude predators. Ample food resources also may not be a unique trait of the Apaura-Vaiane nursery area because foraging success of *C. melanopterus* caught at this site is low (Weideli *et al.*, 2019a). Previous studies also provide evidence that nursery area use is not necessarily associated with prey availability or quality in some populations of scalloped hammerhead (*Sphyrna lewini*; Bush and Holland, 2002; Duncan and Holland, 2006) and blacktip sharks (*Carcharhinus limbatus*; Heupel and Hueter, 2002). Nursery areas were not communal, even though *C. melanopterus* were caught (albeit in considerably low abundance) in the Apaura-Vaiane nursery area for *N. acutidens*. Interspecific competition between these species could, therefore, be a driver of habitat suitability for nursery areas. However, *C. melanopterus* and *N. acutidens* appear to exhibit trophic niche partitioning where they co-occur around Moorea (Matich *et al.*, 2017) and elsewhere (Weideli *et al.*, 2019b). Abiotic characteristics of habitats, including seawater conditions (i.e., temperature, dissolved oxygen, pH, salinity) and substrate are associated with reef shark abundance in some species (Yates *et al.*, 2015; Oh *et al.*, 2017a); although, this has not been previously tested in *N. acutidens* or in the context of shark nursery areas for either species. Because *C. melanopterus* were caught in equal abundance all around the island, it is possible that Moorea serves as a nursery area system for *C. melanopterus*; however, I was not able to test the nursery area criteria at an inter-island scale. Beyond the assumed benefits of nursery areas with direct relation to survival (i.e., starvation, predation), it is possible that *N. acutidens* also derive benefits associated with social learning interactions within nursery areas (Jacoby *et al.*, 2012). To date, no study has quantified sociality in *N. acutidens*; although, it has been suggested among adults (Clua *et al.*, 2010) and among juveniles of the sister species, *N. brevirostris* (Wilson *et al.*, 2015; Keller *et al.*, 2017; Finger

et al., 2018). Moving beyond current nursery theory and refuge benefits, it is possible that sharks derive physiological performance benefits when using nursery areas.

There was no apparent effect of temperature on growth in either species. Changes in body mass in sharks recaptured up to over one year at liberty did not vary with thermal exposure. As thermal exposure and time-at-liberty were tightly correlated, it is possible that sharks did not experience enough variability in thermal exposure for effects on growth *in situ* to be measurable. Around Moorea, water temperatures where sharks were captured vary from ~22-37 °C; however, average summer temperatures only varied 28-32 °C where *C. melanopterus* were caught and ~29-31 °C where *N. acutidens* were caught. Indeed, growth exhibits 'typical' thermal dependence in fishes, where performance increases with temperature up to an optimal temperature (i.e., T_{opt}) and then decreases with further warming to an upper critical limit (i.e., T_{crit}) (Gräns *et al.*, 2014; Payne *et al.*, 2016). The breadth of these 'thermal performance curves', when performance of a trait is within 80-90% of performance at T_{opt}, is generally larger in species that are 'thermal generalists' (Schulte *et al.*, 2011; Nati *et al.*, 2016), such that changes in temperature have minimal impacts on performance. A relatively large thermal performance curve breadth may, therefore, explain why growth was not affected by thermal exposure in *C. melanopterus* or *N. acutidens*. Alternatively, both species exhibit highly variable growth rates in a Seychelles nursery area system (Weideli *et al*., 2019b) and this variability could confound possible thermal effects. Further, neonatal *C. melanopterus* exhibit lower foraging success relative to conspecifics from the Seychelles nursery area system (Weideli *et al*., 2019a), and food availability is also known to interact with temperature to affect growth rate in fishes (McLeod *et al.*, 2013; Cominassi *et al.*, 2020). Indeed, I also demonstrate no effects of temperature on growth rate in *C. melanopterus* between 28 and 31 °C *ex situ* (**Chapter 5**). The adaptive nature of temperature effects on growth rates of neonate and juvenile sharks is further complicated by the direction of selective pressures, where high growth rates are selected against in the congener of *N. acutidens*, *N. brevirostris* (DiBattista *et al.*, 2007; Hussey *et al.*, 2017). Finally, *C. melanopterus* and *N. acutidens* in this study gained mass during Moorea's wet season and, in some cases, from season to season. Lear *et al*. (2020) recorded mass loss in both *C. leucas* and *P. pristis* juveniles during their dry season and predicted unsustainable mass loss under relevant climate change temperature scenarios. Clearly, more research is needed to define thermal dependence of growth in these tropical reef shark neonates; although, this study provides a preliminary understanding.

Routine metabolic rates exhibited minimal temperature sensitivity across an ecologically relevant range. The Q_{10} values across the entire 25-33 °C range were 1.72 and 1.61 in *C. melanopterus* and *N. acutidens*, respectively. These Q_{10} values are lower than the 2.0-3.0 range that is typically observed in other elasmobranch fishes (Di Santo and Bennett, 2011; Lear *et al.*, 2017), meaning that metabolic rates are predicted to increase by less than double when exposed to a 10 °C increase in temperature. Comparatively, juvenile *C. leucas* and *P. pristis* exhibited similarly low Q_{10} values of 1.88 and 1.58, respectively; however, routine metabolic rates were measured *in situ* using a static, annular respirometry

system that allows for swimming, and animals experienced diel temperatures of 20-32 °C (Lear *et al*., 2020). Low or reduced sensitivity of metabolic rates to temperature is an adaptive response for neonatal *C. melanopterus* and *N. acutidens* that can experience daily temperature fluctuations of ~ 4 °C as well as variability in diel fluctuations from as little as 1 °C to 8 °C. However, Q_{10} measured in this study was for temperature change over diel scales and, therefore, does not reflect Q_{10} values over longer (e.g., seasonal) time scales (Schulte *et al.*, 2011). When considering reef sharks' long-term acclimation capacity, *C. melanopterus* and *N. acutidens* would be predicted to exhibit a reduced thermal acclimation response (i.e., a Q_{10} value closer to 1 than 2) because their environment is characterised by large daily fluctuations in temperature, but small seasonal variability (da Silva *et al.*, 2019). Comparing temperatures between the average wet season (i.e., austral summer and parturition season) and dry season during 2015-2019 across all 10 study sites reveals less than 2 °C of difference (wet = 29.6 °C, dry = 28.0 °C) in average seasonal temperatures. Therefore, although seasonal temperatures around Moorea are stable and within a narrow range, unpredictability in daily temperature should produce phenotypes among neonatal *C. melanopterus* and *N. acutidens* that exhibit a relatively small thermal acclimation response (Healy and Schulte, 2012). Alternatively, *C. melanopterus* and *N. acutidens* could exhibit a thermal compensation response (Sandblom *et al.*, 2014). Thus, metabolic costs associated with nursery and non-nursery area habitat in *C. melanopterus* and *N. acutidens* should not increase considerably under ocean warming alone. Yet, it is unclear whether this holds for sharks under future thermal regimes and exposure to multiple global change stressors, including acidification and deoxygenation.

Routine metabolic rates also exhibited variability in thermal sensitivity in *C. melanopterus*, but not in *N. acutidens*. In *C. melanopterus*, Q_{10} was generally higher at cooler temperatures (i.e., 25-28 °C) than at higher temperatures (i.e., 28-33 °C); yet, there was no evidence of directional warming or cooling effects on Q_{10}. There were no apparent effects of temperature range or the direction of temperature change on Q_{10} in *N. acutidens*. Among other possible explanations, observed trends in *C. melanopterus* could reflect metabolic compensation/depression, activity, or stress. A lack of an effect of the direction of temperature change suggests that sharks did not experience metabolic depression that is typically observed by high Q_{10} values much higher than the typical 2-3 range (Hopkins and Cech, 1994; Dabruzzi *et al.*, 2013; Speers-Roesch *et al.*, 2018). Metabolic compensation, where Q_{10} values approach 1 could be at play; although, the lowest Q_{10} for *C. melanopterus* (Q_{10} = 1.43, measured at 28-33 °C) was still considerably greater than 1, and compensation typically occurs over weeks to months (Sandblom *et al*., 2014). Instead, there may have been confounding effects of spontaneous activity during respirometry at each of the different temperatures. For instance, *C. melanopterus* are generally more active that *N. acutidens* that often rest (Baldwin and Wells, 1990). Indeed, spontaneous activity can influence measurement of Q_{10} (Speers-Roesch *et al.*, 2018), and a greater propensity for spontaneous activity could explain the difference in trends between *C. melanopterus* and *N. acutidens*. Further, Q_{10} in *C.*

melanopterus may have been lower at 28-33 °C relative to 25-28 °C because of reduced activity at 33 °C, which is 1-2 °C below the maximum habitat temperature at some sites where *C. melanopterus* occur around Moorea. Finally, confounding effects of stress must be considered, because static respirometry systems are known to induce stress in fishes (Murray *et al.*, 2017), and the first measurements made at 28 °C before cooling the system to 25 °C may have been affected by stress. Whilst the nuances of thermal acclimation capacity and sensitivity in sharks warrant further investigation, this study still corroborates an overall diminished temperature sensitivity of metabolic rates in sharks confined to nursery areas.

In conclusion, these data demonstrate minimal temperature dependence of performance in reef shark neonate populations that use the island of Moorea as a nursery area system. In so doing, this study advances nursery area concepts by providing further evidence to explain how shark neonates exploit shallow, nearshore environments as nursery areas. Moving forward, it will be imperative to define ecophysiological mechanisms that link abiotic conditions and physiological performance to predict effects of climate change for shark nursery areas. This will involve measuring physiological performance traits at current temperatures *in situ* (Payne *et al.*, 2018), testing hypotheses to explain species' vulnerability to ocean warming (e.g., oxygen- and capacity-limited thermal tolerance; Pörtner *et al.*, 2017), and considering the exacerbating effects of multiple global change stressors beyond warming (e.g., ocean acidification and deoxygenation; Boyd *et al.*, 2018). Furthermore, the role of behaviour in moderating global change effects must be considered (Sunday *et al.*, 2012; Pecl *et al.*, 2017). Clearly, sharks in French Polynesia benefit from the establishment of the world's largest protected area for sharks that isolates them from their most prolific threat: overfishing (Dulvy *et al.*, 2014; Ward-Paige and Worm, 2017). As more research emerges to suggest new anthropogenic pathways to reduce fitness in sharks, conservation issues, like climate change, will urgently need to be addressed.

Chapter 3: Estimating oxygen uptake rates to understand stress in sharks and rays

3.1 Summary

Elasmobranch populations face worldwide declines owing to anthropogenic stressors, with lethal and sub-lethal consequences. Oxygen uptake rates ($\dot{M}O_2$, typically measured in mg O_2 kg^{-1} h^{-1}) can be quantified as proxies of whole-organism aerobic metabolic rates and are relevant to fisheries management and conservation through aerobic performance's relationship with fitness and spatial ecology. The purpose of this review was to better understand how $\dot{M}O_2$ has been and can be applied to predict how elasmobranch populations will respond to current and future anthropogenic stressors. I identified 10 studies spanning 9 elasmobranch species that quantified $\dot{M}O_2$ to understand elasmobranch populations' responses to exposure to anthropogenic stressors. Studies measuring responses to climate change stressors (ocean warming and acidification, declining oxygen content, increasing storm frequency) were most common. Studies with relevance to fisheries stressors used $\dot{M}O_2$ to approximate energetic costs of capture and estimate recovery times in bycatch scenarios. Ecotourism encounters were investigated in the context of increases in energetic requirements owing to anthropogenic disruption of diel activity cycles. Furthermore, I discuss how an understanding of $\dot{M}O_2$ in elasmobranchs has been and can be applied to predict populations' responses to anthropogenic stressors with deliverables for improving species management and conservation. Specifically, $\dot{M}O_2$ can be applied to predict population-level responses to stressors by quantifying associations between $\dot{M}O_2$ and fitness-related processes, spatial ecology, and impact on ecosystem function (*via* bioenergetics modelling). This review is meant to serve as a call-to-action to further bridge the gap between experimental biology and elasmobranch conservation in the "good Anthropocene".

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Data availability

There are no data associated with this chapter.

3.2 Introduction

Elasmobranch populations (sharks and rays) have seen worldwide declines that necessitate better protection for threatened populations and improved management for sustainable populations (Dulvy *et al.*, 2017; Simpfendorfer and Dulvy, 2017). Overall, elasmobranchs are one of the most threatened vertebrate taxa, partly owing to their life history characteristics that limit species' abilities to rapidly respond to anthropogenic threats (Dulvy *et al.*, 2014; Stein *et al.*, 2018). Overexploitation and bycatch, habitat loss, and climate change have been identified as predominant threats driving declines in many species (Chin *et al.*, 2010; Dulvy *et al.*, 2014). Population declines can occur as a result of direct mortality (e.g., harvest), but also *via* sub-lethal effects (Wilson *et al.*, 2014). Considering sub-lethal effects is important for understanding outcomes following an animal's exposure to a stressor because sub-lethal effects can have cryptic fitness consequences (Romero *et al.*, 2009). The efficacy of management tools (i.e., ecological risk assessments, stock assessments, etc.) could be improved with physiological data that quantifies sub-lethal responses, thereby allowing for a better understanding of animals' responses to current threats, and the ability to predict responses to anticipated threats (Horodysky *et al.*, 2016; McKenzie *et al.*, 2016). Effectively addressing threats that elasmobranchs are currently facing and predicted to face in the future will require examining species' susceptibility to both lethal and sub-lethal outcomes.

Physiological studies have much to offer elasmobranch conservation. Defining physiological mechanisms underlying conservation problems can provide important information to support management decisions (Cooke *et al.*, 2013), including fisheries management (Horodysky *et al.*, 2016; Illing and Rummer, 2017). Numerous studies on elasmobranch species have taken physiological approaches to address prominent conservation issues, such as characterizing injury and stress from commercial and recreational fisheries capture (Skomal and Mandelman, 2012) to measuring whole-organism responses to climate change (Rosa *et al.*, 2017). Notably, physiologically-informed models of the condition of southern stingrays (*Hypanus americanus*) at an ecotourism site provided managers with evidence suggesting a need to regulate anthropogenic influences on these animals (Semeniuk *et al.*, 2010; Madliger *et al.*, 2016). As such, there is a utility in implementing and refining physiological tools that are both informative and palatable to conservation practitioners and stakeholders (Cooke and O'Connor, 2010; Madliger *et al.*, 2018). In particular, there has been a general call to investigate physiological markers used elsewhere for their applicability as "new" tools to measure stress or predict mortality for elasmobranchs following exposure to anthropogenic stressors (Van Rijn and Reina, 2010; Awruch *et al.*, 2011; Guida *et al.*, 2016a). Such approaches can help to realise the benefits that physiological research can provide toward elasmobranch conservation.

Changes in oxygen uptake rates ($\dot{M}O_2$, typically in mg O_2 kg^{-1} h^{-1}) can be informative of whole-organism responses to stressors. The $\dot{M}O_2$ of fishes have been quantified since the 19th century as a proxy for

metabolic rates and therefore a fundamentally important metric for understanding the behavioural and physiological ecology of an organism (Brown *et al.*, 2004; Nelson, 2016). Conservation-focused studies on fishes have quantified $\dot{M}O_2$ to address conservation problems, such as those relating to species' or populations' vulnerabilities to environmental change and anthropogenic stressors (Horodysky *et al.*, 2016; McKenzie *et al.*, 2016; Illing and Rummer, 2017). For instance, $\dot{M}O_2$ has been used as proxy for various fitness-related metrics to understand vulnerability of many marine ectotherms to climate change (Pörtner *et al.*, 2017; Jutfelt *et al.*, 2018). For elasmobranchs, studies quantifying $\dot{M}O_2$ focus largely on quantifying metabolic costs in a behavioural and physiological ecology context, with relatively few measuring responses to stressors. Indeed, while various studies of stress in elasmobranchs claim that metabolic rates (i.e., $\dot{M}O_2$) should explain species- and population-level variation in elasmobranchs' vulnerability to stressors, a general lack of empirical evidence highlights a mismatch between what conservation-minded studies claim and can support (Skomal and Mandelman, 2012).

The purpose of this review was to better understand the utility of quantifying whole-organism performance (i.e., $\dot{M}O_2$) to predict how elasmobranch populations will respond to current and future anthropogenic stressors. I accomplish this goal in two steps. I first describe studies that quantify $\dot{M}O_2$ with the specific objective of predicting elasmobranch populations' responses to various anthropogenic stressors; in so doing, I briefly discuss the specific $\dot{M}O_2$ traits that were measured. Second, I discuss how $\dot{M}O_2$ data have, and can, be applied to predict elasmobranch populations' responses to various anthropogenic stressors. In so doing, this review is intended to serve as a call to action in further bridging the gap between elasmobranch conservation and experimental biology (Cooke *et al.*, 2017).

3.3 Materials and methods

To achieve my first objective of reviewing how $\dot{M}O_2$ has been quantified to predict elasmobranch populations' responses to stress, I first targeted all studies that directly measured $\dot{M}O_2$. I included the following caveats in my literature search: (1) only whole-organism – including embryos – $\dot{M}O_2$ was considered and not that of perfused tissues; (2) studies measuring $\dot{M}O_2$ of catheterized or perfused animals were also considered. A systematic literature search of all published research since 1965 (i.e., no theses) was conducted on 1 June 2018 using the Thomas Reuter's Web of Science database with the following search terms: (elasmobranch* OR shark* OR chondrichth* OR dogfish*) AND (oxygen* OR metabol* OR transport* OR binding OR capacity OR respir* OR aerobic* OR ventilat* OR o2 OR gas* OR blood OR h*emato* OR h*emoglobin* OR consum* OR scope). I acknowledge that although $\dot{M}O_2$ denotes oxygen uptake strictly in milligrams of O_2 per unit mass per unit time (oxygen uptake was classically measured in millilitres of O_2; $\dot{V}O_2$), $\dot{M}O_2$ will be used throughout to generically refer to oxygen uptake rates (Rummer *et al.*, 2016). Because targeted studies approximated whole-organism metabolic rate *via* rates of oxygen uptake, this study preferentially refers to $\dot{M}O_2$ rather than "metabolic rates" (Nelson, 2016). Indeed, I acknowledge that there are several thorough reviews of $\dot{M}O_2$ in

elasmobranchs (Carlson *et al.*, 2004; Bernal *et al.*, 2012). Herein, I briefly describe the few studies that have quantified $\dot{M}O_2$ in response to targeted stressors, and then describe how these data have been, and can be, applied to predict current and future elasmobranch populations' physiological and behavioural responses.

3.4 Results and discussion

Estimating $\dot{M}O_2$ to understand stress in elasmobranchs

Commonly measured $\dot{M}O_2$ traits to quantify stress

This review identified 81 studies of $\dot{M}O_2$ spanning 35 shark and ray species (of nearly 1200 total species), of which only 10 studies spanning 9 species were relevant to directly quantifying elasmobranch populations' responses to anthropogenic stressors (Table 3.1). Five $\dot{M}O_2$ traits were commonly measured in studies measuring stress responses and are discussed below.

Table 3.1 Studies that use oxygen uptake to understand responses of elasmobranch populations to anthropogenic stress. Lettered superscripts indicate the conditions species were tested under, and numbered superscripts indicate the metrics that were measured. For instance, the notation *Chiloscyllium punctatum*[a,d,3] indicates that $\dot{M}O_{2Min}$ ([3]) was measured in response to pCO_2 ([a]) and temperature ([d]). Abbreviations: aerobic scope, AS; excess post-exercise oxygen consumption, EPOC; maximum oxygen uptake rate, $\dot{M}O_{2Max}$; minimum oxygen uptake rate, $\dot{M}O_{2Min}$; partial pressure of carbon dioxide, pCO_2; partial pressure of oxygen, pO_2; swimming oxygen uptake rate, $\dot{M}O_{2Swim}$.

Stressor	**Treatments**	**Metrics**	**Species (n = 9 of ~1200)**	**References (n = 10 of 81)**
Climate change	pCO_2[a], pO_2[b], salinity[c], temperature[d]	AS[1], EPOC[2], $\dot{M}O_{2Min}$[3], $\dot{M}O_{2Max}$[4], $\dot{M}O_{2Swim}$[5]	*Chiloscyllium punctatum*[a,d,3], *Galeorhinus galeus*[c,3], *Hemiscyllium ocellatum*[a,3], *Leucoraja erinacea*[a,b,d,1-5*], *Mustelus antarcticus*[c,3], *Scyliorhinus canicula*[a,1,3,4]	(Green and Jutfelt, 2014; Heinrich *et al.*, 2014; Rosa *et al.*, 2014; Di Santo, 2015, 2016; Di Santo *et al.*, 2016; Morash *et al.*, 2016)

Fisheries stressors	Exhaustive exercise	AS[1], EPOC[2], $\dot{M}O_{2Min}$[3], $\dot{M}O_{2Max}$[4], $\dot{M}O_{2Swim}$[5]	*Carcharhinus melanopterus*[1-4], *Negaprion brevirostris*[1-5]	(Bouyoucos *et al.*, 2017b, 2018)
Ecotourism encounters	Diel activity	$\dot{M}O_{2Min}$, $\dot{M}O_{2Swim}$	*Triaenodon obesus*	(Barnett *et al.*, 2016)

*$\dot{M}O_{2Swim}$ quantified as tail-beats in *L. erinacea* embryos

The minimum $\dot{M}O_2$ of a quiescent, fasted fish at rest and at stable temperatures represents its $\dot{M}O_{2Min}$ (Chabot *et al.*, 2016b) and can be a proxy for estimating routine/resting or standard metabolic rates. $\dot{M}O_{2Min}$ is the most commonly measured component of elasmobranchs' metabolic phenotype, and is often the only metric measured (Carlson *et al.*, 2004; Bernal *et al.*, 2012). Changes in $\dot{M}O_{2Min}$ in response to stressors can be interpreted as changes in the minimum energetic investment in maintenance, although interpretation is less meaningful without reference to other components of the metabolic phenotype (Hannan and Rummer, 2018). The effects of environmental change on $\dot{M}O_{2Min}$ are well-described elsewhere; briefly, elasmobranchs generally exhibit transient changes in $\dot{M}O_{2Min}$ in response to osmoregulatory and/or acid-base challenges (e.g., hyposalinity or hypercapnia), $\dot{M}O_{2Min}$ is highly sensitive to environmental oxygen saturation, and elasmobranchs are not known to thermally compensate (i.e., restoration of $\dot{M}O_{2Min}$ following temperature change) (Carlson *et al.*, 2004; Tullis and Baillie, 2005; Bernal *et al.*, 2012; Hannan and Rummer, 2018). While $\dot{M}O_{2Min}$ is a desirable metric to measure, it has been argued that, for sharks, the lowest swimming $\dot{M}O_2$ is a more ecologically relevant metric than $\dot{M}O_{2Min}$ because obligate ram-ventilating species cannot achieve $\dot{M}O_{2Min}$ in the wild anyway (Lowe, 2001). Indeed, $\dot{M}O_{2Min}$ can be approximated for obligate-ram ventilating species by extrapolating $\dot{M}O_2$-activity level relationships to zero activity, although this approach may overestimate $\dot{M}O_{2Min}$ (Roche *et al.*, 2013; Di Santo *et al.*, 2017). Alternatively, $\dot{M}O_{2Min}$ can be measured for obligate ram-ventilating animals that have been chemically immobilized, but this is a lethal endpoint (Carlson and Parsons, 2003; Dowd *et al.*, 2006b).

The highest $\dot{M}O_2$ value achievable under sustained maximal activity or following fully exhaustive exercise represents maximum $\dot{M}O_2$ ($\dot{M}O_{2Max}$), which serves as a proxy for maximum metabolic rate (Norin and Clark, 2016). Indeed, as fishes may rely on anaerobic metabolism to support high activity levels, $\dot{M}O_{2Max}$ may include oxygen uptake for supporting aerobic metabolism and resolving physiological disturbances from using anaerobic metabolism (Norin and Clark, 2016). In elasmobranchs, $\dot{M}O_{2Max}$ is limited by the capacity of the cardiorespiratory system for oxygen transport (Hillman *et al.*, 2013), and changes in response to stressors represent changes in the upper limit for

oxygen uptake. Relatively few studies have quantified $\dot{M}O_{2Max}$ for any elasmobranch in the context of characterising metabolic costs, and fewer have quantified changes in $\dot{M}O_{2Max}$ in response to stressors. To date, the available literature suggests equivocal effects of aquatic acidification (*via* carbon dioxide) on $\dot{M}O_{2Max}$ and no temperature effect; no other stressors, like reduced oxygen availability, have been tested (Green and Jutfelt, 2014; Di Santo, 2016). Research on *Carcharhinus melanopterus* corroborates an absent temperature effect on $\dot{M}O_{2Max}$ (IA Bouyoucos, this thesis); although, challenges associated with measuring $\dot{M}O_{2Max}$ for fishes, such as behavioural *versus* physiological fatigue in swimming respirometry chambers or compounding behavioural effects of aquatic acidification should invoke a healthy scepticism of the precision of $\dot{M}O_{2Max}$ estimates, especially for elasmobranchs (Peake and Farrell, 2006; Lefevre, 2016). Clearly, it is difficult to quantify $\dot{M}O_{2Max}$ for many elasmobranchs; $\dot{M}O_{2Max}$ is typically estimated *via* swimming in a swimming respirometry chamber or upon chasing to exhaustion (Norin and Clark, 2016; Rummer *et al.*, 2016). The former swims fish in a flume or swim tunnel over a range of increasing flow velocities for a fixed period of time and constant velocity increment and estimates $\dot{M}O_2$ at each flow velocity; $\dot{M}O_{2Max}$ is typically estimated as the $\dot{M}O_2$ value at the flow velocity when fish recruit anaerobic metabolism to support swimming and fatigue. The latter method encourages fish to burst swim in a small pool for a fixed period of time (usually minutes) or until the fish no longer responds to chasing stimuli, after which animals can be air exposed before being placed in a resting respirometry chamber to measure $\dot{M}O_2$. Some species are too large, even as juveniles, to swim in flume respirometry chambers, or are simply not amenable to forced swimming (Brett and Blackburn, 1978; Lowe, 2001; Sepulveda *et al.*, 2007). Chasing to exhaustion can produce similar $\dot{M}O_{2Max}$ estimates to swimming in a flume for some teleosts (Killen *et al.*, 2017), but it remains to be determined whether chasing is a viable alternative to swimming elasmobranchs in a flume to generate accurate $\dot{M}O_{2Max}$ estimates. Anecdotally, $\dot{M}O_{2Max}$ measured after exhaustion in a flume was higher than measured after chasing for juvenile *Negaprion brevirostris* (Bouyoucos *et al.*, 2017b, 2017a).

Aerobic scope (AS) represents the oxygen available to support multiple oxygen-demanding processes above $\dot{M}O_{2Min}$ and can be calculated as the difference between (or ratio of) $\dot{M}O_{2Max}$ and $\dot{M}O_{2Min}$ (Clark *et al.*, 2013). Changes in AS are often interpreted as changes in an organism's capacity to use oxygen for fitness-related processes (e.g., growth, reproduction, etc.) (Fry, 1947; Claireaux and Lefrançois, 2007; Farrell, 2016). Among teleost fishes, studies have documented associations between AS and species' ecology (e.g., life-history traits), and can even explain variation among physiological and behavioural traits (Clark *et al.*, 2013; Killen *et al.*, 2016; Metcalfe *et al.*, 2016). Aerobic scope was first referenced in the elasmobranch literature in 1988, or 41 years after its inception (Fry, 1947; Du Preez *et al.*, 1988). Several studies provide enough information to calculate AS *post-hoc* (Brett and Blackburn, 1978; J. B. Graham *et al.*, 1990; Lowe, 2001; Bouyoucos *et al.*, 2017a, 2018), but only two have directly tested factors that affect AS in the context of climate change (Green and Jutfelt, 2014; Di Santo, 2016). Stress physiology studies have suggested that the intensity and magnitude of the elasmobranch stress

response during fishing capture is associated with AS, yet none have directly tested this hypothesis (Skomal and Mandelman, 2012). The $\dot{M}O_{2Min}$ data appear to support this idea indirectly, where sluggish, benthic species with low $\dot{M}O_{2Min}$ experience lower mortality than active or ram-ventilating species (Skomal and Bernal, 2010; Dapp *et al.*, 2016). Furthermore, lamnid and sphyrnid sharks are predicted to have the highest AS among elasmobranchs (Lowe, 2001; Sepulveda *et al.*, 2007); yet, lamnid sharks generally exhibit high resilience to stress and high post-release survivorship, whereas sphyrnid sharks experience tremendously high at-vessel and post-release mortality (Marshall *et al.*, 2012; Gallagher *et al.*, 2014; Butcher *et al.*, 2015; French *et al.*, 2015). Thus, studies that directly quantify AS are necessary to fully support or refute this hypothesis.

Excess post-exercise oxygen consumption (EPOC) represents an increase in $\dot{M}O_2$ to resolve a physiological stress response following exhaustive exercise (Gaesser and Brooks, 1984; Wood, 1991; Milligan, 1996). Studies of sharks and rays have measured EPOC as a proxy of anaerobic exercise capacity, to quantify the energetic cost of an activity, and as a means for estimating time to recover following exhaustive exercise (Brett and Blackburn, 1978; Bouyoucos *et al.*, 2017a; Di Santo *et al.*, 2017). The presence of EPOC alone can be indicative of a stress response, and changes in the magnitude of EPOC and recovery time can reflect differences in the intensity of stressors (e.g., exhaustive exercise *versus* fishing capture) and/or changes in an organism's capacity to respond to stress. However, sharks and rays can incur EPOC simply by swimming at routine activity levels; indeed, some elasmobranchs recruit anaerobic metabolism to support sub-maximal swimming (Piiper *et al.*, 1977; Di Santo and Kenaley, 2016; Di Santo *et al.*, 2017). The duration and magnitude of EPOC measured for sharks and rays is variable and appears to be related to the duration and intensity of activity. For instance, exhaustion following maximal aerobic swimming resulted in a shorter recovery period and smaller EPOC than exhaustive chasing for juvenile *N. brevirostris* (Bouyoucos *et al.*, 2017b, 2017a). In addition, EPOC and recovery time exhibit sensitivity to environmental stressors; juvenile *Leucoraja erinacea* take longer to recover following exhaustive exercise under aquatic acidification conditions (Di Santo, 2016). Previously, studies have referred to animals experiencing – or paying back – an "oxygen debt" from relying on anaerobic metabolic pathways during exercise (Piiper *et al.*, 1977; Brett and Blackburn, 1978). It should be noted that the term "oxygen debt" implies a causal mechanism underlying the increase in metabolic rate post-exercise; "EPOC" avoids such confusion (Gaesser and Brooks, 1984).

The range of submaximal $\dot{M}O_2$ values of swimming sharks and rays (i.e., swimming $\dot{M}O_2$) can be the most energetically costly and variable component of an elasmobranch's energy budget (Lowe, 2001). As such, changes in swimming $\dot{M}O_2$ can represent changes in energy allocation within the available AS, where more time and energy invested in activity over possible fitness-related processes can increase an individual's susceptibility to mortality (Priede, 1977). At routine activity levels, available data suggest that sharks' swimming $\dot{M}O_2$ accounts for 25-46% of their AS (Brett and Blackburn 1978; Lowe 2001;

Dowd et al. 2006; Bouyoucos et al. 2017b); similar data do not exist for rays. Increases in swimming $\dot{M}O_2$ with activity become proportionally less at higher temperatures; although, swimming $\dot{M}O_2$ at a given velocity increases with temperature (Du Preez *et al.*, 1988; Whitney *et al.*, 2016; Lear *et al.*, 2017). Furthermore, the swimming speed with the lowest cost of transport increases with temperature, making life at higher temperatures inherently more costly (Iosilevskii and Papastamatiou, 2016). Swimming $\dot{M}O_2$ also changes in sharks exposed to hypoxia, but responses likely reflect changes in activity levels rather than changes in the cost of activity; obligate ram-ventilating species generally increase activity levels to minimize time in the hypoxic zone, and buccal-pumping species appear to decrease activity (Parsons and Carlson, 1998; Carlson and Parsons, 2001). Finally, bio-logging and biotelemetry technologies have made it possible to estimate swimming $\dot{M}O_2$ *in situ* by calibrating $\dot{M}O_2$ across an ecologically relevant range of activity levels (and temperatures) with electronic tag outputs (e.g., acceleration) (Barnett *et al.*, 2016; Bouyoucos *et al.*, 2017a; Lear *et al.*, 2017).

Climate change

Climate change is a threat to elasmobranch populations, and studies have quantified $\dot{M}O_2$ to better understand the extent of physiological impairment elasmobranchs may experience (Rosa *et al.*, 2017). Ocean acidification is the most-investigated stressor (Green and Jutfelt, 2014; Heinrich *et al.*, 2014; Rosa *et al.*, 2014; Di Santo, 2015, 2016), despite only recently being considered a threat to elasmobranchs (Chin *et al.*, 2010; Rosa *et al.*, 2017; Rummer and Munday, 2017). Thus far, ocean warming has only been investigated alongside acidification (Rosa *et al.*, 2014; Di Santo, 2015, 2016). The effects of declining oxygen levels on $\dot{M}O_2$ have been investigated to understand species' hypoxia tolerance in the context of climate change (Heinrich *et al.*, 2014; Di Santo *et al.*, 2016). Similarly, the effects of salinity on $\dot{M}O_2$ have received attention, but only one study to date has investigated the effects of changing salinity in the context of increasing storm frequency with climate change (Carlson *et al.*, 2004; Morash *et al.*, 2016). The paucity of available literature highlights knowledge gaps regarding changes in aerobic (i.e., $\dot{M}O_{2Max}$ and AS) and anaerobic (e.g., EPOC) capacity in response to key climate change stressors (i.e., ocean acidification and warming, declining oxygen content). In addition, there are a lack of studies investigating species in high trophic positions (e.g., large mesopredators or apex predators), including tropical or deep-sea species (Rosa *et al.*, 2017). Finally, studies have investigated elasmobranch's capacity for developmental and reversible acclimation, but no study to date has investigated sharks' or rays' capacity for transgenerational acclimation (Donelson *et al.*, 2018).

Ocean acidification and warming are predicted to have negative consequences for oxygen uptake rates ($\dot{M}O_{2Min}$, $\dot{M}O_{2Max}$, and AS) in embryonic, juvenile, and adult sharks and rays. Both temperate and tropical species are expected to have higher $\dot{M}O_{2Min}$ and are predicted to develop – from the early embryonic stages – faster under warming and acidification conditions, but neonates may exhibit reduced body condition and lower survival than conspecifics reared under current-day conditions (Rosa et al.

2014; Di Santo 2015; Gervais et al. 2016). The effects of acidification alone on $\dot{M}O_{2Min}$, $\dot{M}O_{2Max}$, and AS are equivocal (Green and Jutfelt, 2014; Heinrich *et al.*, 2014; Hannan and Rummer, 2018). However, acidification appears to lengthen recovery from exhaustive exercise, and exacerbates the effects of warming on $\dot{M}O_{2Min}$, $\dot{M}O_{2Max}$, and AS (Rosa *et al.*, 2014; Di Santo, 2015, 2016; Lefevre, 2016). There is also evidence of population-level differences in $\dot{M}O_2$ and responses to warming and acidification (Di Santo, 2015, 2016). Temperature sensitivity of $\dot{M}O_2$ in elasmobranchs should be expected; mitochondrial ATP production becomes increasingly inefficient at high temperatures (i.e., increased proton leak across mitochondrial membranes), ectothermic species have relatively temperature-sensitive haemoglobins, and no species has been documented to exhibit thermal compensation of $\dot{M}O_{2Min}$ (Tullis and Baillie, 2005; Schulte, 2015; Bernal *et al.*, 2018). Furthermore, elasmobranchs' apparent resilience to some of the effects of ocean acidification may be a result of elasmobranchs' high plasma buffering capacities and ability to maintain haemoglobin-oxygen affinity (i.e., weak or absent Bohr-shifts) under acidotic conditions, relative to teleosts (Berenbrink *et al.*, 2005; Morrison *et al.*, 2015). Indeed, elasmobranchs appear to possess physiological mechanisms to maintain $\dot{M}O_2$ under acidotic conditions; although, the mechanism is different to that in teleosts and has not yet been identified (Hannan and Rummer, 2018).

The effects of hypoxia on $\dot{M}O_2$ in the context of climate change have been investigated for elasmobranchs with confined distributions (i.e., benthic species and embryos in egg cases). Briefly, a common hypoxia tolerance metric is the oxygen level at which $\dot{M}O_{2Min}$ can no longer be regulated, and decreases with declining oxygen content (Wood, 2018). The epaulette shark, *Hemiscyllium ocellatum*, a species renowned among elasmobranchs for its hypoxia tolerance, did not experience changes in $\dot{M}O_2$ or hypoxia tolerance under elevated pCO_2 (Heinrich *et al.*, 2014). Egg-bound embryonic *L. erinacea* reared at 15 °C were found to reduce $\dot{M}O_2$ but increase tail-beating activity (i.e., for ventilation) at moderately low oxygen saturations, possibly supporting their activity anaerobically (Di Santo *et al.*, 2016). Indeed, metabolic scope (tail-beating $\dot{M}O_2$ - $\dot{M}O_{2Min}$) for embryonic *L. erinacea* was higher at 18 °C and 20 °C relative to 15 °C, which suggests embryonic *L. erinacea* could have a greater capacity for tail-beating and tolerating hypoxia under warming (Di Santo, 2015; Di Santo *et al.*, 2016). Elasmobranchs are generally considered to have poor hypoxia tolerance (Routley *et al.*, 2002); elasmobranch haemoglobins generally have higher oxygen affinities than teleost haemoglobins, but lack comparable mechanisms to improve oxygen delivery, such as increases in haematocrit, strong pH-sensitivity of haemoglobins, or a β-adrenergic stress response at the red blood cells (Brill and Lai, 2015; Morrison *et al.*, 2015). Clearly, more research is needed to characterize the responses of elasmobranchs to hypoxia in the context of climate change.

The effects of changing salinity on elasmobranchs are variable and dependent on the duration of exposure. The frequency of storm and drought events are predicted to increase as climate change progresses, and these environmental challenges could have consequences for salinity exposure (e.g.,

acute changes in $\dot{M}O_{2Min}$), particularly in coastal and estuarine environments (Morash *et al.*, 2016; Tunnah *et al.*, 2016). Sharks (*Galeorhinus galeus* and *Mustelus antarcticus*) that were acutely exposed to changes in salinity over a 48-hour period exhibited changes in $\dot{M}O_{2Min}$ (Morash *et al.*, 2016; Tunnah *et al.*, 2016). Increases in $\dot{M}O_{2Min}$ can represent increased osmoregulatory maintenance costs, while reduced $\dot{M}O_{2Min}$ may relate to reductions in Hb-O_2 affinity. Notably, the potential mechanism underlying changes in $\dot{M}O_{2Min}$ in response to osmotic challenges, the osmorespiratory compromise, has not been identified in elasmobranchs (Tunnah *et al.*, 2016). Given the relevance of increased frequency and severity of storm events with climate change, further investigation into the effects of osmotic challenges on elasmobranchs is warranted.

Fisheries stressors

The energetic costs and recovery times associated with fisheries capture have been estimated by quantifying EPOC. Chasing protocols supplemented with air exposure are often employed in teleost stress studies because these techniques induce a similar physiological disturbance as fishing capture and handling (Clark *et al.*, 2012; Currey *et al.*, 2013). For example, EPOC estimated by chasing juvenile *N. brevirostris* to exhaustion was paired with swimming $\dot{M}O_2$ estimated from acceleration data from juveniles hooked on experimental longlines to produce an estimate of the total energetic cost of a longline capture event (Bouyoucos *et al.*, 2017b). In this case, capture resulted in a 58% increase in energy expenditure during a one-hour capture event; although, the estimated five-hour recovery costs only represented a 2% increase in daily activity energy expenditure (Bouyoucos *et al.*, 2017b). Alternatively, the cost of the initial struggling period during capture can be estimated by immediately transferring animals to respirometry chambers *in situ*; using this approach, gill-net capture was estimated to increase the daily activity energy expenditure of juvenile *C. melanopterus* by 15% and required almost nine hours of recovery (Bouyoucos *et al.*, 2018). Estimates of $\dot{M}O_2$ may also partially explain inter-specific variation in stress responses following capture. When compared to *N. acutidens*, the reduction in blood pH following gill-net capture and delayed mortality rates were both less pronounced (Bouyoucos *et al.*, 2018).

Ecotourism encounters

Finally, $\dot{M}O_2$ has been applied to understand the energetic costs of human-wildlife encounters. Human-wildlife encounters, such as those mediated through ecotourism, can be a source of stress for elasmobranchs (Brena *et al.*, 2015; Gallagher *et al.*, 2015). Interacting with wildlife tourism operations has been demonstrated to affect $\dot{M}O_2$ in elasmobranchs through changes in diel activity levels (Barnett *et al.*, 2016). Specifically, changes in activity levels have been related to swimming $\dot{M}O_2$ and $\dot{M}O_{2Min}$ through calibrated relationships between $\dot{M}O_2$ and telemetry device output (i.e., overall dynamic body acceleration as a proxy of $\dot{M}O_2$ and metabolic rate). For instance, provisioning was documented to increase oxygen uptake rates of whitetip reef sharks (*Triaenodon obesus*) by increasing activity levels

during the day when sharks would normally rest (Barnett *et al.*, 2016). Although this is the only instance where $\dot{M}O_2$ has been applied to understand elasmobranchs' responses to stress associated with human-wildlife encounters, it is also possible to quantify $\dot{M}O_2$ as it applies to provisioning *via* specific dynamic action (SDA; the increase in $\dot{M}O_2$ during digestion and assimilation of food). Large meals, such as those that might occur for bold sharks that consistently feed or sharks that gorge during competitive interactions, may reduce aerobic capacity by reducing the available AS for other oxygen-demanding processes (Norin and Clark, 2017). Indeed, single provisioning events can satiate sharks for days (Brunnschweiler *et al.*, 2018). Whilst feeding is not a stressful event, the consequences of reduced AS following ingestion of a large meal (e.g., less aerobic capacity for swimming) on the bioenergetics of provisioned sharks ought to be considered. Given concerns that human-wildlife encounters can have consequences for the health of sharks and rays (Semeniuk *et al.*, 2010), $\dot{M}O_2$ can be applied to quantify stress, especially in the context of bioenergetics, that is associated with ecotourism.

Predicting population-level responses from $\dot{M}O_2$ data

To date, laboratory studies of $\dot{M}O_2$ in elasmobranchs have largely taken a basic approach to characterising metabolic costs. While the studies presented in this review take an applied approach in estimating $\dot{M}O_2$ to achieve conservation-minded objectives, these studies' application toward predicting species- or population-level responses to the investigated anthropogenic stressors can be vague. Herein, I attempt to demonstrate how estimates of $\dot{M}O_2$ for elasmobranch populations can be of relevance to elasmobranch fisheries management and conservation. Specifically, I discuss how $\dot{M}O_2$ estimates (with an emphasis on AS) can be applied to predict changes in fitness, spatial ecology, and bioenergetics for elasmobranch populations (Claireaux and Lefrançois, 2007; Horodysky *et al.*, 2016).

Fitness

Aerobic scope is thought to correlate with fitness-related processes (i.e., growth and reproduction) in some populations of teleost fishes, such that AS can be measured along with other performance traits to understand and predict population-level responses to stressors (Claireaux and Lefrançois, 2007; Farrell, 2016). For some species and populations (e.g., some salmonids), AS is optimized under specific environmental conditions; deviation from optimal conditions can result in a decrease in AS that may translate to a decrease in organismal fitness (Fry, 1947). As abiotic conditions of the ocean are predictably changing owing to global climate change, AS has been widely measured for predicting changes in fitness as climate change progresses (Farrell, 2016; Pörtner *et al.*, 2017). Indeed, an optimum temperature and upper thermal limit for AS (i.e., a "bell-shaped" thermal performance curve), is central to the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis, that has been applied to predict species' responses to ocean warming and acidification, and has been consulted by managing bodies like the Intergovernmental Panel on Climate Change (IPCC; Pörtner et al. 2017). Therefore, elasmobranch species' or populations' vulnerability to climate change stressors can (potentially) be

assessed by constructing thermal performance curves for AS and related fitness metrics (e.g., growth rates, reproductive investment, etc.), provided that the OCLTT hypothesis applies to this group of fishes. Such an approach has, thus far, only been applied to two populations of *L. erinacea*, with a focus on embryos and juveniles (Di Santo, 2015, 2016; Lefevre, 2016). Applying the OCLTT hypothesis for these two populations of *L. erinacea* suggested that temperatures exceeding the thermal optimum for aerobic performance in embryos was associated with increased mortality, while juveniles experienced sub-lethal reductions in aerobic (i.e., AS) and anaerobic (i.e., escape endurance) performance (Di Santo, 2015, 2016). However, AS is a controversial metric for predicting the vulnerability of ectotherms to climate change, and indeed, the ubiquity of the OCLTT hypothesis is highly controversial (Jutfelt *et al.*, 2018). It has also been suggested that multiple performance metrics ought to be considered, given that different metrics may be optimized under different conditions (Clark *et al.*, 2013). However, given the general lack of measurement of AS in elasmobranchs, further studies to improve our understanding of hypotheses like the OCLTT for predicting elasmobranch populations' responses to climate change are warranted (Lefevre, 2016).

As a consequence of ocean warming, fishes are predicted to achieve smaller maximum body sizes according to the temperature-size rule, and measuring $\dot{M}O_2$ can help elucidate the extent of the "shrinkage" threat to elasmobranchs. The temperature-size rule highlights a well-documented negative correlation between body size and temperature, and although the specific underlying mechanism is unclear, it may be related to oxygen supply and, therefore, $\dot{M}O_2$ (Forster *et al.*, 2012; Audzijonyte *et al.*, 2019). A highly controversial hypothesis (gill-oxygen limitation, or GOL) posits that a mismatch between oxygen demand at elevated temperatures and capacity for supply across the gills owing to geometric constraints limits fishes' maximum body size (Pauly and Cheung, 2017). In other words, for a sufficiently large fish, AS is entirely devoted to $\dot{M}O_{2Min}$, and increases in temperature increase $\dot{M}O_{2Min}$, thereby reducing the size when AS is zero. Empirical evidence from elasmobranch studies suggests that $\dot{M}O_{2Min}$ is proportionally less in larger fishes, and that gill surface area scales with a similar exponent (Wegner, 2015; Bigman *et al.*, 2018). Substantial evidence from teleost literature suggests that $\dot{M}O_{2Max}$ scales with body mass with a similar exponent as $\dot{M}O_{2Min}$, such that AS could not be reduced to zero (i.e., to only support $\dot{M}O_{2Min}$) as fish grow (Killen *et al.*, 2016; Lefevre *et al.*, 2017, 2018). Mass- and temperature-scaling (i.e., Q_{10}, the exponential increase in $\dot{M}O_2$ over a 10 °C increment) data for $\dot{M}O_{2Max}$ do not exist for elasmobranchs. Indeed, there is a dearth of information on mass- and temperature-scaling data for $\dot{M}O_2$ in elasmobranchs, and these data would be of great importance for understanding the mechanism by which elasmobranchs may be expected to shrink as climate change progresses and for modelling the potential apparent shrinkage. Given that hypotheses like GOL have received support from management bodies like the International Union for Conservation of Nature (IUCN), there is certainly interest in applying $\dot{M}O_2$ data to understand how climate change will affect elasmobranch populations.

Spatial ecology

Temperature's profound effect on $\dot{M}O_2$ of ectotherms has been used to link aerobic performance with habitat use and species redistributions, partially in the context of climate change (Pörtner and Farrell, 2008; Sunday *et al.*, 2012). Indeed, various performance metrics, including AS, are thought to be linked with populations' thermal niche, such that populations may occur at temperatures near their optimal temperature for performance (Payne *et al.*, 2016; Speers-Roesch and Norin, 2016). Latitudinal shifts in boundary temperatures (i.e., where performance is reduced) may, therefore, coincide with shifts in species' distributions. Studies of elasmobranchs have provided indirect evidence of relationships between performance metrics related to $\dot{M}O_2$ (i.e., activity) and distribution, with one suggesting distribution shifts for *Galeocerdo cuvier* with ocean warming as sharks "follow" water temperatures that optimize activity performance (Payne *et al.*, 2016, 2018). Studies have also provided evidence of shifts in the distribution of pelagic and coastal sharks with warming, although without offering evidence of links to aerobic performance (Hazen *et al.*, 2013; Bangley *et al.*, 2018). Overall, there is much interest for management and conservation in characterising species redistributions for managing changes in ecosystem function and even human wellbeing (Pecl *et al.*, 2017). Indeed, species redistributions can be explained or even predicted by the influence of factors like temperature on $\dot{M}O_2$. Most notably, a physiologically-informed habitat suitability model, centred around estimates of AS, predicted vertical habitat compression for commercially important *Thunnus albacares* in response to climate change driven warming, acidification, and deoxygenation of the pelagic environment (Del Raye and Weng, 2015). Ultimately, field and laboratory studies that take an ecophysiological approach to measuring whole-animal performance (e.g., aerobic scope) and energy expenditure (e.g., $\dot{M}O_{2Min}$, routine or field $\dot{M}O_2$) in elasmobranchs can generate meaningful model inputs for predicting changes in species' and populations' habitat use in response to anthropogenic stressors like climate change.

Bioenergetics

Finally, $\dot{M}O_2$ has direct application to elasmobranch fisheries management and application, through its input value in bioenergetics models. For most simplified bioenergetics models, consumption requirements are modelled as the sum of energy invested in metabolism (i.e., $\dot{M}O_2$), generation of waste products, and somatic investment (i.e., growth and reproduction) (Sundström and Gruber, 1998; Lowe, 2002). Specifically, $\dot{M}O_{2Min}$ and swimming $\dot{M}O_2$ are valuable model inputs; swimming $\dot{M}O_2$ can be the most variable and energetically costly activity in a fish's daily regime (Lowe and Goldman, 2001; Bernal and Lowe, 2015). Data on at least mass- and temperature-scaling of $\dot{M}O_2$ should be available to generate precise model estimates that are sensitive to environmental change (Dowd *et al.*, 2006a; Chen *et al.*, 2008; Dale *et al.*, 2013). Bioenergetics models can then be applied to predict consumption requirements of current populations with the goal of quantifying a population's influence on ecosystem function; for high trophic-level species, models can suggest the extent to which populations exert top-

down control and contribute to mortality of other commercially important species (Dowd *et al.*, 2006a; Barnett *et al.*, 2017). Furthermore, models can be applied to predict changes in consumption requirements for populations in response to threats like climate change, so that inference can be drawn about future ecosystem function (Luongo and Lowe, 2018). Therefore, there is clear support from the literature for applying $\dot{M}O_2$ to create bioenergetics models for elasmobranch populations to support management and conservation goals.

3.5 Conclusions and future directions

As this review highlights, there is potential for measuring $\dot{M}O_2$ to predict responses of elasmobranch species and populations to anthropogenic stressors. Indeed, aquatic respirometry techniques and best-practice guidelines for measuring $\dot{M}O_2$ are becoming increasingly accessible (Chabot *et al.*, 2016a). Resources are readily available to construct respirometry chambers, automated pump systems, and even oxygen meters (Svendsen *et al.*, 2016). Studies have even overcome restrictions for working with large animals that have precluded $\dot{M}O_2$ measurement for some species (J. B. Graham *et al.*, 1990; Sepulveda *et al.*, 2007; Ezcurra *et al.*, 2012; Payne *et al.*, 2015); although, many species of sharks do not grow larger than one meter as adults.

Is there utility in estimating $\dot{M}O_2$ to understand and predict elasmobranch species' and populations' responses to stressors? As this review demonstrates, $\dot{M}O_2$ has application to understanding species' responses to various anthropogenic stressors (e.g., climate change, fisheries stressors, and ecotourism encounters), and can ultimately be applied to predict changes in organismal fitness, spatial ecology, and impact on ecosystem function (i.e., *via* bioenergetics modelling). However, without a foundation of empirical evidence, elasmobranch conservation efforts may be missing critical physiological information underlying species' or populations' responses to current and future anthropogenic stressors (Chin *et al.*, 2010; Lefevre, 2016). For instance, I am unaware of any study to date that relates aspects of aerobic performance in elasmobranchs to behavioural traits, such as boldness, that may influence risk-taking or even catchability in fisheries (Redpath *et al.*, 2010; Robert J Lennox *et al.*, 2017). Elasmobranchs are certainly a group of fishes that have borne the brunt of anthropogenic influence in the natural world. Diseminating tried-and-tested techniques with tangible deliverables to improve management and conservation of imperilled taxa is paramount to transitioning from an Anthropocene extinction to a "good Anthropocene" (Madliger *et al.*, 2017).

Chapter 4: Evaluating the physiological status and survival of neonatal reef sharks under stress

4.1 Summary

Marine protected areas (MPAs) can protect shark populations from targeted fisheries, but resident shark populations may remain exposed to stressors like capture as bycatch and environmental change. Populations of young sharks that rely on shallow coastal habitats, e.g., as nursery areas, may be at risk of experiencing these stressors. The purpose of this study was to characterize various components of the physiological stress response of neonatal reef sharks following exposure to an exhaustive challenge under relevant environmental conditions. To accomplish this, I monitored markers of the secondary stress response and measured oxygen uptake rates ($\dot{M}O_2$) to compare to laboratory-derived baseline values in neonatal blacktip reef (*Carcharhinus melanopterus*) and sicklefin lemon sharks (*Negaprion acutidens*). Measurements occurred over three hours following exposure to an exhaustive challenge (gill-net capture with air exposure). Blood lactate concentrations and pH deviated from baseline values at the three-hour sample, indicating that both species were still stressed three hours after capture. Evidence of a temperature effect on physiological status of either species was equivocal over 28-31 °C. However, aspects of the physiological response were species-specific; *N. acutidens* exhibited a larger difference in blood pH relative to baseline values than *C. melanopterus*, possibly owing to higher minimum $\dot{M}O_2$. Neither species experienced immediate mortality during the exhaustive challenge; although, single instances of delayed mortality were documented for each species. Energetic costs and recovery times could be extrapolated for *C. melanopterus via* respirometry; sharks were estimated to expend 9.9 kJ kg^{-1} (15% of energy expended on daily swimming) for a single challenge and could require 8.4 hours to recover. These data suggest that neonatal *C. melanopterus* and *N. acutidens* are resilient to brief gill-net capture durations, but this was under a narrow temperature range. Defining species' vulnerability to stressors is important for understanding the efficacy of shark conservation tools, including MPAs.

Associated publication

Bouyoucos IA, Weideli OC, Planes S, Simpfendorfer CA, Rummer JL (2018) Dead tired: evaluating the physiological status and survival of neonatal reef sharks under stress. *Conserv Physiol* 6: coy053.

Data availability

Data presented in this manuscript are available from the Research Data Repository (Tropical Data Hub) at James Cook University: http://dx.doi.org/10.25903/5dfc22d73d4c4

4.2 Introduction

Marine protected areas (MPA), including shark sanctuaries, can be important conservation tools for protecting threatened shark populations. Indeed, some shark populations face declines worldwide, owing to overexploitation in fisheries (Dulvy *et al*., 2014). One strategy to potentially reduce the threat of fishing to shark populations is through the creation of MPAs with specific regulations that protect shark populations. For instance, "shark sanctuaries" ban targeted shark fisheries within a country's exclusive economic zone (EEZ) (Cramp *et al.*, 2018). A general concern regarding protected habitats for sharks and other top predators is that other significant threats, like bycatch or environmental change, are not adequately managed (Ward-Paige and Worm, 2017). Incidental capture, or bycatch, affects shark populations through fishing-induced mortality and negative sub-lethal outcomes (Skomal and Mandelman, 2012; Wilson *et al.*, 2014; Ellis *et al.*, 2017). Climate change is resulting in ocean warming and acidification and can affect shark populations through local extirpation as conditions become too extreme in addition to negative sub-lethal outcomes (Rosa *et al.*, 2017; Payne *et al.*, 2018). Protected shark populations may be inherently at risk of experiencing negative outcomes associated with these stressors because virtually all shark sanctuaries are in the tropics. Here, environmental conditions may border species' limits to optimal physiological performance and therefore impede resolving stressors (Rummer *et al.*, 2014). Furthermore, populations that rely on shallow coastal waters during key parts of their life histories (e.g., neonates in nursery areas) are already facing quite variable environmental conditions and can also be risk of fishing interactions (Knip *et al.*, 2010). Therefore, developing an understanding of shark populations' resilience to stressors that they are still expected to face within MPAs can provide valuable information for improving the efficacy of these conservation tools (Chin *et al.*, 2010; Illing and Rummer, 2017).

Neonatal and juvenile shark populations that rely on nearshore habitats may be vulnerable to bycatch. Shallow coastal environments are important for young sharks as nursery areas (Heupel *et al*., 2007). Alternatively, non-nursery areas can provide stability to young shark populations that typically utilize a diversity of habitats (Yates *et al.*, 2012). While shallow waters may offer young sharks protection from predators, proximity to the coastline increases the probability of fishing interactions (Knip *et al.*, 2010). Specifically, young sharks can be caught as bycatch in artisanal and recreational fisheries. Depending on the type of fishery (e.g., hook-and-line or net fishing), different species have varying susceptibilities to lethal or sub-lethal outcomes (Dapp *et al.*, 2016). Capture is generally associated with vigorous escape attempts that can drive a physiological stress response (Brooks *et al.*, 2012; Guida *et al.*, 2016; Gallagher *et al.*, 2017). The stress response is generally characterized by a release of hormones (e.g., adrenaline and noradrenaline), the accumulation of by-products of anaerobic metabolism (e.g., lactate) that drive declines in tissue pH, and resultant osmotic and ion imbalances (Skomal and Mandelman, 2012). Capture is also associated with an increased rate of energy expenditure (Bouyoucos *et al.*, 2017b). Physiological stress and depleted energy reserves following fisheries capture

can even contribute to exhaustion-induced mortality or post-release predation (Danylchuk *et al.*, 2014; Robert J. Lennox *et al.*, 2017). Additional lethal stressors can be problematic because young sharks may already experience high mortality rates during their first year of life (Gruber *et al.*, 2001; Heupel and Simpfendorfer, 2002).

Young shark populations in shallow coastal habitats must also contend with stressors associated with variable environmental conditions. Shallow coastal environments can be prone to seasonal and tidal variations in environmental conditions, such as temperature, salinity, and dissolved oxygen concentrations that affect the abundance and distribution of various species of sharks (Knip *et al*., 2010; Schlaff *et al*., 2014; Oh *et al*., 2017b). Changes in abundance and distribution may be partially attributed to physiological costs associated with variable environmental conditions. Increases in temperature decrease oxygen's solubility in water. Oxygen uptake rates (a proxy for metabolic rate) also increase, along with concomitant decreases in haemoglobin-oxygen (Hb-O_2) affinity (Bernal *et al*., 2012, 2018). In addition, parameters associated with sharks' stress response to capture vary with temperature, such that capture at high temperatures can be fatal for some species (Hoffmayer *et al*., 2012; Danylchuk *et al*., 2014; Guida *et al*., 2016). While sharks may attempt to maintain a preferred body temperature or boundaries to their critical thermal limits, life history stages (e.g., neonates) that derive specific benefits from confined habitats (e.g., predator avoidance within nursery areas) must be able to tolerate local conditions (Knip *et al.*, 2010; Payne *et al.*, 2016, 2018). However, sharks in the tropics are expected to be adapted to a narrow range of temperatures and, therefore, to have a low tolerance for variable environmental temperature conditions (Rummer and Munday, 2017). While there is a paucity of data on thermal tolerance limits for sharks, it is likely that sharks within coastal habitats in tropical latitudes may already be living close to their thermal tolerance limits (Rummer *et al.*, 2014).

The purpose of this study was to characterize various components of the stress response of neonatal reef sharks following an exhaustive challenge. Specifically, I sought to measure the physiological status of neonatal blacktip reef sharks (*Carcharhinus melanopterus*) and sicklefin lemon sharks (*Negaprion acutidens*) at multiple points in time following *in situ* gill-net capture. The objectives of this study were to (1) characterize physiological responses in neonatal reef sharks following capture, (2) predict the effect of changes in environmental temperatures on physiological status, (3) assess the differential vulnerability of co-occurring neonatal reef shark species to stress-induced physiological impairment, and (4) estimate the energetic cost of an exhaustive challenge in the context of routine energy requirements. Studies of this nature are necessary for understanding whether stressors hold lethal or sub-lethal consequences under predictable environmental conditions in important habitats like shark nursery areas. As such, these data will have management applications to better support conservation initiatives for reef sharks (Illing and Rummer, 2017).

4.3 Materials and methods

All experiments were approved by James Cook University Animal Ethics Committee protocol A2089. Research on sharks in French Polynesia was approved under Arrêté N° 9524 issued by the Ministère de la Promotion des Langues, de la Culture, de la Communication et de l'Environnement of the French Polynesian government on 30 October 2015.

Study site, animal collection and husbandry

Fieldwork was conducted from shore around Moorea, French Polynesia (17°30'S, 149°51'W), where targeted shark fishing in the country's EEZ has been banned since 2012 (Ward-Paige and Worm, 2017). Newborn *C. melanopterus* and *N. acutidens* are abundant during parturition months from September through February (Mourier and Planes, 2013; Mourier *et al*., 2013a, b). Sharks were collected during November and December 2016 using monofilament gill-nets (50.0 m × 1.5 m, 5.0 cm mesh) fished at dusk (17:00-20:00). Captured sharks were immediately identified and removed from the net in under five minutes. Prior to release, biological data (total length, mass, and sex) were collected from all sharks. Individuals were tagged with coloured T-bar anchor tags (Hallprint, Hindmarsh Valley, SA, Australia) to avoid repeatedly sampling recaptured animals for this study. Only animals in good condition (e.g., without open or healing bite wounds or retained fish hooks) were sampled for this study. Environmental temperatures were recorded every ten minutes with one or two temperature data loggers (UA-002-64, Onset Computer Corporation, Bourne, MA, USA) that were deployed in a transect parallel to the gill-net.

A subset of sharks was transported to the Centre de Recherches Insulaires et Observatoire de l'Environnement (CRIOBE) by vehicle in 200.0 L insulated coolers of aerated seawater (Chin *et al*., 2015). Before transport, sharks were retained in individual flow-through mesh bags (0.2 m diameter and 1.0 m long) for no more than one hour prior to transport. Including transport, sharks were typically confined for under 90 minutes post-capture before arriving at the CRIOBE's holding facility. Sharks were housed in 1250 L circular flow-through tanks (2-3 sharks per tank), and *C. melanopterus* and *N. acutidens* were separated. Tanks were covered with 60% shade cloth, continuously aerated, and supplied filtered seawater from an offshore pump. The holding facility was covered and open-sided, exposing sharks to a natural photoperiod. Sharks were held for at least one week before experimentation and were fed 5.0% of their body mass in fresh tuna every other day (Chin *et al.*, 2015) with the exception of a 48-hour fasting period prior to their use in experiments. All sharks were released to their original capture site after no more than four weeks in captivity.

Quantifying physiological responses

Physiological responses to an exhaustive challenge were quantified for *C. melanopterus* and *N. acutidens*. The exhaustive challenge used throughout the entire study included approximately three

minutes of gill-net capture (3.4 ± 1.2 minutes S.D.) and one minute of air exposure. Gill-net capture has been demonstrated to induce exhaustion in elasmobranchs (Fric*k et al*., 2009, 2010, 2012) – including juvenile *C. melanopterus* and *N. acutidens* (Dapp *et al*., 2017) – and a standardized duration of air exposure is commonly employed along with an exhaustive challenge to maximally exhaust fish and to simulate handling of fish out of water by fishers (Clark *et al.*, 2013; Rummer *et al.*, 2016). Values for physiological metrics were generated from unique individuals subjected to one of four treatments. One group of laboratory-acclimated sharks was phlebotomized in a quiescent state after 2-4 weeks in captivity and a 48-hour fasting period to generate minimally-stressed values ("baseline" treatment). A quiescent state was achieved by subjecting sharks to 24 hours of respirometry, which allowed for minimal handling and rapid sampling upon removing sharks from respirometry chambers. A second group of sharks was phlebotomized immediately following the exhaustive challenge in the field ("immediate" treatment). The third group of sharks faced the same exhaustive challenge and was retained in flow-through mesh bags in the field for three hours before phlebotomy ("three-hour" treatment). A final group of sharks was sampled after three hours in a respirometry chamber that was used to estimate energetic costs and recovery times for the exhaustive challenge ("respirometry" treatment). All blood samples were processed immediately following phlebotomy.

Sharks were phlebotomized via caudal puncture using heparin-rinsed 23.0 gauge 3.8 cm needles. Five parameters were measured using point-of-care analytical devices: blood glucose concentration (mmol L^{-1}), blood lactate concentration (mmol L^{-1}), blood pH, haemoglobin concentration ([Hb]; g dL^{-1}), and haematocrit (Hct). Blood was first transferred from syringes directly to two 70 µL microcapillary tubes that were run in parallel in a microhaematocrit centrifuge (ZIPocrit, LW Scientific, Lawrenceville, GA, USA) for two minutes at 4400 *g* (Danylchuk *et al*., 2014). Whole blood glucose and lactate concentrations were measured with 10 µL samples of whole blood using an Accutrend Plus (Roche Diagnostics Ltd., Rotkreuz, Switzerland), with ranges of 1.1-33.3 mmol L^{-1} and 0.8-22.0 mmol L^{-1}, respectively (Butcher *et al.*, 2015). Readings that were outside the measurement range were reported as the value of the upper or lower device limit for statistical analyses. Haemoglobin concentration was measured with a HemoCue Hb 201 System (Australia Pty Ltd, Victoria, Australia) using 10 µL of whole blood, and was corrected using a calibration equation generated for fish that has previously been applied to sharks (Clark *et al.*, 2008; Heinrich *et al.*, 2014). Haemoglobin concentration was then converted to tetramer Hb concentration (Hb_4, in mmol L^{-1}) using conversions generated for tropical reef species in order to calculate mean cell haemoglobin concentration (MCHC; mmol L^{-1}), as Hb_4 divided by Hct (Rummer *et al.*, 2013; Heinrich *et al.*, 2014). Blood pH was measured using a HI98165 pH meter (Hanna Instruments, Victoria, Australia), and raw pH values were converted to values derived from the conventional i-STAT system using a correction formula generated for juvenile lemon sharks (*N. brevirostris*) at 25.6-31.3 °C (Talwar *et al.*, 2017).

Estimating energetic costs and recovery

To estimate costs of an exhaustive challenge and recovery times, individuals from another subset of sharks (*C. melanopterus*) were, transferred to individual field respirometry chambers immediately after capture and air exposure so that oxygen uptake rates ($\dot{M}O_2$, in mg O_2 kg^{-1} h^{-1}) could be measured over three hours. To do this, two respirometry chambers (24.0 cm diameter and 70.0 cm long, 32.0 L volume including tubing) were submerged in a 400.0 L circular pool positioned approximately 3.0 m from the shoreline. Water in the pool was continuously aerated, and was supplied at a rate of 4800.0 L h^{-1} from a pump approximately 5.0 m offshore in at least 0.3 m of water. Respirometry chambers were configured for intermittent-flow respirometry with 2500.0 L h^{-1} flush and recirculating pumps (Rummer *et al*., 2016; Svendsen *et al*., 2016). Dissolved oxygen concentration (DO, in mg L^{-1}) was measured every second with fibre optic probes that were mounted within chambers and connected to a Firesting Optical Oxygen Meter (PyroScience, Aachen, Germany). Probes were calibrated to fully-aerated freshwater (100.0% saturation) before each use and to 0.0% saturation with sodium sulphite as needed. Flush pumps were manually operated to cycle flush (9.1 ± 6.5 minutes SD) and measurement periods (11.5 ± 6.1 minutes) such that DO remained above 80.0% air saturation. The timing of cycles was determined by watching DO in real-time on a laptop computer. Sharks were placed into the chambers immediately upon capture, and therefore the time from the onset of capture to the beginning of the first measurement was 4.4 ± 1.2 minutes (i.e., the length of the exhaustive challenge). Each field respirometry trial consisted of 6-12 measurement periods over three hours. Then, immediately after removal from respirometry chambers, all sharks were phlebotomized to determine whether undergoing respirometry influenced the stress response (the "respirometry" treatment).

Oxygen uptake rates were estimated by first calculating rates of DO decline every 30 seconds during each measurement using LabChart (7.3.8, ADInstruments, Dunedin, New Zealand). Specifically, $\dot{M}O_2$ was calculated as $\dot{M}O_2 = SV_{Resp}M^{-1}$ where S is the slope of the linear decline in DO (in mg O_2 L^{-1} s^{-1}), V_{Resp} is the volume of the respirometer minus the shark's volume (in L), and M is the mass of the fish (in kg). Background respiration was accounted for by modelling the linear increase in background $\dot{M}O_2$, measured before and after each trial in chambers without fish, and subtracting proportional background $\dot{M}O_2$ from each $\dot{M}O_2$ measurement (Rodgers *et al*., 2016; Rummer *et al*., 2016). The highest $\dot{M}O_2$ during each measurement period was selected, and these values were fit with an exponential decay curve (recovery curve). The highest $\dot{M}O_2$ value for each shark was recorded as its maximum $\dot{M}O_2$ ($\dot{M}O_{2Max}$).

Oxygen uptake rates of minimally-stressed, resting sharks (*C. melanopterus* and *N. acutidens*; the same animals used in for the "baseline" treatment) were measured in the laboratory. The same respirometry chambers described above were placed in holding tanks, and flush pumps were automated with a custom-built data acquisition system and software (National Instruments, Austin, Texas, USA). Flush pumps were automated to shut off for 5 minutes every 12 minutes for *C. melanopterus*, and 5 minutes

every 15 minutes for *N. acutidens*, yielding at least 120 measurements for *C. melanopterus* and at least 96 measurements for *N. acutidens* over 24 hours. Shorter measurement periods and longer flush periods were deemed necessary for *N. acutidens* because all individuals were larger than the *C. melanopterus* used for this study and had higher $\dot{M}O_2$. One slope (*S*) was calculated for each measurement. Minimum $\dot{M}O_2$ ($\dot{M}O_{2Min}$) was calculated as the mean of the lowest 10% of $\dot{M}O_2$ values, excluding values outside of the mean ± 2 SD (Clark *et al*., 2013). All sharks were phlebotomized immediately after removal from respirometry chambers to generate baseline values.

Statistical and data analyses

Underlying physiological responses were characterized by comparing values of physiological parameters over time after an exhaustive challenge, and against baseline values. The influence of temperature on physiological status (i.e., values of physiological and oxygen uptake parameters) was assessed by including temperature as a covariate in models. Physiological parameters (i.e., blood glucose and lactate concentrations, blood pH, [Hb], Hct, and MCHC) were fit with linear models to observe variation in responses with treatment (fixed effect), temperature and mass (covariates) for both species. For *C. melanopterus*, the factor "treatment" had four levels (i.e., baseline, immediate, three-hour, and respirometry). It was not possible to catch comparable numbers of *N. acutidens*, and as a result the factor "treatment" only had three levels (i.e., baseline, immediate, and three-hour). All possible interactions (two-way and three-way) were included in these models for *C. melanopterus*. Samples sizes were too small to include interactions for *N. acutidens*. Post-hoc multiple comparisons were made with Tukey's highly significant difference (HSD) tests. Models were validated with Q-Q plots of model residuals, and by plotting residuals against treatment and fitted values (Zuur *et al*., 2007). For all tests, the acceptable Type I error rate (α) was 0.05, and all analyses were conducted using the R Stats Package (R Core Team, 2018).

Recovery times and costs were estimated for *C. melanopterus* using respirometry data. The mean value of $\dot{M}O_{2Min}$ that was derived from the laboratory was used as a baseline for estimating the excess post-exercise oxygen consumption (EPOC, in mg O_2 kg^{-1}) of individual sharks from field respirometry. Recovery times were estimated for individual sharks as the time when the recovery curve intersected the upper 95% confidence interval limit of $\dot{M}O_{2Min}$ (Bouyoucos *et al*., 2017a). Excess post-exercise oxygen consumption, which represents the cost of recovery from exhaustive activity (Gaesser and Brooks, 1984), was calculated as the area bound by individual sharks' recovery curves, $\dot{M}O_{2Min}$, the time of the first $\dot{M}O_2$ measurement, and the time of recovery (Bouyoucos *et al.*, 2017a). Oxygen uptake parameters (i.e., $\dot{M}O_{2Min}$, $\dot{M}O_{2Max}$, EPOC, and recovery time) were fit with linear models to observe variation with temperature and mass, including interactions.

4.4 Results

Quantifying physiological responses

Morphometric data for *C. melanopterus* are presented in Table 4.1. Sharks exhibited significant changes in blood glucose and lactate concentrations as well as blood pH across treatments (Table 4.2). Blood glucose concentrations at three hours were higher than baseline values (Tukey's HSD, $t = 4.387$, $p < 0.001$) and values for immediately sampled sharks (Tukey's HSD, $t = 4.062$, $p = 0.002$) (Figure 4.1a). Blood glucose concentrations also had a positive linear relationship with temperature (Linear regression, $R^2 = 0.27$, $F_{1, 29} = 10.82$, $p = 0.003$; 27.9-30.9 °C; Figure 4.2) across treatments (Supplementary Table 4.2). Baseline and immediately-sampled values for blood lactate concentrations did not differ (Tukey's HSD, $t = 1.436$, $p = 0.489$), and values after three hours in recovery bags and respirometry chambers were not different (Tukey's HSD, $t = -0.639$, $p = 0.918$). Blood lactate concentrations were at least 14-fold higher three hours post-capture relative to baseline and immediately sampled values (Tukey's HSD, $p < 0.001$) (Figure 4.1b). Lastly, blood pH was uniformly reduced across all treatments relative to baseline values (Tukey's HSD, $p < 0.001$) (Figure 4.1c). No significant differences in [Hb] (4.48 ± 0.77 g dL^{-1}), Hct (0.17 ± 0.03), or MCHC (4.20 ± 0.58 mmol L^{-1}) were detected.

Table 4.1 Morphometric data (mean ± S.D.), samples sizes by sex, and water temperatures by experimental treatment. Baseline values were taken from quiescent, fasted sharks ("baseline"). Other sharks were phlebotomized immediately following exhaustive gill-net capture ("immediate"), after three hours in a recovery bag ("three-hour"), or after three hours in a field respirometry chamber ("respirometry").

Species	Treatment	n (f:m)	Total length (mm)	Mass (kg)	Water Temperature (°C)
Carcharhinus melanopterus	Baseline	6:2	577.88 ± 30.13	1.08 ± 0.16	29.66 ± 0.69
	Immediate	3:5	578.63 ± 31.08	1.08 ± 0.12	28.77 ± 0.49
	Three-hour	1:7	587.75 ± 32.34	1.18 ± 0.18	29.72 ± 0.83
	Respirometry	3:5	559.13 ± 20.15	1.02 ± 0.12	30.06 ± 1.28
Negaprion acutidens	Baseline	1:2	688.67 ± 17.01	1.55 ± 0.26	29.29 ± 0.75
	Immediate	2:6	647.50 ± 35.40	1.45 ± 0.23	30.14 ± 0.49
	Three-hour	1:3	680.00 ± 5.29	1.49 ± 0.18	29.73 ± 0.25

Table 4.2 Linear model output for physiological parameters (response) fit with treatment, mass, and temperature as factors for blacktip reef sharks (*Carcharhinus melanopterus*). Oxygen uptake parameters

(response) were fit with temperature and mass as covariates. Abbreviations: excess post-exercise oxygen consumption (EPOC), haematocrit (Hct), haemoglobin concentration (Hb), maximum oxygen uptake rate ($\dot{M}O_{2Max}$), mean cell haemoglobin concentration (MCHC), minimum oxygen uptake rate ($\dot{M}O_{2Min}$).

Response	**Factor**	**D.F.**	***F*-value**	***p*-value**
Glucose	**Treatment**	**3, 15**	**14.75**	**< 0.001**
	Temperature	**1, 15**	**6.77**	**0.020**
	Mass	1, 15	2.77	0.117
	Treatment × Temperature	3, 15	1.16	0.359
	Treatment × Mass	3, 15	2.06	0.148
	Temperature × Mass	1, 15	0.67	0.424
	Treatment × Temperature × Mass	3, 15	1.82	0.186
Lactate	**Treatment**	**3, 15**	**340.91**	**< 0.001**
	Temperature	1, 15	1.39	0.256
	Mass	1, 15	0.94	0.348
	Treatment × Temperature	3, 15	1.86	0.180
	Treatment × Mass	3, 15	0.44	0.729
	Temperature × Mass	1, 15	0.00	0.985
	Treatment × Temperature × Mass	3, 15	1.21	0.340
pH	**Treatment**	**3, 15**	**11.58**	**< 0.001**
	Temperature	1, 15	0.03	0.865
	Mass	1, 15	0.27	0.609
	Treatment × Temperature	3, 15	0.73	0.551
	Treatment × Mass	3, 15	0.07	0.977
	Temperature × Mass	1, 15	0.01	0.923
	Treatment × Temperature × Mass	3, 15	0.76	0.533
Hb	Treatment	3, 15	0.17	0.914
	Temperature	1, 15	0.27	0.613
	Mass	1, 15	0.40	0.127
	Treatment × Temperature	3, 15	0.37	0.771
	Treatment × Mass	3, 15	0.87	0.480
	Temperature × Mass	1, 15	0.04	0.846
	Treatment × Temperature × Mass	3, 15	1.32	0.306
Hct	Treatment	3, 15	0.14	0.934
	Temperature	1, 15	0.34	0.570

	Mass	1, 15	1.63	0.221
	Treatment × Temperature	3, 15	0.78	0.526
	Treatment × Mass	3, 15	0.41	0.764
	Temperature × Mass	1, 15	0.13	0.719
	Treatment × Temperature × Mass	3, 15	0.54	0.662
MCHC	Treatment	3, 15	0.76	0.536
	Temperature	1, 15	0.04	0.855
	Mass	1, 15	0.03	0.873
	Treatment × Temperature	3, 15	1.56	0.240
	Treatment × Mass	3, 15	0.53	0.669
	Temperature × Mass	1, 15	0.00	0.941
	Treatment × Temperature × Mass	3, 15	0.43	0.736
$\dot{M}O_{2Max}$	Temperature	1, 4	0.527	0.508
	Mass	1, 4	0.018	0.901
	Temperature × Mass	1, 4	1.786	0.252
$\dot{M}O_{2Min}$	Temperature	1, 4	0.053	0.829
	Mass	1, 4	0.160	0.710
	Temperature × Mass	1, 4	2.446	0.191
EPOC	Temperature	1, 4	0.654	0.464
	Mass	1, 4	0.483	0.525
	Temperature × Mass	1, 4	5.014	0.088
Recovery Time	Temperature	1, 4	1.192	0.336
	Mass	1, 4	0.009	0.931
	Temperature × Mass	1, 4	2.949	0.161

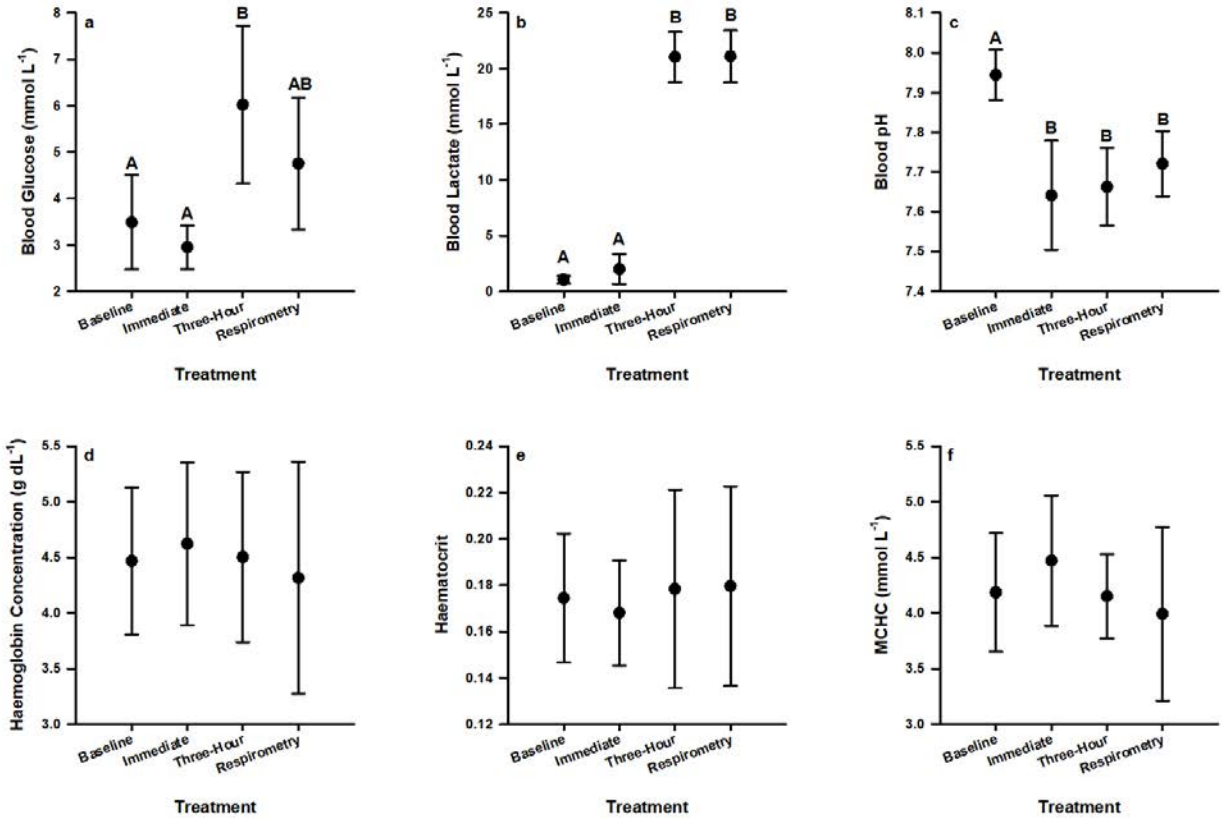

Figure 4.1 Indicators of the stress response in juvenile blacktip reef sharks (*Carcharhinus melanopterus*) following an exhaustive challenge in situ. Baseline values were taken from quiescent, fasted sharks ("baseline"). Other sharks were phlebotomized immediately following exhaustive gill-net capture ("immediate"), after three hours in a recovery bag ("three-hour"), or after three hours in a field respirometry chamber ("respirometry"). Differing letters denote statistically significant differences. Abbreviation: mean cell haemoglobin concentration (MCHC).

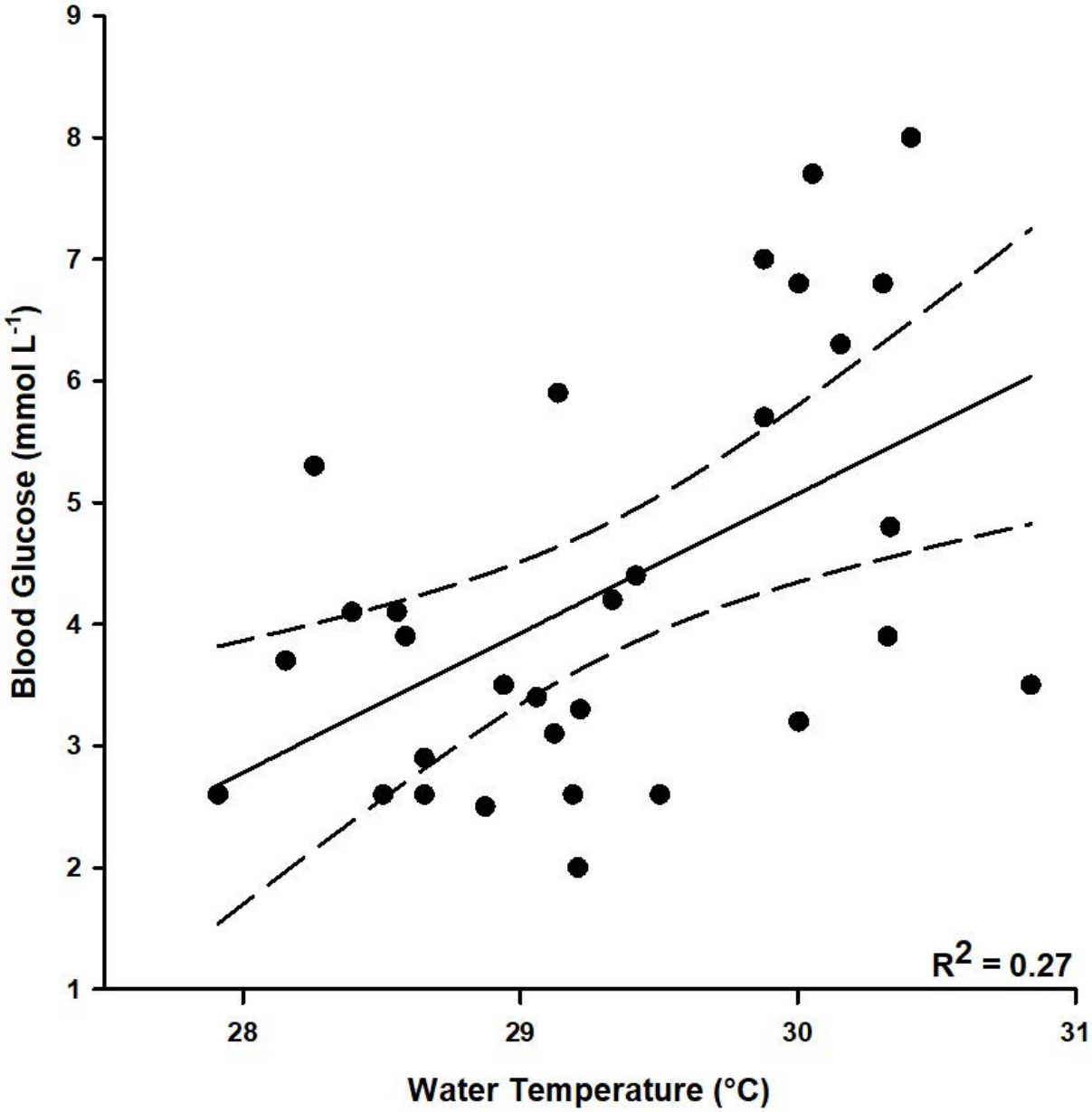

Figure 4.2 Relationship between temperature and physiological status (blood glucose concentrations) for blacktip reef sharks (*Carcharhinus melanopterus*).

Morphometric data for *N. acutidens* are presented in Table 1. Differences between treatments were only detected for blood lactate concentration and blood pH (Table 4.3). Blood lactate concentrations were at least 6-fold higher for *N. acutidens* three hours after capture relative to baseline (Tukey's HSD, t = 9.128, $p < 0.001$) and immediately-sampled values (Tukey's HSD, t = 8.407, $p < 0.001$), which were not different (Tukey's HSD, t = -1.679, p = 0.269) (Figure 4.3b). In addition, blood pH was significantly reduced for sharks sampled immediately (Tukey's HSD, t = -3.153, p = 0.014) and three hours post-

capture (Tukey's HSD, t = -2.940, p = 0.037) relative to baseline pH (Figure 4.3c). Blood pH values sampled immediately or three hours post-capture were not different (Tukey's HSD, t = 0.185, p = 0.981). There were no significant differences across treatments for blood glucose concentration (5.22 ± 0.88 mmol L^{-1}), Hb (5.15 ± 0.83 g dL^{-1}), Hct (0.19 ± 0.03), or MCHC (4.26 ± 0.77 mmol L^{-1}) for *N. acutidens*. No physiological parameter varied with mass (Table 4.3), and Hb had a positive linear relationship with temperature (Linear regression, $R^2 = 0.37$, $F_{1, 13} = 7.59$, $p = 0.016$; 29.5-30.9 °C; Figure 4.4).

Table 4.3 Linear model output for physiological parameters (response) fit with treatment, temperature and mass as factors for sicklefin lemon sharks (*Negaprion acutidens*). Abbreviations: haematocrit (Hct), haemoglobin concentration (Hb), mean cell haemoglobin concentration (MCHC).

Response	**Factor**	**D.F.**	***F*-value**	***p*-value**
Glucose	Treatment	2, 8	1.551	0.270
	Temperature	1, 8	0.305	0.596
	Mass	1, 8	0.504	0.498
Lactate	**Treatment**	**2, 8**	**50.211**	**< 0.001**
	Temperature	1, 8	1.866	0.209
	Mass	1, 8	0.135	0.723
pH	**Treatment**	**2, 8**	**6.593**	**0.018**
	Temperature	1, 8	0.002	0.968
	Mass	1, 8	0.631	0.449
Hb	Treatment	2, 8	1.018	0.404
	Temperature	**1, 8**	**6.318**	**0.036**
	Mass	1, 8	0.167	0.693
Hct	Treatment	2, 8	0.795	0.484
	Temperature	1, 8	0.157	0.703
	Mass	1, 8	0.070	0.797
MCHC	Treatment	2, 8	0.372	0.701
	Temperature	1, 8	2.780	0.134
	Mass	1, 8	0.052	0.826

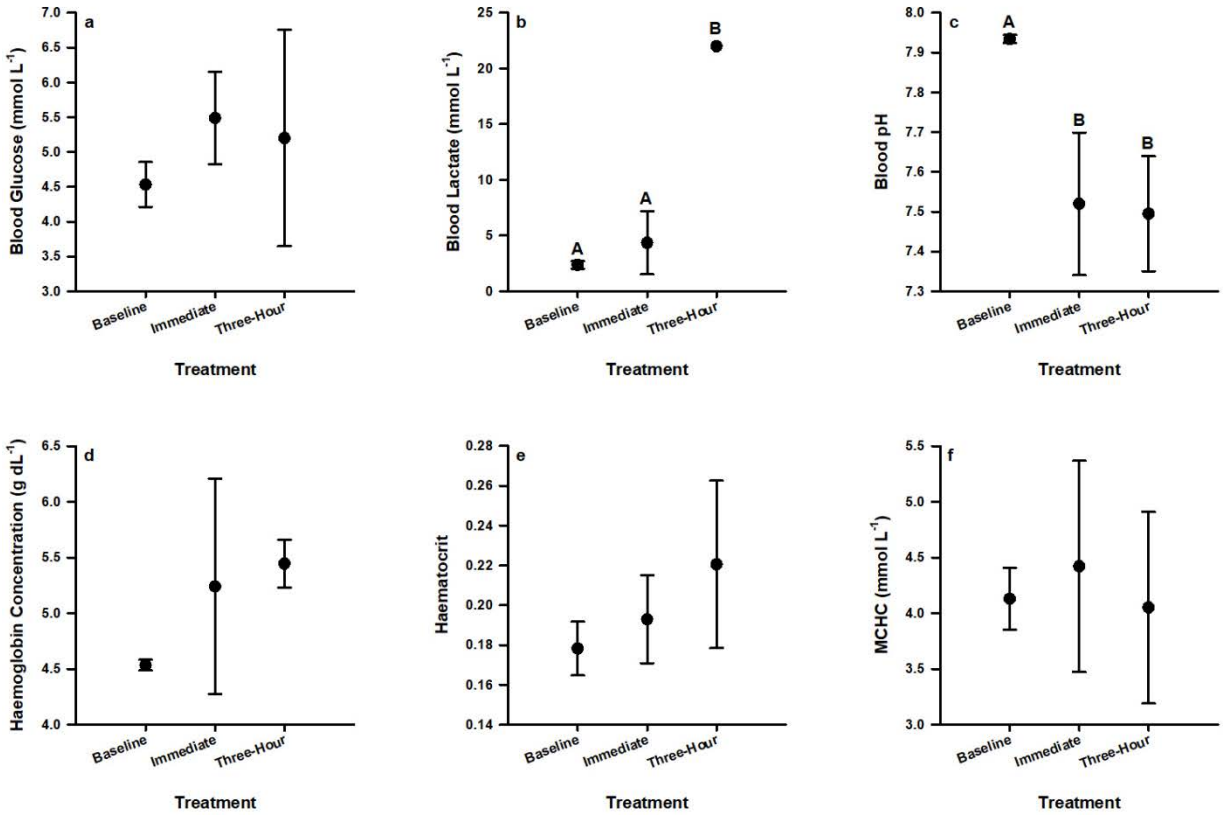

Figure 4.3 Indicators of the stress response in juvenile sicklefin lemon sharks (*Negaprion acutidens*) following an exhaustive challenge in situ. Baseline values were taken from quiescent, fasted sharks ("baseline"). Other sharks were phlebotomized immediately following exhaustive gill-net capture ("immediate") or after three hours in a recovery bag ("three-hour"). Differing letters denote statistically significant differences. Abbreviation: mean cell haemoglobin concentration (MCHC).

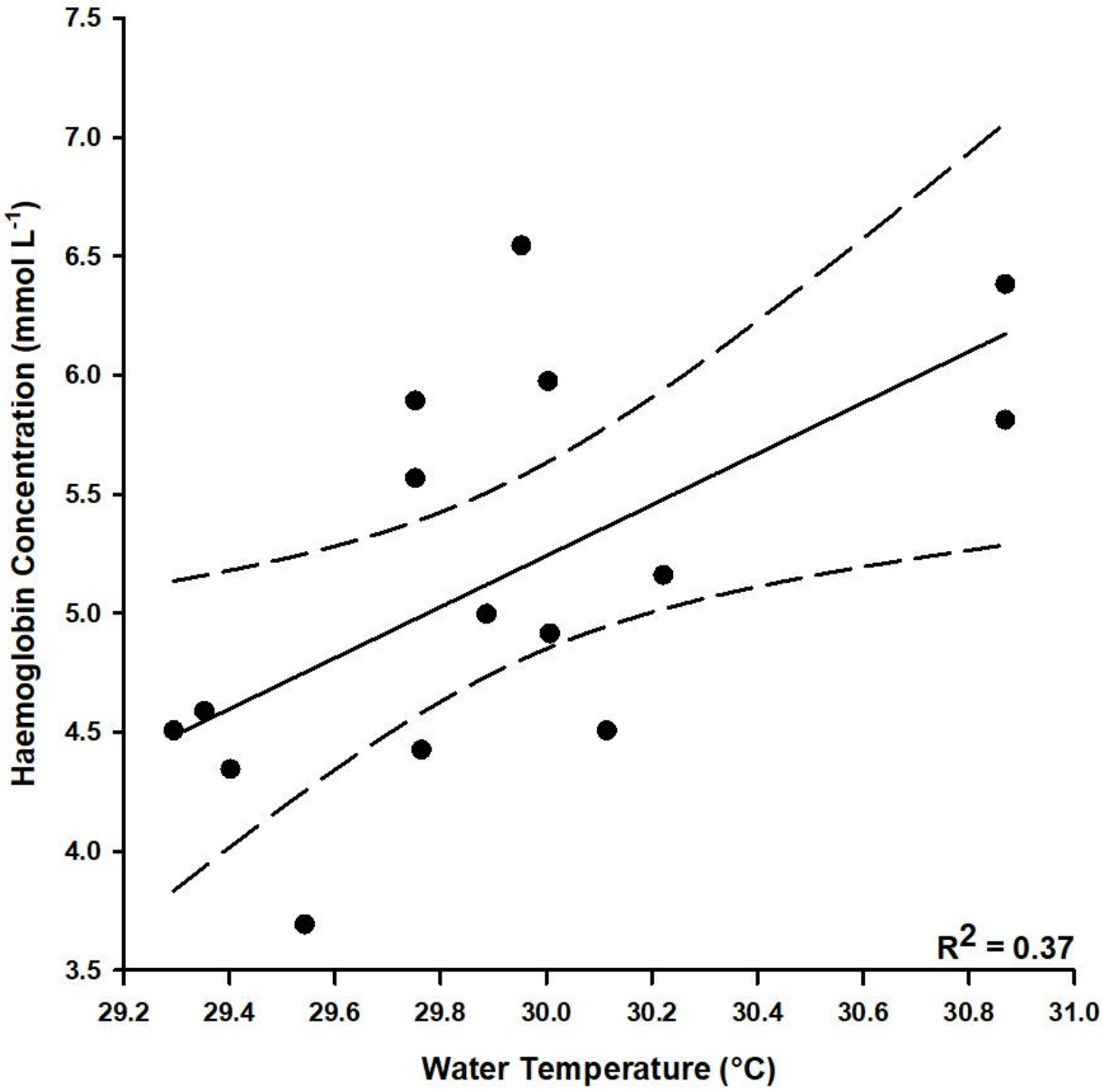

Figure 4.4 Relationship between temperature and physiological status (haemoglobin concentrations) for sicklefin lemon sharks (*Negaprion acutidens*).

Estimating energetic costs and recovery

Mean $\dot{M}O_{2Max}$ was 322.91 ± 72.93 mg O_2 kg^{-1} h^{-1}, and EPOC was 703.72 ± 361.53 mg O_2 kg^{-1} at 30.06 ± 1.28 °C (Figure 4.5a). From laboratory measurement for *C. melanopterus*, $\dot{M}O_{2Min}$ was 100.92 ± 11.30 mg O_2 kg^{-1} h^{-1} at 29.66 ± 0.69 °C, and estimated aerobic scope (AS = $\dot{M}O_{2Max} - \dot{M}O_{2Min}$) was 221.98 mg O_2 kg^{-1} h^{-1}. No shark had recovery curves that intersected the upper 95% CI limit of $\dot{M}O_{2Min}$ (110.38 mg O_2 kg^{-1} h^{-1}) in under three hours, and extrapolated recovery times ranged from 3.1-19.8 hours (8.42 ±

5.78 hours). None of the oxygen uptake parameters varied with temperature, mass, or their interaction (Table 4.2). Lastly, only three *N. acutidens* were brought to the CRIOBE to generate baseline values for this species, where $\dot{M}O_{2Min}$ was determined to be 139.95 ± 12.07 mg O_2 kg^{-1} h^{-1}.

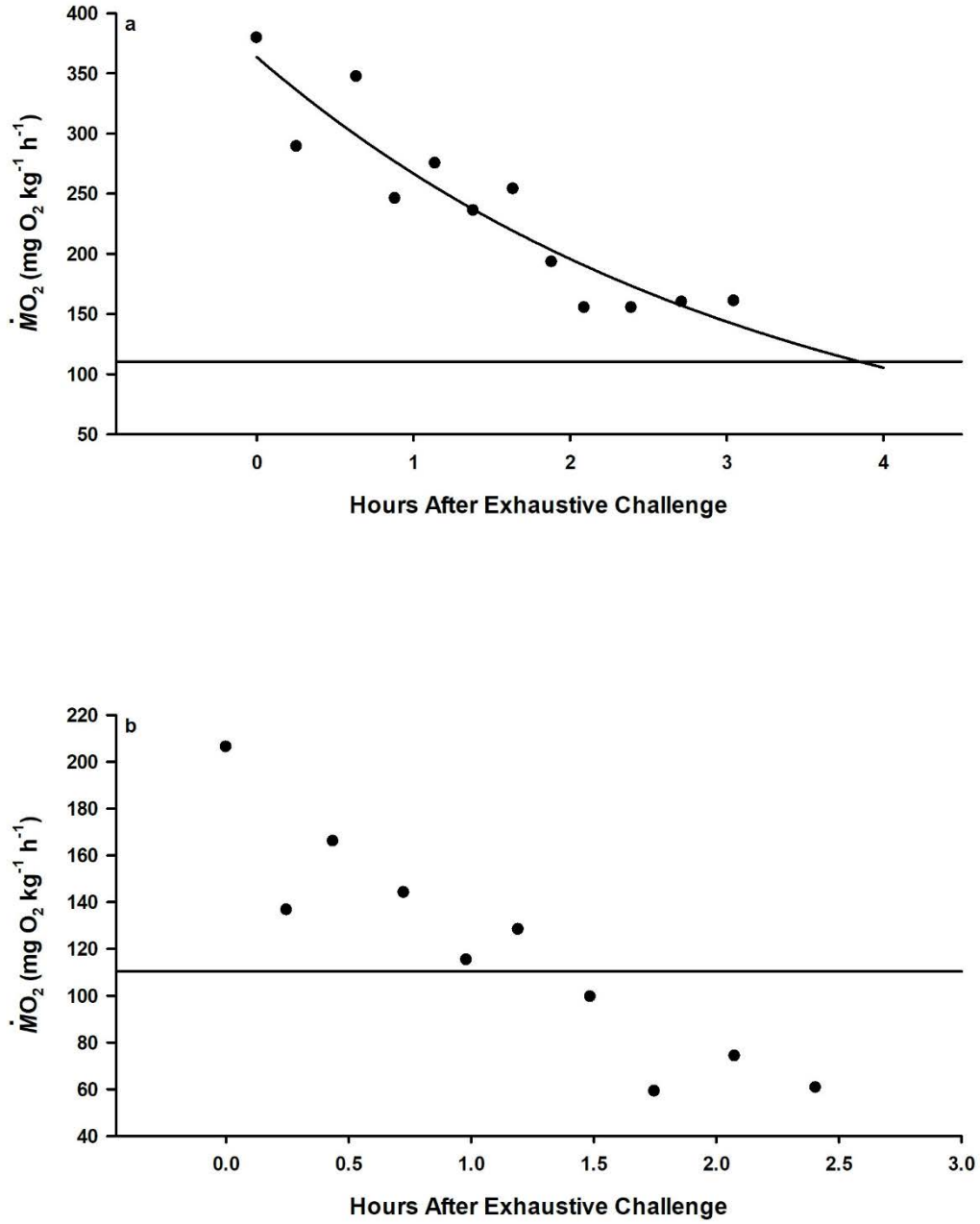

Figure 4.5 Representative traces of excess post-exercise oxygen consumption (EPOC). Data are presented for individual *Carcharhinus melanopterus* in good condition (a) and moribund (b). Oxygen uptake rates ($\dot{M}O_2$) were measured for three hours after sharks were caught in gill-nets. Recovery time was extrapolated by fitting $\dot{M}O_2$ with an exponential decay function. The upper 95% confidence interval limit of minimum $\dot{M}O_2$ (horizontal line) was estimated from captive *C. melanopterus*, and the

intersection of these two lines represent an individual's extrapolated recovery time. The data in the lower panel are not fit with a recovery curve, because this individual exhibited aerobic failure when $\dot{M}O_2$ dropped below its estimated "recovered" value.

Observed mortality

Immediate mortality was 0% for both species, but delayed mortality was observed for both *C. melanopterus* and *N. acutidens*. A single *C. melanopterus*, which was caught at 32.33 °C, was moribund upon release from its field respirometry chamber. Oxygen uptake data suggest that this animal experienced aerobic failure at approximately 1.5 hours following the exhaustive challenge (Figure 4.5b). Including this animal, delayed mortality for *C. melanopterus* was 5.9% (1/17). The body of one *N. acutidens* was recovered the day after release from a recovery bag, suggesting that delayed mortality for this species was 25.0% (1/4); this animal was caught at 29.75 °C.

4.5 Discussion

Neonatal *C. melanopterus* and *N. acutidens* were still stressed three hours after facing an exhaustive challenge. Values for blood glucose, lactate, and pH taken three hours after the exhaustive challenge deviated from baseline values for both species (except blood glucose concentrations in *N. acutidens*). These physiological responses are characteristic of the elasmobranch secondary stress response (Skomal and Mandelman, 2012; Wilson *et al*., 2014). Vigorous attempts by sharks to escape fishing gear are generally supported by anaerobic metabolic pathways that are partially characterized by increases in blood glucose and lactate concentrations and a resultant drop in blood pH (Guida *et al.*, 2016; Bouyoucos *et al.*, 2017b; Gallagher *et al.*, 2017). Furthermore, sharks entangled in gill nets may not be able to ventilate, thereby driving further declines in blood pH by restricting carbon dioxide offloading, and relying on anaerobic metabolic pathways while oxygen uptake is impeded (Dapp *et al.*, 2016). Even if a shark that is restrained in a net can actively ventilate, for example *via* buccal pumping, this strategy could be a far less efficient method for gas exchange and may actually exacerbate the stress response (Parsons and Carlson, 1998; Brooks *et al.*, 2011). Many shark species also lack mechanisms to modulate haematological parameters related to improving oxygen delivery during a stress response (Brill and Lai, 2015). It is important to consider whether the inability of sharks to move in recovery bags and respirometry chambers prolonged recovery to over three hours. Indeed, previous studies of free-swimming sharks have also documented that sharks facing brief exhaustive challenges can take over three hours to recover (Brooks *et al.*, 2011, 2012). While I documented neonatal sharks experiencing various aspects of the stress response, it was beyond the scope of this study to determine exactly how detrimental the levels of stress experienced were (i.e., changes in recovery times or risk of experiencing mortality). Interestingly, recapture rates for both species have been relatively high within a given parturition season (~15-30 %), but low recapture rates from over five years of annual surveys

around Moorea suggest that natural mortality (e.g., starvation or predation) is quite high among these populations (S.P. unpublished results). Size classes between neonates and adults are notably absent from gill-net and hook-and-line surveys; although, variable habitat use or size-selective gears may appear to suggest high juvenile mortality in the absence of natural mortality rate estimates for this population (Mourier *et al.*, 2013b). Around Moorea, exhaustive challenges are expected in the form of artisanal and recreational fisheries bycatch and predator-prey interactions (Chin *et al.*, 2015; Mourier *et al.*, 2017; Thiault *et al.*, 2017). Although French Polynesia is a shark sanctuary, implementing and enforcing management strategies to mitigate fishing pressure during parturition months could reduce neonatal sharks' chance of facing exhaustive challenges (i.e., fishing capture).

Evidence of an effect of temperature on the physiological status of *C. melanopterus* and *N. acutidens* was equivocal over a narrow, albeit ecologically relevant temperature range. Blood glucose concentrations doubled, on average, over a 3.0 °C range for *C. melanopterus* (27.9-30.9 °C), and [Hb] increased with temperature by approximately 23% over a 1.4 °C range (29.5-30.9 °C) in *N. acutidens*. Some markers of physiological status may respond to changing environmental temperatures for elasmobranchs because of temperature's influence on the metabolic rates of ectothermic organisms (Hoffmayer *et al*., 2012; Guida *et al*., 2016). Conversely, temperature-associated changes in blood glucose concentrations of *C. melanopterus* could simply reflect increased activity levels of sharks in warmer water, as opposed to a temperature-mediated metabolic response (Payne *et al.*, 2016, 2018; Whitney *et al.*, 2016). Increases in [Hb] of *N. acutidens* with increasing temperature may be a compensatory mechanism as Hb-O_2 affinity increases (i.e., a reverse temperature effect) or decreases (Bernal *et al.*, 2018). Alternatively, the apparent correlation between [Hb] and temperature may have been spurious, as changes in [Hb] ultimately did not result in variation in MCHC or Hct. However, *N. acutidens* in warmer waters may have had smaller red blood cells (RBCs) or immature RBCs in greater circulation but with similar [Hb] to sharks at cooler temperatures that would appear as an increase in [Hb] without affecting other haematological variables. No other physiological or oxygen uptake parameters that were measured displayed variations with temperature. Metabolic compensation, where an organism maintains consistent $\dot{M}O_2$ with temperature acclimation, has not been documented for elasmobranchs (Tullis and Baillie, 2005). It is likely, however, that, even for seasonally-acclimated elasmobranchs, variations in temperature exceeding 3.0 °C may be necessary to elicit an observable response (Carlson and Parsons, 1999; Neer *et al.*, 2006). Moorea's neonatal shark populations face summer temperatures that average 30 °C during parturition months, daily variations of up to 8 °C, and extreme temperatures ranging 26-36 °C (J.L.R. unpublished results). For juvenile sharks, facing an exhaustive challenge in shallow coastal waters when temperatures are high can be lethal (Danylchuk *et al.*, 2014). The only *C. melanopterus* to die in this study was, coincidentally, captured at >32 °C, but I could not confirm whether this single mortality was related to temperature. Both *C. melanopterus* and *N. acutidens* exhibit some degree of philopatry to natal areas around Moorea and elsewhere, such that

extreme temperature events in these potential nursery areas could put neonates at risk of mortality after facing exhaustive challenges (Mourier and Planes, 2013; Mourier *et al*., 2013b; Oh *et al*., 2017a). Without controlled studies to investigate the effect of temperature on reef sharks' resilience to stress, it is unclear whether thermal stressors like ocean warming brought on by climate change could be problematic for neonatal sharks in tropical nearshore habitats.

Physiological status before and after the exhaustive challenge was species-specific. Notably, *N. acutidens* exhibited a larger difference in blood pH relative to baseline values, did not exhibit variation in blood glucose concentrations across the samples, and had high baseline lactate concentrations compared to *C. melanopterus*. Overall trends in blood lactate concentrations, blood pH, and haematological parameters, however, were similar for both species and consistent with what has been reported for other elasmobranchs (Lowe *et al.*, 1995; Richards *et al.*, 2003; Brill *et al.*, 2008). The higher $\dot{M}O_{2Min}$ observed for *N. acutidens* could explain the larger drop in blood pH (ΔpH = 0.44) following the exhaustive challenge relative to *C. melanopterus* (ΔpH = 0.28). It is hypothesised that the magnitude and severity of a stress response is related to $\dot{M}O_2$ for elasmobranchs (Skomal and Mandelman, 2012). While it was not possible to calculate AS for both species, *Carcharhinus melanopterus* are generally regarded as stronger aerobic swimmers than *N. acutidens*, which are less active and known to rest (Baldwin and Wells, 1990; Wells *et al*., 1992). High blood glucose concentrations and resting blood lactate concentrations in *N. acutidens* could be a result of this species recruiting anaerobic metabolism to support bouts of swimming that are interspersed with periods of resting (Piiper *et al.*, 1977). Blood-oxygen transport properties ([Hb], Hct, MCHC) were not affected by exercise, and were similar between the two species, as has been previously reported (Wells and Baldwin, 1990; Wells *et al.*, 1992). However, it is possible that measuring baseline values in restrained sharks confounded possible stress effects on Hct relative to baseline values generated for free-swimming animals (Schwieterman *et al.*, 2019a). Juvenile *C. melanopterus* and *N. acutidens* from Heron Island (on the Great Barrier Reef) were reported to exhibit similarly pH-insensitive haemoglobins, suggesting that Hb-O_2 affinity and oxygen transport are not greatly affected by an acidosis for these species (Baldwin and Wells, 1990; Wells *et al.*, 1992). Taken together, these data suggest that each species has a unique physiological response to stress in relation to their behaviour and aerobic capacity, where *N. acutidens* may have experienced a more intense stress response owing to a potential greater reliance on anaerobic metabolism to support activity. It would be informative to characterize the full physiological response from initiation to resolution (i.e., recovery or mortality) to determine how differently these two species respond to capture.

There was no observable immediate mortality for *C. melanopterus* and *N. acutidens*. However, delayed mortality rates were higher for *N. acutidens* (25%) than *C. melanopterus* (5.9%), although this study's experimental design precluded quantification of robust mortality rates for either species. Adult *C. melanopterus* around Moorea appear to be quite resilient to hook-and-line capture (Mourier *et al.*,

2017), and neonate and juvenile *C. melanopterus* and *N. acutidens* both exhibited near 0% mortality following gill-net and hook-and-line capture in the Mangrove Bay Sanctuary Zone on Ningaloo Reef, Australia (Oh *et al.*, 2017a). In contrast, however, another study out of Western Australia reported that, when facing unspecified capture durations in gill-nets, juvenile and adult *C. melanopterus* were more susceptible to immediate mortality than *N. acutidens* (Dapp *et al.*, 2017). Local adaptation to environmental conditions at the population level may influence these contrasting trends (Eliason *et al.*, 2011; Di Santo, 2016); although, temperature data were not reported from the Western Australia study (Dapp *et al.*, 2017). The model generated by Dapp *et al*. (2017) to estimate immediate mortality of *C. melanopterus* would have predicted 100% mortality for sharks in this study using only total length as a predictor. It is possible that differences in the duration of capture led this study to conclude that immediate mortality was 0%, whereas difficulty in identifying capture events by Dapp *et al*. (2017) could have allowed for sufficiently long capture durations and more realistic immediate mortality estimates. Alternatively, stress resulting from this study's shorter capture durations may simply not have been fatal (Oh *et al*., 2017a). Differences in mortality estimates for *N. acutidens* may have been related to size; although, sizes of *N. acutidens* were not reported by Dapp *et al*. (2017). It, therefore, seems likely that these apparently contrasting findings resulted from differences in the nature and duration of the stressor (e.g., capture duration, supplementing air exposure, local environmental conditions, etc.).

This study's exhaustive challenge was associated with a large energetic cost and long recovery for *C. melanopterus*. The mean estimated EPOC was 703.72 mg O_2 kg^{-1}, and recovery was estimated to take 8.42 hours. Comparatively, chasing juvenile lemon sharks (*N. brevirostris*) to exhaustion without air exposure resulted in an EPOC of 154.10 mg O_2 kg^{-1} and 5.40 hours of recovery at 30 °C (Bouyoucos *et al*., 2017b). Gill-net capture and air exposure may result in a larger EPOC than exhaustive chasing because oxygen uptake is impeded, such that recovery cannot begin until oxygen uptake is resumed; a chased fish in water can still meet some of its energy demand aerobically and even begin to recover. Further, EPOC and recovery time may be lower and shorter, respectively, in sharks that swim during recovery as opposed to restrained, resting animals (Cordero *et al.*, 2019). The EPOC estimated for *C. melanopterus* is much larger than measured for other elasmobranchs (Brett and Blackburn, 1978; Bouyoucos *et al.*, 2017a, b), but it is similar to values reported for a tropical coral reef fish (*Pomacentrus amboinensis*) at comparable temperatures (28-29 °C) (Killen *et al.*, 2014). However, recovery times for *P. amboinensis* were under one hour, which likely relates to this species' impressive AS that is almost ten times that of *C. melanopterus* (Killen *et al.*, 2014). Assuming that $\dot{M}O_2$ scales with swimming speeds similarly among carcharhinid sharks (Carlson *et al.*, 2004), a routine swimming $\dot{M}O_2$ of 195.87 mg O_2 kg^{-1} h^{-1} can be estimated for *C. melanopterus* using a power-performance slope of 0.36, a routine swimming speed of 0.80 body lengths s^{-1} for captive *C. melanopterus*, and this study's estimate of $\dot{M}O_{2Min}$ (Webb and Keyes, 1982; Bushnell *et al.*, 1989). Applying an oxygen equivalent of 14.14 J mg

O_2^{-1}, *C. melanopterus* would have a daily metabolic rate of 66.47 kJ kg^{-1} d^{-1} for swimming alone, and an EPOC of 9.95 kJ kg^{-1} from an exhaustive challenge would increase daily energy expenditure for swimming by 14.9% (Elliott and Davison, 1975). Around Moorea, neonatal *C. melanopterus* (and *N. acutidens*) must quickly transition from relying on endogenous fuel stores to energy acquired through hunting (Matich *et al.*, 2015). Energetically costly one-off exhaustive challenges, like incidental capture, could precede starvation in neonatal sharks, especially for populations with high natural mortality.

In conclusion, within a narrow range of temperatures, neonatal *C. melanopterus* and *N. acutidens* are resilient to brief durations of gill-net capture. However, I am unaware of these species' physiological resilience to longer durations of capture with different gear types, to longer periods of air exposure, or at temperatures beyond 28-31 °C. As such, artisanal and recreational fisheries bycatch mortality could still pose a threat to Moorea's neonate and juvenile shark populations. Indeed, longer gill-net capture durations could be fatal, at least for *C. melanopterus* (Dapp *et al*., 2017). Moving forward, studies are needed to define environmental conditions that limit physiological performance and to fully characterize recovery following a challenge. Furthermore, defining changes in routine energy requirements and reserves of neonates exposed to stressors in relation to the quality and availability of shelter and prey will be important for estimating sharks' likelihood of facing predation and starvation, respectively. Together, these data have the potential to improve our understanding of how anthropogenic and environmental stressors affect the survivorship of neonate and juvenile reef sharks in important habitats like shark nursery areas. Understanding the vulnerability of shark populations to manageable stressors, like fishing pressure, is an important step toward improving the efficacy of MPAs as conservation tools for sharks, globally.

Chapter 5: Testing physiological responses to temperature in a reef shark

5.1 Summary

Thermal dependence of metabolic rates and growth can influence thermal preference and tolerance in marine ectotherms. Ocean warming is predicted to act on these traits with consequences for fitness; yet, little is known about thermal dependence of whole-organism physiological performance in sharks. Here, I quantified thermal dependence of performance and tolerance traits, and observed thermal preference in blacktip reef shark (*Carcharhinus melanopterus*) neonates. I measured oxygen uptake rates ($\dot{M}O_2$) as proxies for metabolic rate (minimum and maximum $\dot{M}O_2$, aerobic scope, excess post-exercise oxygen consumption and recovery), tolerance traits (critical thermal maximum, CT_{Max}; thermal safety margin, TSM; hypoxia tolerance), and growth performance (growth rate, body condition, and food conversion efficiency) at two ecologically relevant acclimation temperatures (28 and 31 °C). Furthermore, I measured the effects of temperature (25, 30, and 35 °C) and pH on *in vitro* haemoglobin-oxygen (Hb-O_2) affinity of wild-caught sharks, and logged body temperatures in free-ranging sharks to test thermal preference *in situ*. Oxygen uptake rates and growth did not differ between acclimation temperatures. Temperature and hypoxia tolerance increased with acclimation and were positively associated, whilst TSM (the difference between CT_{Max} and acclimation temperature) decreased with acclimation. Haemoglobin-oxygen affinity of wild-caught sharks was lower at 25 °C compared to 30-35 °C, and a strong effect of pH on Hb-O_2 affinity at 30 °C was absent at 25 and 35 °C. Free-ranging sharks avoided 31 °C *in situ*. Together, these data suggest that *C. melanopterus* neonates have both physiological and behavioural capacities to tolerate temperature change and possibly ocean warming.

Associated publication

Bouyoucos IA, Morrison PR, Weideli OC, Jacquesson E, Planes S, Simpfendorfer CA, Brauner CJ, Rummer JL (2020) Thermal tolerance and hypoxia tolerance are associated in blacktip reef shark (*Carcharhinus melanopterus*) neonates. *J Exp Biol* 223: jeb221937.

Data availability

Data presented in this manuscript are available from the Research Data Repository (Tropical Data Hub) at James Cook University: http://dx.doi.org/10.25903/5e16c585c2b51

5.2 Introduction

Temperature is a powerful controlling factor of physiological performance in aquatic ectotherms. In fishes, oxygen uptake rates ($\dot{M}O_2$) are commonly measured whole-organism performance traits (Clark *et al.*, 2013). Studies generally measure minimum $\dot{M}O_2$ ($\dot{M}O_{2Min}$, the oxygen cost of maintaining homeostasis and a proxy of standard metabolic rate; Chabot et al., 2016). Fewer studies also measure maximum $\dot{M}O_2$ ($\dot{M}O_{2Max}$, the upper limit to oxygen uptake and a proxy of maximum metabolic rate; Norin and Clark, 2016) to calculate aerobic scope (AS, an organism's ability to match oxygen supply with demand; Farrell, 2016). A fish's metabolic phenotype also includes traits related to anaerobic capacity and environmental stress tolerance (Zhang *et al.*, 2018), including excess post-exercise oxygen consumption (EPOC, the oxygen cost of recovery from anaerobic exercise; Svendsen et al., 2010) and hypoxia tolerance. The metabolic phenotype is predictably affected by temperature, where $\dot{M}O_{2Min}$ increases with temperature at a higher rate than $\dot{M}O_{2Max}$ (Sandblom *et al.*, 2016), such that AS should plateau or even decrease with increasing temperature (Schulte, 2015); although AS can increase across an ecologically relevant range (Lefevre, 2016). Where AS is documented to decrease with increasing temperature, studies have sought to predict upper thermal limits for AS (i.e., where AS is reduced to zero at the critical thermal maximum, CT_{Max}), assuming that thermal tolerance is oxygen-dependent (Payne *et al.*, 2016; Pörtner *et al.*, 2017). Further, thermal dependence of $\dot{M}O_2$ is hypothesised to explain thermal dependence of growth (Pörtner and Knust, 2007). Indeed, multiple physiological traits should be considered to adequately predict the effects of temperature on organismal fitness (Clark *et al.*, 2013; Gangloff and Telemeco, 2018; Kellermann *et al.*, 2019).

Properties of the oxygen transport cascade suggest several mechanisms to explain thermal dependence of whole-organism traits, including oxygen uptake rates and environmental tolerance traits. Haemoglobin-oxygen (Hb-O_2) affinity (*via* $p50$, the blood-oxygen partial pressure to achieve 50% haemoglobin saturation) typically decreases with increases in temperature (Nikinmaa, 2011; Morrison et al., 2015); although, reverse temperature effects are evident (Clark *et al.*, 2010; Bernal *et al.*, 2018). Teleost fishes have a remarkable capacity to enhance O_2 unloading from haemoglobins under acidotic conditions (Rummer and Brauner, 2015), and this mechanism has been suggested to explain increases in $\dot{M}O_{2Max}$ and AS in tropical damselfishes faced with mild aquatic acidification (Couturier *et al.*, 2013; Rummer *et al.*, 2013b). Hypoxia tolerance, another trait of the metabolic phenotype, is also associated with Hb-O_2 affinity, where higher Hb-O_2 affinity supports greater hypoxia tolerance in teleost (Mandic *et al.*, 2009) and elasmobranch fishes (Speers-Roesch *et al.*, 2012). Interestingly, associations have been documented between oxygen transport properties and critical thermal maximum (CT_{Max}), a thermal tolerance metric; although, evidence is mixed. For instance, studies have found an association between CT_{Max} and haematocrit, which is the ratio of erythrocytes to plasma in whole blood (Beers and Sidell, 2011; Wang *et al.*, 2014). In addition, cardiac myoglobin (Anttila *et al.*, 2013) and haemoglobin concentration (Rodgers and De Boeck, 2019) exhibit associations with CT_{Max}. Further, hypoxia

tolerance and thermal tolerance are associated in some fishes (Anttila *et al.*, 2013), which suggests a common mechanism that is possibly linked to the oxygen transport cascade (McBryan *et al.*, 2013); although, there is some evidence to suggest that whilst environmental tolerance traits may be associated, they are independent, polygenic traits (Healy *et al.*, 2018). Thus, oxygen transport properties may provide a mechanistic, albeit non-comprehensive, explanation for thermal dependence of whole-organism oxygen uptake and tolerance traits.

The thermal dependence of physiological traits can explain thermal preference in wild fishes. It is a long held assumption that an ectotherm's preferred temperature in the wild should approximate temperatures that maximise physiological performance, and that animals ought to achieve body temperatures that maximise fitness (Angilletta et al., 2006; Huey and Bennett, 1987). Several studies offer indirect evidence of preference for temperatures that support maximal performance in fish populations. For instance, fish abundance (a proxy for preferred temperature) is highest at temperatures that support the highest routine activity levels (Gannon *et al.*, 2014; Payne *et al.*, 2018). Further, thermal dependence of aerobic scope has been suggested to predict thermal preference (*via* habitat suitability; Del Raye and Weng, 2015) and upper thermal limits (*via* CT_{Max}; Payne et al., 2016; Speers-Roesch and Norin, 2016). It has also been suggested that a population's preferred temperature falls below optimal temperatures for performance because performance should predictably decline with warming beyond the optimal temperature (Martin and Huey, 2008). Fish may also strive to maintain a sufficient thermal safety margin (TSM, the difference between mean habitat temperature and upper thermal limit), as has been documented by the progressive poleward displacement of marine ectotherm populations with increasing sea surface temperatures (Sunday *et al.*, 2012). Thermal safety margins predictably decreases with acclimation to increasing temperatures (Sandblom *et al.*, 2016), and tropical species have inherently narrower TSMs than temperate species (Comte and Olden, 2017). In combination, characterizing thermal dependence of physiological performance and thermal tolerance can contribute to an understanding of a population's thermal preference.

Elasmobranch fishes (sharks and rays) represent a knowledge gap in our understanding of thermal dependence of physiological performance in aquatic ectotherms. An interesting and unique trait of shark cardiorespiratory physiology is the apparent reduced ability of many species to substantially elevate oxygen uptake rates relative to most teleost fishes (Brill and Lai, 2015). Like teleosts, sharks' capacity for maximal oxygen uptake is limited by cardiovascular function, but unlike teleosts, sharks generally do not increase their heart rate substantially, they lack pH-sensitive haemoglobins and adrenergically-sensitive red blood cells, and do not alter haematocrit to a great degree (Hillman *et al.*, 2013; Randall *et al.*, 2014; Brill and Lai, 2015; Morrison *et al.*, 2015). For instance, where teleost fishes can elevate their oxygen uptake rates as high as 22 times routine oxygen uptake rates (i.e., factorial aerobic scope, "typical" range = 2-to-12-fold), sharks have been documented to increase oxygen uptake rates by a factor of three or less (Bernal *et al.*, 2012; Fulton *et al.*, 2013; Killen *et al.*, 2016). Studies on the effects

of temperature on whole-organism physiological traits are lacking; yet, of the few studies that investigate thermal dependence of aerobic scope in sharks and rays, all document no effect of temperature on $\dot{M}O_{2Max}$ (Di Santo, 2016; Crear *et al.*, 2019; Schwieterman *et al.*, 2019b). Moreover, ocean warming is viewed as a potential threat to tropical sharks (Rosa *et al.*, 2017), albeit less than the massive, immediate threat of overfishing (Dulvy *et al.*, 2014). The tropical New Caledonia catshark (*Aulohalaelurus kanakorum*) is listed as threatened by climate change (Dulvy *et al.*, 2014), and several other tropical sharks demonstrate reduced physiological performance and fitness under simulated ocean warming conditions (Rosa *et al.*, 2014; Gervais *et al.*, 2018). Thus, an understanding of thermal dependence of whole-organism physiological traits in tropical reef sharks can assist in predicting population-level responses to climate change.

The purpose of this study was to identify physiological and behavioural responses to temperature in a tropical reef shark neonate population. Using the blacktip reef shark (*Carcharhinus melanopterus*) as a representative high trophic level mesopredator and model reef shark, I tested for physiological responses to temperature over various timescales (acute exposure and thermal acclimation) to explain behavioural responses to temperature *in situ*. I hypothesised that 1) physiological performance traits (i.e., oxygen uptake rates, thermal and hypoxia tolerance traits, and growth performance) are affected by temperature acclimation across an ecologically relevant range, 2) whole blood-oxygen affinity is reduced with acute warming, and 3) sharks exhibit thermal preference and avoidance behaviours *in situ*. Hypotheses were tested using laboratory and field approaches. Specific laboratory objectives were to test for the effects of two ecologically relevant acclimation temperatures on oxygen uptake rates ($\dot{M}O_{2Min}$, $\dot{M}O_{2Max}$, AS, EPOC, and recovery), tolerance traits (CT_{Max}, TSM, and hypoxia tolerance), and growth performance (growth rate, body condition, food conversion efficiency) to test hypothesis 1, and to define temperature and pH sensitivity of whole blood-oxygen affinity ($p50$) of wild sharks *in vitro* to test hypothesis 2. Specific field objectives were to identify preference or avoidance of acclimation temperatures of wild sharks *in situ* to test hypothesis 3. Taken together, this study is a comprehensive investigation of the thermal biology of an elasmobranch fish model that improves our understanding of associations between temperature, physiology, and behaviour in fishes. These data are also significant to understanding and predicting population-level consequences of ocean warming in a high trophic level fish and in this case, a protected species.

5.3 Materials and methods

Ethics

Research on sharks in the French Polynesian shark sanctuary was approved by the Ministère de la Promotion des Langues, de la Culture, de la Communication, et de l'Environnement (Arrêté 9524), and James Cook University's Animal Ethics Committee (A2394). Globally, *C. melanopterus* are classified as "near threatened" by the International Union for Conservation of Nature Red List (Heupel, 2009),

and the target population is protected (*c*. 2006) within a so-called "shark sanctuary" (Ward-Paige, 2017). I aimed to test the smallest reasonable sample of sharks because of ethical considerations of working with protected species (Sloman *et al.*, 2019) and restrictions on the number of sharks that could be sampled under the research permit.

Animal collection

Sharks were collected from shore around the island of Moorea, French Polynesia (S 17°30'; W 149°50') from October 2017 to March 2018. Gillnets (50.0 m by 1.5 m, 5.0 cm mesh) were deployed at dusk and capture durations were brief (3 ± 3 minutes; values presented are means ± standard deviation unless noted otherwise). Animals were transported to a holding facility (Centre de Recherches Insulaires et Observatoire de l'Environnement; CRIOBE) in 200 L coolers of aerated seawater. Sharks were habituated under natural photoperiod in 1250 L, flow-through circular tanks for one week and fed 5% of their body mass in fresh tuna (*Thunnus* spp.) every other day. A 48-hour fast was used prior to experimentation. Following experimentation, sharks were released at their site of capture.

Thermal dependence of performance

I quantified thermal dependence of oxygen uptake rates (i.e., $\dot{M}O_{2Min}$, $\dot{M}O_{2Max}$, AS, EPOC, and recovery), hypoxia tolerance and thermal tolerance traits (i.e., CT_{Max} and TSM), and growth (i.e., growth rate, body condition, and food conversion efficiency) at two ecologically relevant acclimation temperatures. Following habituation to the laboratory, sharks were held at acclimation temperatures for four weeks and tested to quantify performance metrics over the following week (Table 5.1). Acclimation temperatures were 28 °C (n = 10 sharks, 6 females and 4 males; 1.05 ± 0.19 kg) and 31 °C (n = 9 sharks, 4 females and 5 males; 1.20 ± 0.15 kg), which represent an average dry season temperature and a warm wet season temperature in neonatal *C. melanopterus* habitat, respectively. A higher acclimation temperature (33 °C; an ocean warming scenario) was abandoned due to high post-exercise mortality (4/5 sharks); although, mortality did occur at 31 °C (3/10 sharks). Target acclimation temperatures were achieved by 0.5 °C day^{-1} (Rummer *et al.*, 2014) changes using commercially available heaters (Jager, Eheim GmbH & Co. KG, Deizisau, Germany) or chillers (TK-1000, Teco S.r.l., Ravenna, Italy).

Table 5.1 Descriptive data for blacktip reef sharks (*Carcharhinus melanopterus*; sample size, sex, and mass) and holding tanks (water temperature) for replicate groups within temperature acclimation treatments.

Acclimation temperature	Parameter	Replicate 1	Replicate 2	Replicate 3

28 °C (average dry season temperature)	*n* (sharks)	4 (2 females, 2 males)	3 (2 females, 1 male)	3 (2 females, 1 male)
	Mass (kg)	0.93 ± 0.13	1.20 ± 0.07	1.07 ± 0.23
	Temperature	28.0 ± 0.4	28.1 ± 0.3	28.2 ± 0.3
31 °C (warm wet season temperature)	*n* (sharks)	3 (3 females)	3 (3 males)	3 (1 female, 2 males)
	Mass (kg)	1.22 ± 0.19	1.22 ± 0.15	1.17 ± 0.16
	Temperature	31.1 ± 0.7	30.9 ± 0.6	30.6 ± 0.5

Data are presented as means ± standard deviation.

Oxygen uptake

Sharks were subjected to an exhaustive exercise protocol to generate a range of oxygen uptake rates ($\dot{M}O_2$) that are characteristic of the full aerobic range. Immediately prior to respirometry, sharks were chased for three minutes in a 100 L, 1 m diameter pool at acclimation temperatures followed by one minute of air exposure. Three minutes is sufficiently long to fully exhaust *C. melanopterus* neonates; sharks no longer exhibited burst swimming, which indicates physiological exhaustion during chase protocols (Clark *et al.*, 2012), after 83 ± 25 s (range = 40-150 s). A chase protocol and static respirometry could be used because *C. melanopterus* are not an obligate ram-ventilating shark. Further, chase protocols are commonly used to elicit $\dot{M}O_2$ values representative of maximum $\dot{M}O_2$ (i.e., $\dot{M}O_{2Max}$; Clark et al., 2013; Norin and Clark, 2016); although, $\dot{M}O_{2Max}$ estimated using this method could still be an underestimate (Roche *et al.*, 2013; Rummer *et al.*, 2016), as this technique has not been validated against swimming respirometry for sharks.

Oxygen uptake rates were measured using intermittent-flow respirometry. Dissolved oxygen concentration (DO, in mg L^{-1}) was measured using a Firesting Optical Oxygen Meter (PyroScience GmbH, Aachen, Germany) that corrects for temperature, barometric pressure, and salinity. An oxygen probe was placed inside the overflow outlets of individual respirometry chambers (24.0 cm diameter, 70.0 cm long, 32.0 L), and a single temperature probe was placed in the water bath. Probes were calibrated to 100% air saturation before each trial, and to 0% using sodium sulphite as needed (Rummer *et al.*, 2016). For each trial, up to four chambers – each containing one shark – were placed in a water bath set to the respective acclimation temperatures (i.e., 28 °C or 31 °C). Chambers were completely opaque except for a 5.0 cm by 10.0 cm window on the top of chambers; sharks could not see each other, and tanks were covered with 60% shade cloth to minimize external disturbances. Recirculating pumps (2500 L h^{-1}; Eheim GmbH & Co. KG, Deizisau, Germany) continuously recirculated water within

individual chambers, and flush pumps intermittently flushed oxygenated water from the water bath into each chamber. A single recirculating pump and flush pump were connected to each chamber. Measurement phases (i.e., when flush pumps are disabled) were five minutes long and flush phases (i.e., when flush pumps are enabled) were seven minutes long. Determinations of $\dot{M}O_2$ were made during measurement phases. The intermittent cycling of measurement and flush phases was automated with a custom-built data acquisition system and software (National Instruments, Austin, TX, USA), yielding 120 determinations over 24 hours. Sharks were weighed immediately upon removal from respirometry chambers. Background $\dot{M}O_2$ was measured in empty chambers before and after testing animals (Rummer *et al.*, 2016).

First, $\dot{M}O_2$ (in mg O_2 kg^{-1} h^{-1}) was calculated using the equation:

$$\dot{M}O_2 = SV_{Resp}M^{-1}$$

where S is the slope of the linear decline in DO (in mg O_2 L^{-1} s^{-1}) with a coefficient of determination greater than 0.95, V_{Resp} is the volume of water in the respirometry chamber (in L), and M is the mass of the shark (in kg). Slopes were calculated in LabChart (7.3.8, ADInstruments, Dunedin, New Zealand). I accounted for background respiration by fitting a line between the two background $\dot{M}O_2$ measurements, interpolating the background respiration value at each $\dot{M}O_2$ determination, and subtracting that value from each shark $\dot{M}O_2$ determination (Rummer *et al.*, 2016). Because sharks exhibited approximately twofold variation in mass (range = 0.75-1.41 kg), $\dot{M}O_2$ was scaled to the mass of a 1.0 kg shark using an intraspecific metabolic scaling exponent of 0.89 (Lefevre *et al.*, 2017; Jerde *et al.*, 2019) and the following equation:

$$\dot{M}O_{2Scaled} = \dot{M}O_{2Measured} \cdot \left(M_{Measured} \cdot {M_{Scaled}}^{-1}\right)^{(1-b)}$$

where $\dot{M}O_{2Scaled}$ is the mass-adjusted $\dot{M}O_2$, $\dot{M}O_{2Measured}$ is the value calculated using the equation above, $M_{Measured}$ is the shark's body mass, M_{Scaled} is the desired body mass of 1.0 kg, and b is the mass-scaling exponent of 0.89 (Norin *et al.*, 2019). Because $\dot{M}O_2$ was scaled to a 1.0 kg fish, values are simply presented in mg O_2 L^{-1}. Six oxygen uptake rate metrics were then estimated from $\dot{M}O_2$ data. Minimum $\dot{M}O_2$ ($\dot{M}O_{2Min}$; 1) was estimated with the Mean of the Lowest Normal Distribution method with the 'mclust' R package (Fraley and Raftery, 2002; Chabot *et al.*, 2016b; Scrucca *et al.*, 2016). Maximum $\dot{M}O_2$ ($\dot{M}O_{2Max}$; 2) was estimated as the highest $\dot{M}O_2$ calculated in sequential 30-second intervals during the first five $\dot{M}O_2$ determinations after exhaustive exercise (Zhang *et al.*, 2019). Absolute aerobic scope (AAS; 3) was calculated as the difference between $\dot{M}O_{2Max}$ and $\dot{M}O_{2Min}$, and factorial aerobic scope (FAS; 4) was calculated as $\dot{M}O_{2Max}$ divided by $\dot{M}O_{2Min}$. Excess post-exercise oxygen consumption (EPOC, in mg O_2; 5) was estimated for each shark by first fitting an exponential decay curve to $\dot{M}O_{2Max}$ and mean hourly $\dot{M}O_2$. The value of EPOC was calculated as the area bound by the exponential decay curve, $\dot{M}O_{2Min}$, the time of the first measurement, and the time when the curve intersected the upper

95% confidence interval limit for $\dot{M}O_{2Min}$ (Bouyoucos *et al.*, 2018). The curve intersection was recorded as each sharks' recovery time (in hours; 6).

Tolerance traits

Following respirometry, sharks were fed normally over four days before undergoing a hypoxia tolerance test. Hypoxia tolerance was assessed using a modified loss-of-equilibrium (LOE) test (Wood, 2018). Individual sharks were transferred from holding tanks to a 100 L, 1 m diameter circular pool with an aquarium pump for continuous mixing. Sharks were tested at their acclimation temperature and were habituated to the pool for five minutes. Oxygen saturation (%) was monitored with a Firesting Optical Oxygen Meter with a fibre-optic probe and temperature probe attached to the wall of the pool. At the beginning of the test, oxygen saturation in the pool was lowered with nitrogen gas at a rate of 6.2 ± 0.8 % min^{-1} (Jung *et al.*, 2019). Traditional endpoints (loss of equilibrium or righting response, cessation of ventilation, aquatic surface respiration) could not be identified for *C. melanopterus*, and death was not an acceptable endpoint for a protected species. Only the onset of spasms (OS), which was defined as any rapid convulsion originating from the trunk of the animal, was determined to be a reliable and non-lethal endpoint (Lutterschmidt and Hutchison, 1997a). The oxygen saturation at OS was recorded as an individual's critical saturation minimum (CS_{Min}) that is likely intermediate of the hypoxia tolerance metrics, critical oxygen threshold (i.e., P_{crit}) and incipient lethal oxygen threshold (Zhang *et al.*, 2018). Sharks were immediately returned to their holding tanks after achieving OS without any apparent effects. Recovery from this test was assumed to occur in under 48 hours because previous data suggest that fishes acclimated to normoxia and acutely exposed to hypoxia (i.e., 1-12 hours at ~10 % air saturation) require approximately five hours of recovery (Svendsen *et al.*, 2012; Borowiec *et al.*, 2018). Further, data from this thesis suggests that *C. melanopterus* require up to nine hours to recover from gill-net capture air exposure – a technique that impedes ventilation – supplemented with air exposure (**Chapter 4**). Sharks accepted food within hours of the hypoxia tolerance test.

Thermal tolerance was assessed 48 hours after quantifying hypoxia tolerance in the same sharks. The experimental setup was identical, except the pool was continuously aerated to achieve > 80% air saturation. After a five-minute habituation period, water temperature was increased at a consistent rate of 0.28 ± 0.01 °C min^{-1} by the addition of ~ 70 °C water from a tap at the periphery of the pool (Zhang *et al.*, 2018). This heating rate is comparable to other studies of sharks and rays (Fangue and Bennett, 2003; Dabruzzi *et al.*, 2013; Gervais *et al.*, 2018); however, heating rates of ~ 0.1 °C h^{-1} have been suggested for larger fish to allow for core body temperature to change at the same rate (Messmer *et al.*, 2017). The water temperature at which sharks exhibited OS was recorded as an individual's critical thermal maximum (CT_{Max}; Lutterschmidt and Hutchison, 1997b; Lutterschmidt and Hutchison, 1997a). Thermal safety margin (TSM) was calculated as CT_{Max} minus acclimation temperature (Sandblom *et*

al., 2016). Sharks were immediately transferred to their holding tanks after reaching CT_{Max} without any apparent effects.

Growth

Growth performance was quantified after sharks spent approximately four weeks at their acclimation temperatures. Sharks were weighed (i.e., wet weight in kg) and measured (i.e., total length in mm) at two weeks of acclimation and again at four weeks. Three growth performance metrics were calculated. First, specific growth rate (SGR, in % day^{-1}) was calculated as

$$SGR = \left((\ln M_F - \ln M_I) \cdot t^{-1}\right) \cdot 100$$

where M_F and M_I are wet weight measured at four and two weeks of acclimation, respectively, and t is the number of days (t = 14) between measurements (Norin and Clark, 2017). Second, body condition was quantified as Fulton's condition index (K)

$$K = (M \cdot TL^{-3}) \cdot 100$$

where M is wet weight (in g) measured at four weeks of acclimation and TL is the animal's total length (in cm) measured at four weeks of acclimation. Third, conversion efficiency (in %) was estimated by dividing sharks' difference in wet weight between two and four weeks of acclimation and the approximate wet weight of food consumed (Norin and Clark, 2017).

Oxygen transport properties

I characterised *in vitro* temperature and pH sensitivity of whole blood-oxygen (Hb-O_2) binding of a subset of wild-caught, non-acclimated sharks (n = 6, 5 females and 1 male; 1.10 ± 0.14 kg) to estimate thermal performance of oxygen binding *in situ*. Whole blood Hb-O_2 affinity was estimated *in vitro* at ecologically relevant assay temperatures (25, 30, and 35 °C; Bouyoucos et al., 2018) and physiologically-relevant CO_2 tensions (0.25 and 1.00 %; Morrison et al., 2015). Water temperatures at capture sites of these sharks during the week prior to their capture was 27.15 ± 0.23 °C. Sharks were sampled within 1-3 days of their arrival at the CRIOBE at 26.4 ± 0. 1 °C. Blood (5.5 ± 0.7 mL) was drawn in less than 1 minute from each shark from the caudal vasculature using 23-gauge, heparin-washed needles into 10 mL syringes. Samples were flown on ice by researchers from Tahiti (French Polynesia) to Vancouver (Canada) within 24 hours of collection. Blood was then refrigerated and subsequently analysed at the University of British Columbia (UBC) within 4-5 days of collection.

Haemoglobin-oxygen saturation

Oxygen equilibrium curves (OEC) were generated following previously described techniques (Lilly *et al.*, 2013). Approximately 6.0 µL of whole blood was sealed between two sheets of polyethylene film that were secured to a metal ring with O-rings to make a microcuvette. Up to 18 microcuvettes were

loaded into a custom-built tonometer that was designed to fit in a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Optical density (OD) was measured at 430 nm (a maximum for deoxygenated Hb), and 390 nm (an isosbestic point where OD is independent of Hb-O_2 saturation). The microplate reader was set to the relevant temperature (25, 30, and 35 °C) prior to loading microcuvettes. Samples within the tonometer were equilibrated with 100% N_2 for at least 30 minutes to fully deoxygenate Hb. After reaching a stable OD, the CO_2 tension was set to either 0.25 or 1.00%, and the oxygen tension was increased by 11 increments of O_2 balanced with N_2. Desired gas mixtures (O_2, CO_2, and N_2) were obtained using a Wösthoff DIGAMIX gas-mixing pump (H. Wösthoff Messtechnik, Bochum, Germany). Optical density was recorded following equilibration at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 10.0, 15.0, and 21.0% O_2. A final OD was recorded at 30% O_2 and 0% CO_2 where it was assumed that Hb-O_2 saturation was 100%. For each equilibration step the OD difference (ΔOD) between 430 nm and 390 nm was used to determine Hb-O_2 fractional saturations by relating the change in ΔOD between deoxy Hb and each equilibration step to the maximal change in ΔOD between fully deoxygenated and oxygenated Hb. At least three microcuvettes were run for each shark at each temperature and CO_2 combination; although, in some cases, only one reliable OEC could be generated for each individual at each temperature and CO_2 combination.

Oxygen equilibrium curves were generated by fitting oxygen partial pressure (pO_2 in Torr, where 1.0 % O_2 = 7.6 Torr = 7.6 mm Hg = 1.013 kPa) and Hb-O_2 saturation with a three-parameter logistic function of the form:

$$y = d \cdot \left(1 + 2.718^{b(log_{10}pO_2 - log_{10}e)}\right)^{-1}$$

where y is Hb-O_2 saturation, and d, b, and e are the fitted parameters. The pO_2 at 50 % Hb-O_2 saturation (i.e., $p50$) was estimated for each OEC by inserting the fitted parameters into a rearranged form of equation 2. The cooperativity of Hb-O_2 subunit binding (i.e., the Hill coefficient, n_{50}) was estimated by differentiating the Hill equation at $p50$.

Whole blood pH

Extracellular blood pH (pH_e) and total CO_2 content (TCO_2) were measured in approximately 500 µL of blood equilibrated for 60 minutes with 21% O_2, and 0.25 or 1.00% CO_2 (balanced with N_2) in rotating Eschweiler glass tonometers thermostatted at 25, 30, or 35 °C. Blood was then drawn into a gas tight syringe, and pH_e was measured by drawing about 200 µL of blood through a Microelectrodes 16-705 flow-thru pH electrode in combination with a 16-702 flow-thru reference electrode (Microelectrodes Inc., Bedford, NH, USA) thermostatted at the experimental temperature. The remaining 300 µL of blood was then centrifuged and TCO_2 (mM) was measured in triplicate 50 µL samples of the separated plasma (i.e., true plasma) using a Corning 965 Carbon Dioxide Analyser (Ciba Corning Diagnostics Corp., Medfield, USA).

Haematological parameters

Haematocrit (Hct, in %) was measured as the percentage of packed red blood cells relative to whole blood volume after centrifuging samples at 11,500 rpm for five minutes. Haemoglobin concentration ([Hb], in mM) was determined by the cyanomethaemoglobin method using Drabkin's Reagent (Sigma-Aldrich, St. Louis, MO, USA) and a haem-based extinction coefficient of 11.01 $mmol^{-1}$ cm^{-1} at a wavelength of 540 nm (Völkel and Berenbrink, 2000). Both Hct and [Hb] were measured in triplicate. Mean corpuscular haemoglobin concentration (MCHC, in mM) was calculated as [Hb] divided by Hct.

Thermal preference

I deployed temperature data-loggers on a subset of sharks (n = 6 sharks, 1 female and 5 males; 1.12 ± 0.14 kg) and in the environment (n = 2 locations) to test thermal preference for 28 and 31 °C *in situ* in neonatal *C. melanopterus.* Two locations, Haapiti and Maharepa, were selected because recapture rates of sharks at these sites are high relative to other locations around Moorea (Figure 5.1). Weekly attempts were made to recapture sharks to recover data-loggers and retrieve body temperature (T_b) data. Data-loggers were deployed in Haapiti and Maharepa before commencing the study and were collected at the end of the experiment to retrieve environmental temperature (T_e) data. Sharks used in this part of the study were collected before thermal performance experiments in early 2017 (January – February) and during the present study (November 2017 – January 2018). These animals were not used in any other part of the study.

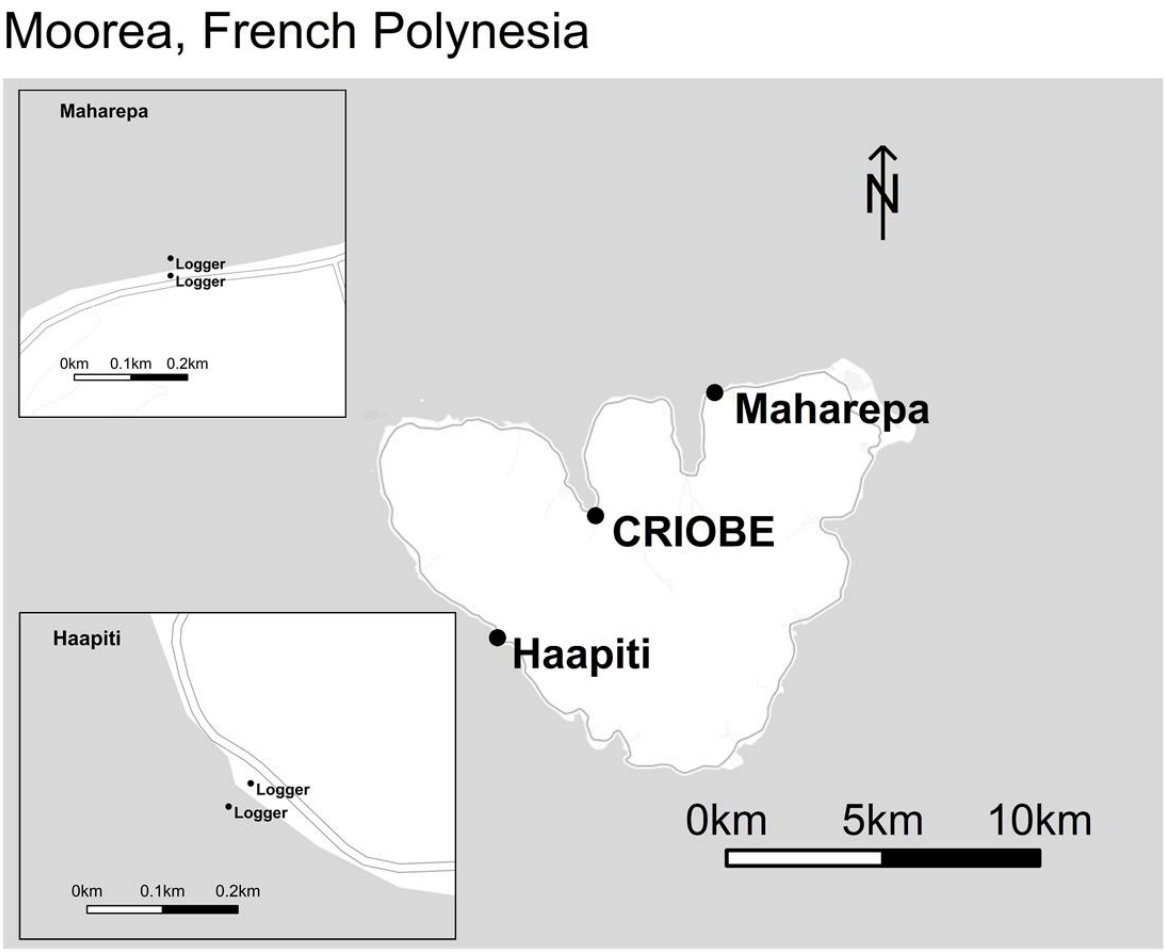

Figure 5.1 The study site, Moorea, French Polynesia (S 17°30'; W 149°50'). Neonatal blacktip reef sharks (*Carcharhinus melanopterus*) were collected from all around the island and brought to the Centre de Recherches Insulaires et Observatoire de l'Environnement (CRIOBE) for experimentation. Additionally, neonates were tagged with temperature-data loggers and recaptured to retrieve data-loggers at two sites, Maharepa and Haapiti. Within these sites, two temperature data-loggers were deployed to characterise environmental temperatures (inset panels). Note that loggers deployed very close to shore (e.g., within mangrove or rock microhabitat) appear to be on land.

Body temperature

Temperature data-loggers (iButton, DS1922L, Maxim Integrated Products, Inc., San Jose, CA, USA) were programmed to record temperatures every 10 minutes at 0.5 °C resolution. Data-loggers were waterproofed in heat shrink tubing and affixed to a rigid foam backing plate; the entire tag package was 3.0 cm by 2.0 cm by 0.8 cm (frontal cross-sectional area = 1.6 cm^2) and weighed 4.2 g in water. As such, the package was approximately 4.9 % of the frontal area of sharks (calculate from the shark's circumference at the pectoral fin insertion), and 9.5 % of the apparent submerged weight of sharks

(assuming submerged weight is 4.1 % of mass; Baldridge, 1970). Data-loggers were attached to the first dorsal fin by making pilot holes at the base of the fin with 18-gauge needles, passing braided nylon fishing line attached to the tag package through the pilot holes, and tying the line taught across a second foam backing plate on the other side of the fin with multiple surgeon's knots. I considered external attachment to be a far less invasive technique than surgically implanting tags inside the body cavity. Attachment took under five minutes and did not require anaesthesia. Further, differences between muscle and water temperature are minimal for ectothermic sharks, and externally measured temperatures are reliable proxies of T_b, especially for small (~1.0 kg) sharks (Bernal *et al.*, 2012).

Environmental temperature

Environmental temperatures were recorded using temperature data-loggers (HOBO UA-002-64, Onset Computer Corporation, Bourne, MA, USA) deployed at the same capture sites as logger-instrumented sharks. Two loggers were deployed at each site in shallow (~20 cm) and deep (~100 cm) water no more than 50.0 m (perpendicular) from shore. Loggers were synchronised using a delayed start feature and logged temperatures every 10 minutes. Because neonatal *C. melanopterus* have small home ranges (0.02 km^2) and were not caught at adjacent fishing sites, it was assumed that sharks stayed within the area of data loggers, such that the loggers recorded temperatures that were representative of the sharks' core habitat (personal observation).

Statistical analyses

Thermal dependence of performance

Thermal dependence of oxygen uptake rates, environmental tolerance traits, and growth was assessed using general linear models. Oxygen uptake rates ($\dot{M}O_{2Max}$, $\dot{M}O_{2Min}$, AAS, FAS, EPOC, and recovery time), tolerance traits (CT_{Max}, TSM, and CS_{Min}), and growth (SGR, Fulton's *K*, and conversion efficiency) were fit with acclimation temperature and replicate group ID as fixed effects. Replicate ID group was included as a fixed effect because of the small number of replicates and sample sizes within replicates. If significant effects of acclimation temperature were detected, linear regression was used to test for correlations between traits. Statistical significance was determined using 95% confidence intervals (CI). Briefly, CIs for the effect size of fixed effects were generated by running 1000 posterior simulations of each fixed effect using the 'arm' R package (Gelman and Su, 2018). Models were validated by visually inspecting plots of residuals against each fixed effect (independence), residuals against fitted values (heterogeneity), and a quantile-quantile plot of residuals (normality) (Zuur *et al.*, 2007). Analyses were conducted using the 'Stats' R package (R Core Team, 2018).

Oxygen transport properties

The pH sensitivity of Hb-O_2 affinity of whole blood from *in vitro* treatments was assessed first by using general linear models. I tested for differences in the slope of the linear relationship between log $p50$ and

pH_e at each assay temperature (25, 30, and 35 °C). Log $p50$ was fit as a response variable with pH (continuous) and assay temperature (categorical) as interacting fixed effects, and shark ID was added as a separate fixed effect. Bohr coefficients (Φ) were calculated from separate linear regressions between log $p50$ and pH_e at each assay temperature following:

$$\Phi = \Delta \log p50 \cdot \Delta pH_e^{-1}$$

The temperature sensitivity of Hb-O_2 affinity was assessed by calculating enthalpies of oxygenation (ΔH^o, in kJ mol O_2) at a standardized pH_e. Log $p50$ was first estimated at a pH of 7.15 – the average pH of plasma by the time it was measured at UBC – using Bohr plots generated at each assay temperature for each treatment group. If no Bohr coefficients could be calculated (i.e., a non-significant linear relationship between log $p50$ and pH_e; an absent Bohr effect), the average $p50$ was used. Enthalpies were calculated according to the van't Hoff isochore:

$$\Delta H^o = 2.303 \cdot R \cdot \Delta \log p50 \cdot \Delta \frac{1}{T}$$

where R is the gas constant (0.008314 kJ K^{-1} mol^{-1} O_2), and T is the temperature in Kelvin (Wyman, 1964).

Thermal preference

Differences between the distributions of T_b and T_e were tested for individual sharks with Kolmogorov-Smirnov tests (Dubois *et al.*, 2009). For each location, the mean T_e was calculated between the two data loggers. For each shark, the corresponding T_e values were selected that were measured at the same time as T_b. The D test statistic of Kolmogorov-Smirnov tests was used as an individual's thermal selection index (TSI), where larger TSI values indicate larger differences between distributions of T_b and T_e (Dubois *et al.*, 2009). Temperature preference was assessed by comparing TSI to the mean of the absolute value of deviations (d_b) of T_b from a preferred temperature, T_{pref} (i.e., $d_b = |T_b - T_{pref}|$), using simple linear regression; a negative linear relationship suggests preference, in that a larger TSI is associated with a smaller d_b (Hertz *et al.*, 1993; Dubois *et al.*, 2009). I used 28 and 31 °C as possible T_{pref} values to generate d_b (two values for each shark). This approach made it possible to test for preference or avoidance of the acclimation temperatures that were used in the thermal performance study.

5.4 Results

Thermal dependence of performance

There was no effect of acclimation temperature on any oxygen uptake rate metric (Figure 5.2; Table 5.2). However, all tolerance traits were affected by temperature acclimation (Table 5.2). Sharks had higher CT_{Max} and lower TSM at 31 °C relative to 28 °C (Figure 5.3A-B). Further, *C. melanopterus* had

a lower CS_{Min} at 31 °C relative to 28 °C (Figure 5.3C). Across treatments, CT_{Max} and CS_{Min} were significantly, negatively associated (mean effect size = -1.85, 95% CI = -3.02 – -0.59; Figure 5.3D), which suggests that individuals with greater thermal tolerance (i.e., higher CT_{Max}) had greater hypoxia tolerance (i.e., lower CS_{Min}). Growth performance metrics (SGR, Fulton's *K*, and conversion efficiency) were unaffected by acclimation temperature (Figure 5.4; Table 5.2).

Table 5.2 Linear model outputs of the effects of acclimation temperature and replicate group on mass-corrected oxygen uptake rates, tolerance traits, and growth rate in neonatal blacktip reef sharks (*Carcharhinus melanopterus*).

Response	Parameter	Mean effect size	95% confidence interval
Minimum oxygen uptake rate (mg O_2 h^{-1})	Intercept	153.71	110.79 – 193.59
28 °C acclimation (*n* = 9) 31 °C acclimation (*n* = 7)	31 °C acclimation	42.21	-22.71 – 104.66
	31 °C replicate group 1	40.12	-35.62 – 113.23
	28 °C replicate group 2	-19.11	-98.13 – 57.52
	31 °C replicate group 2	-10.67	-90.05 – 64.19
	28 °C replicate group 3	17.09	-48.49 – 79.54
Maximum oxygen uptake rate (mg O_2 h^{-1})	Intercept	361.19	291.74 – 432.21
28 °C acclimation (*n* = 10) 31 °C acclimation (*n* = 9)	31 °C acclimation	88.98	-16.93 – 191.91
	31 °C replicate group 1	244.20	121.98 – 374.84
	28 °C replicate group 2	96.66	-25.34 – 224.63
	31 °C replicate group 2	65.25	-56.95 – 196.24
	28 °C replicate group 3	43.77	-71.68 – 143.33
Absolute aerobic scope (mg O_2 h^{-1})	Intercept	207.44	135.22 – 283.23
28 °C acclimation (*n* = 9) 31 °C acclimation (*n* = 7)	31 °C acclimation	46.02	-70.42 – 147.78
	31 °C replicate group 1	204.00	71.00 – 332.05
	28 °C replicate group 2	115.98	-9.69 – 240.99
	31 °C replicate group 2	78.83	-40.52 – 198.77
	28 °C replicate group 3	26.10	-83.87 – 137.32
Factorial aerobic scope	Intercept	2.36	1.83 – 2.86
28 °C acclimation (*n* = 9) 31 °C acclimation (*n* = 7)	31 °C acclimation	0.05	-0.81 – 0.85
	31 °C replicate group 1	0.59	-0.38 – 1.62

	28 °C replicate group 2	1.03	0.17 – 1.93
	31 °C replicate group 2	0.38	-0.56 – 1.36
	28 °C replicate group 3	0.03	-0.76 – 0.82
Excess post-exercise oxygen consumption (mg O_2)	Intercept	166.82	36.38 – 301.34
28 °C acclimation (*n* = 7) 31 °C acclimation (*n* = 6)	31 °C acclimation	91.63	-164.89 – 320.28
	31 °C replicate group 1	66.49	-205.21 – 351.72
	31 °C replicate group 2	69.36	-186.25 – 373.09
	28 °C replicate group 3	15.34	-191.81 – 231.24
Recovery time (h)	Intercept	7.17	3.56 – 11.04
28 °C acclimation (*n* = 7) 31 °C acclimation (*n* = 6)	31 °C acclimation	2.37	-4.06 – 8.47
	31 °C replicate group 1	-0.58	-7.65 – 6.31
	31 °C replicate group 2	-0.64	-7.88 – 6.57
	28 °C replicate group 3	-2.65	-7.81 – 3.08
Critical thermal maximum (°C)	Intercept	37.00	36.46 – 37.55
28 °C acclimation (*n* = 9) 31 °C acclimation (*n* = 7)	31 °C acclimation	1.97	1.14 – 2.78
	31 °C replicate group 1	-0.06	-1.05 – 0.91
	28 °C replicate group 2	-0.18	-1.15 – 0.80
	31 °C replicate group 2	-0.28	-1.32 – 0.71
	28 °C replicate group 3	0.06	-0.82 – 0.89
Thermal safety margin (°C)	Intercept	8.98	8.42 – 9.53
28 °C acclimation (*n* = 9) 31 °C acclimation (*n* = 7)	31 °C acclimation	-1.03	-1.90 – -0.19
	31 °C replicate group 1	-0.05	-1.08 – 0.98
	28 °C replicate group 2	-0.23	-1.23 – 0.76
	31 °C replicate group 2	-0.23	-1.26 – 0.70
	28 °C replicate group 3	0.09	-0.70 – 0.90
Critical saturation minimum (% air saturation)	Intercept	25.79	23.05 – 28.48
28 °C acclimation (*n* = 9) 31 °C acclimation (*n* = 7)	31 °C acclimation	-5.23	-9.26 – -1.22
	31 °C replicate group 1	1.05	-3.84 – 5.94
	28 °C replicate group 2	-1.95	-6.32 – 2.91

	31 °C replicate group 2	-0.37	-4.99 – 4.85
	28 °C replicate group 3	-3.21	-7.23 – 0.74
Specific growth rate (% day^{-1})	Intercept	0.82	0.32 – 1.30
28 °C acclimation (n = 10) 31 °C acclimation (n = 9)	31 °C acclimation	-0.03	-0.77 – 0.75
	31 °C replicate group 1	-0.29	-1.12 – 0.43
	28 °C replicate group 2	-0.49	-1.25 – 0.28
	31 °C replicate group 2	-0.20	-1.03 – 0.65
	28 °C replicate group 3	-0.25	-1.02 – 0.48
Fulton's condition index (K)	Intercept	0.51	0.44 – 0.55
28 °C acclimation (n = 10) 31 °C acclimation (n = 9)	31 °C acclimation	-0.01	-0.08 – 0.04
	31 °C replicate group 1	-0.05	-0.02 – 0.11
	28 °C replicate group 2	0.02	-0.03 – 0.08
	31 °C replicate group 2	0.03	-0.04 – 0.10
	28 °C replicate group 3	-0.02	-0.0 – 0.04
Conversion efficiency (%)	Intercept	38.53	14.56 – 61.88
28 °C acclimation (n = 10) 31 °C acclimation (n = 9)	31 °C acclimation	0.79	-34.58 – 35.85
	31 °C replicate group 1	-19.05	-57.58 – 16.84
	28 °C replicate group 2	-23.92	-62.29 – 11.89
	31 °C replicate group 2	-11.51	-49.33 – 26.88
	28 °C replicate group 3	-10.34	-49.17 – 25.79

Sample sizes are listed for each response variable measured at each acclimation temperature.

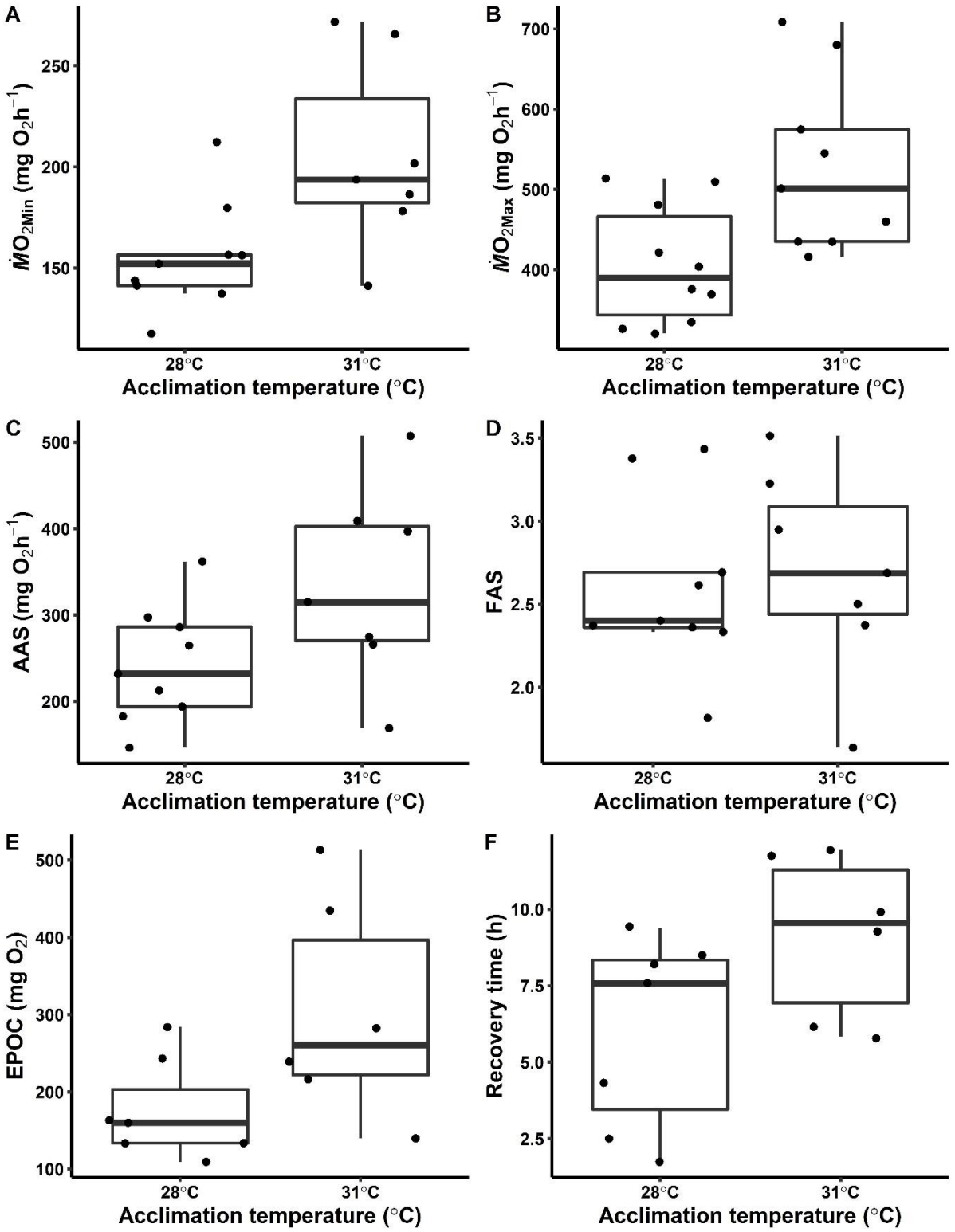

Figure 5.2 Effects of acclimation temperature on oxygen uptake rates and recovery in neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Individual points represent observations for individual sharks. Abbreviations: absolute aerobic scope, AAS; excess post-exercise oxygen consumption, EPOC; factorial aerobic scope, FAS; maximum oxygen uptake rate, $\dot{M}O2_{Max}$; minimum oxygen uptake rate, $\dot{M}O2_{Min}$.

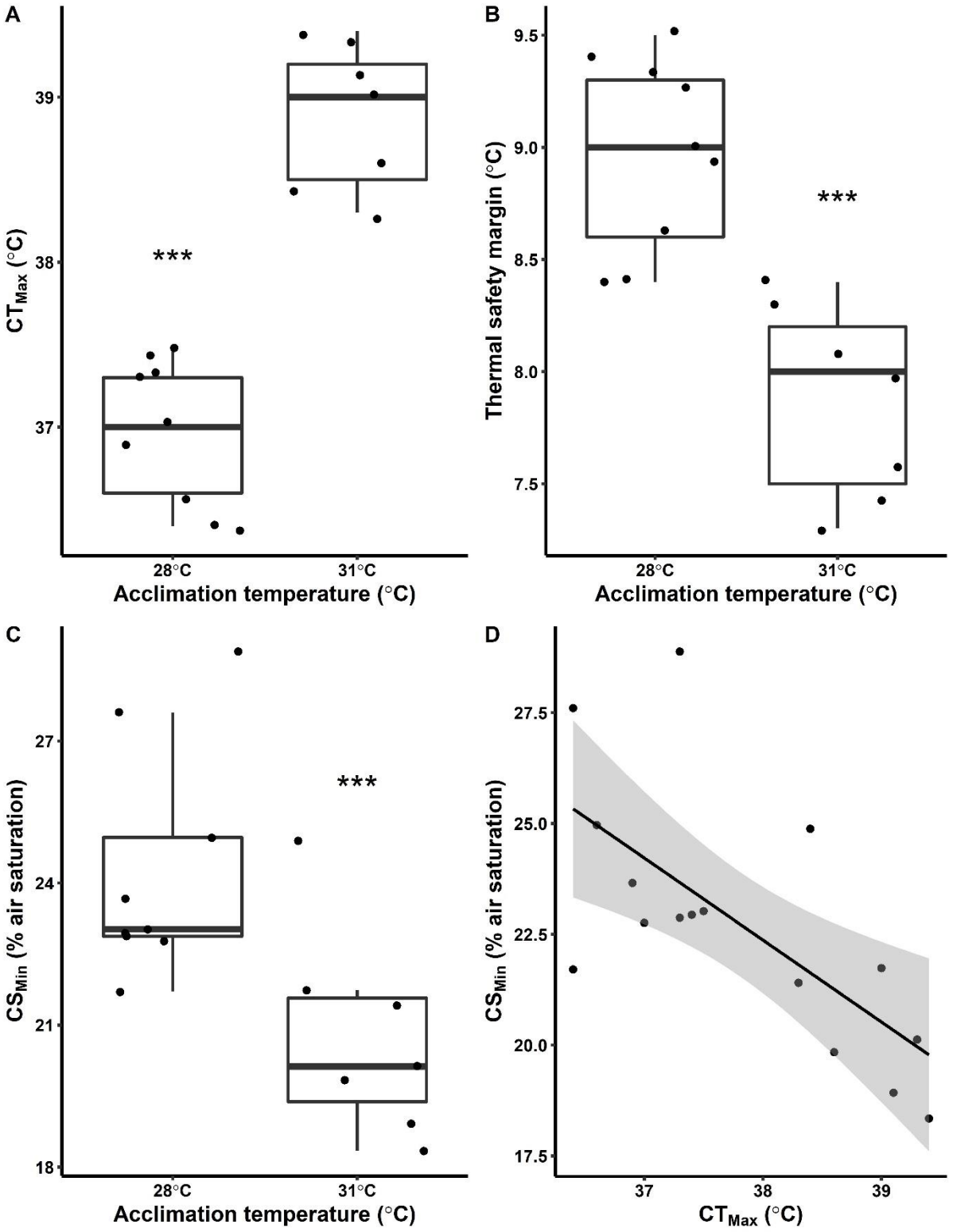

Figure 5.3 Effects of acclimation temperature on tolerance traits of neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Asterisks denote statistically significant effects of acclimation temperature on critical thermal maximum (CT_{Max}; A), thermal safety margin (CT_{Max} minus acclimation temperature; B), and critical saturation minimum (CS_{Min}; C), a hypoxia tolerance metric.

Further, CT_{Max} and CS_{Min} were significantly correlated (D); 95% error bars are represented by the shaded area. Individual points represent observations for individual sharks.

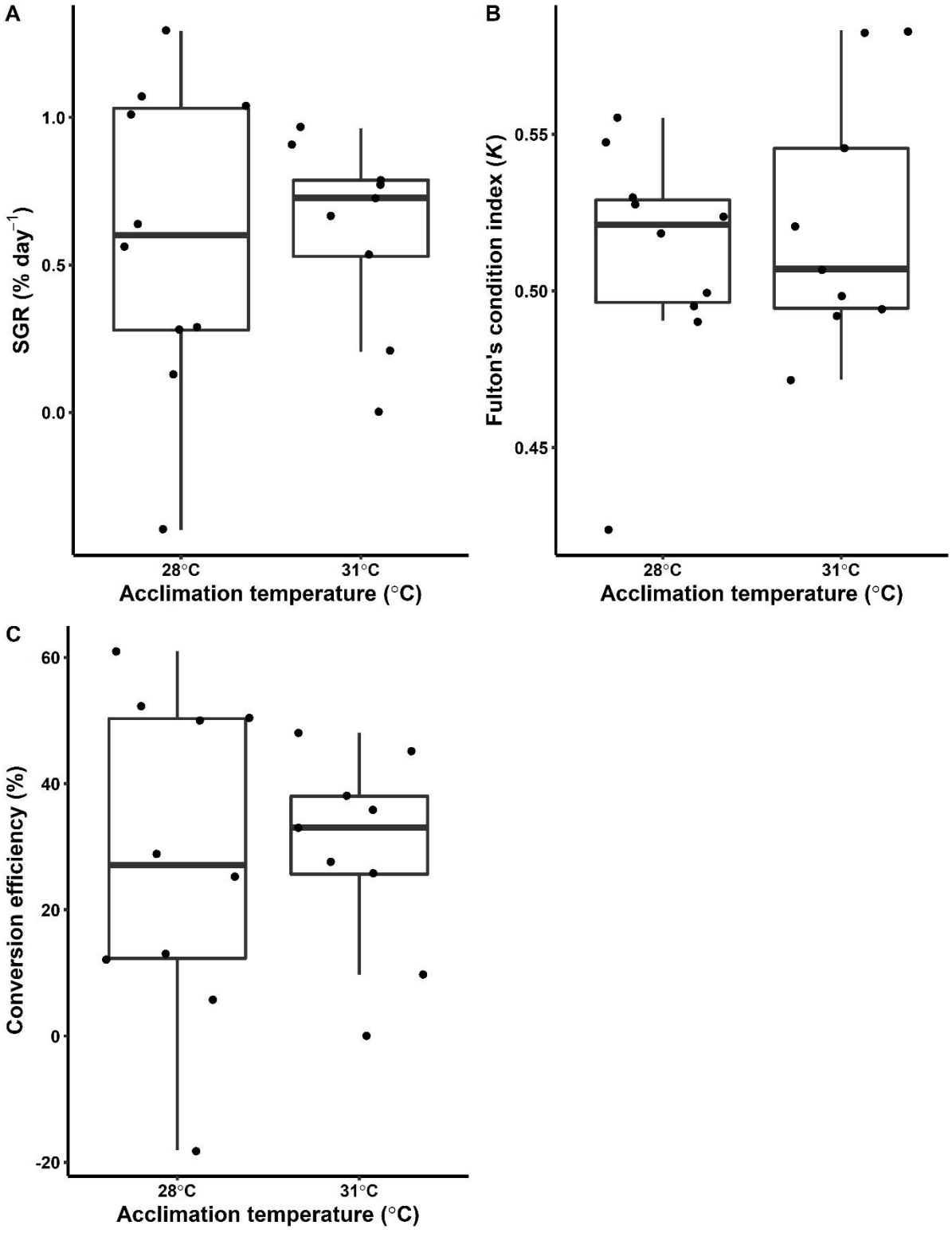

Figure 5.4 Effect of acclimation temperature on growth performance in neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Specific growth rate (SGR; A), Fulton's condition index (*K*; B), and conversion efficiency (C) were not affected by temperature acclimation. Individual points represent observations for individual sharks.

Oxygen transport properties

Haemoglobin-oxygen affinity *in vitro* for wild-caught sharks was affected by assay temperature and the interaction between assay temperature and pH_e. Log $p50$ increased (i.e., Hb-O_2 affinity decreased) from 25 to 30 °C (mean effect size = 6.89, 95% CI = 1.66 – 11.82), but was not different between 30 and 35 °C (mean effect size = 0.42, 95% CI = -5.52 – 6.53). There was evidence of a strong Bohr effect, but only at 30 °C (mean effect size = -0.95, 95% CI = -1.63 – -0.21). Oxygen equilibrium parameters, temperature and CO_2 conditions, Bohr coefficients, enthalpies, and haematological metrics are presented in Table 5.3.

Table 5.3 Oxygen equilibrium parameters, enthalpies of oxygenation, and haematological parameters of wild-caught blacktip reef sharks (*Carcharhinus melanopterus*).

Metric	**25 °C**		**30 °C**		**35 °C**	
CO_2 (%)	**0.25**	**1.00**	**0.25**	**1.00**	**0.25**	**1.00**
pCO_2 (mm Hg)	**1.9**	**7.6**	**1.9**	**7.6**	**1.9**	**7.6**
TCO_2 (mM^1)	2.86 ± 0.78	3.84 ± 0.52	2.53 ± 0.78	4.51 ± 0.49	2.32 ± 0.23	1.96 ± 0.28
pH_e	7.24 ± 0.08	7.14 ± 0.05	7.14 ± 0.07	7.05 ± 0.04	7.12 ± 0.04	7.07 ± 0.05
$p50$ (mm Hg)	14.47 ± 1.19	12.36 ± 2.59	17.84 ± 4.99	18.72 ± 2.32	21.94 ± 3.89	19.33 ± 3.04
n_{50}	1.61 ± 0.30	1.81 ± 0.29	1.87 ± 0.21	1.97 ± 0.26	2.05 ± 0.38	1.84 ± 0.21
Φ ($\Delta\log p50 \cdot \Delta\log pH_e^{-1}$)	N.S.		-0.74		N.S.	
ΔH^o (kJ mol O_2) at pH_e = 7.15	**25-30 °C**		**30-35 °C**		**25-35 °C**	
	-19.43		-45.16		-32.08	
Haematocrit	21.42 ± 1.09					
[Hb] (mM)	0.64 ± 0.06					
MCHC (mM)	2.99 ± 0.19					

Values are presented as means ± standard deviation. Abbreviations: Bohr coefficient, Φ; CO_2 partial pressure, pCO_2; enthalpy of oxygenation, ΔH^o; extracellular pH, pH_e; haemoglobin concentration, [Hb]; Hill coefficient, n_{50}; mean corpuscular haemoglobin concentration, MCHC; oxygen partial pressure at 50% haemoglobin saturation, $p50$; total CO_2, TCO_2. Bohr coefficients are presented unless the slope of the linear relationship between pH_e and log $p50$ was not statistically significant. Enthalpies are presented from 25 to 30 °C, 30 to 35 °C, and 25 to 35 °C. Haematocrit and [Hb] were not measured under specific temperature or CO_2 conditions and were used to calculate MCHC.

Thermal preference

Temperature data-loggers were retrieved from sharks (n = 6) after 4-45 days at liberty (19 ± 13 days). Body temperature distributions (29.6 ± 1.2 °C; range = 26.1-34.1 °C) were significantly different from environmental temperature distributions (29.8 ± 1.6 °C; range = 25.5-35.9 °C) in all six sharks (Kolmogorov-Smirnov test, D = 0.21-0.33, $P < 0.001$; Figure 5.5). Sharks did not exhibit preference or avoidance of 28 °C *in situ* (mean effect size = -7.04, 95% CI = -16.65 – 1.72; Figure 5.6A). However, sharks did exhibit avoidance of 31 °C *in situ*, as demonstrated by a significant positive relationship between d_b and D (mean effect size = 4.16, 95% CI = 1.44 – 6.79; Figure 5.6B).

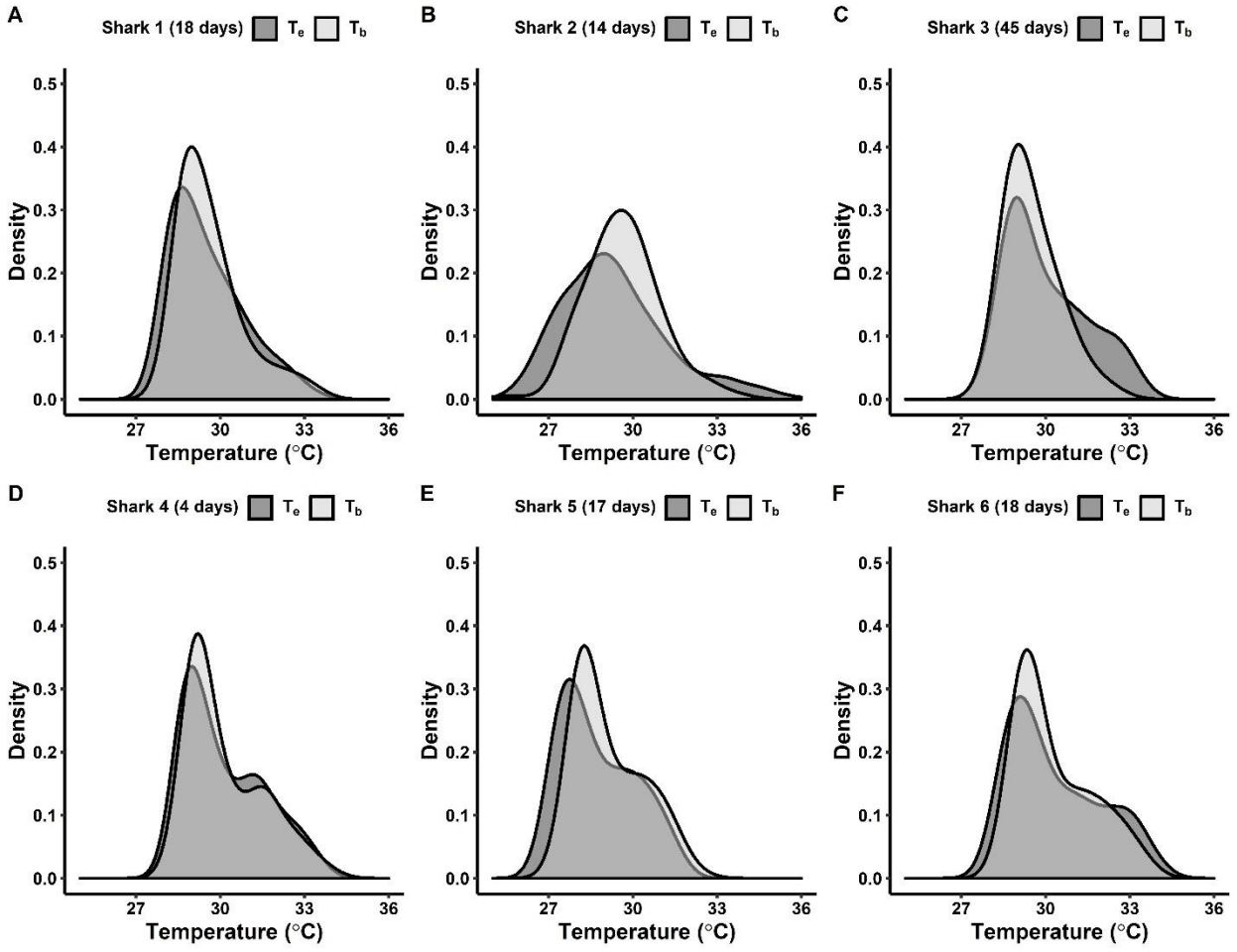

Figure 5.5 Density plots of body temperatures (T_b) of neonatal blacktip reef sharks (*Carcharhinus melanopterus*) and environmental temperatures (T_e). Density is calculated as the relative time a logger recorded a temperature within 0.5 °C bins. Each panel represents an individual shark and the temperature

of its habitat during measurement. The duration of measurement is indicated in parentheses beside shark ID.

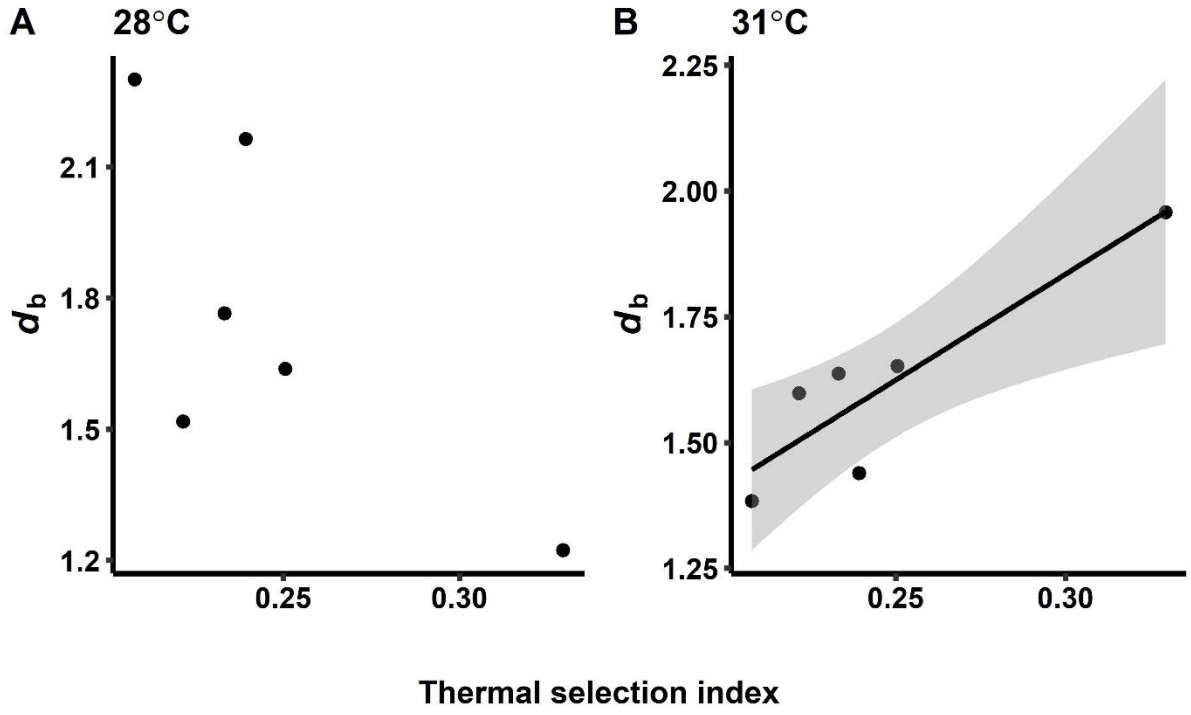

Figure 5.6 Evidence of in situ thermal preference in neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Sharks with a large thermal selection index had body temperatures that differed greatly from environmental temperatures. Deviation from body temperature (d_b) represents the difference in mean body temperature from 28 °C (A) and 31 °C (B). A significant positive relationship was found at 31 °C and suggests avoidance of 31 °C in situ. Individual points represent observations for individual sharks, and shading represents 95% error bars for regression lines.

5.5 Discussion

The purpose of this study was to investigate thermal dependence of performance, thermal preference, and thermal tolerance in a tropical reef shark to understand a population's physiological and behavioural responses to temperature. I tested the hypotheses that 1) oxygen uptake rates, environmental tolerance traits, and growth performance of neonatal *C. melanopterus* would be affected by thermal acclimation across an ecologically relevant range, 2) whole blood-oxygen affinity is reduced with warming *in vitro*, and 3) sharks exhibit thermal preference and avoidance behaviour *in situ*. These data offer varying support of my first

hypothesis. Oxygen uptake rates and growth performance were not affected by acclimation temperature; although, thermal and hypoxia tolerance both increased with acclimation and were even associated across individuals. In support of my second hypothesis, *C. melanopterus* exhibited high thermal sensitivity of whole blood-oxygen binding *in vitro*, where Hb-O_2 affinity decreased with increasing temperature from 25-35 °C. Further, haemoglobins exhibited strong pH sensitivity (i.e., Bohr effect) at 30 °C, but pH sensitivity was lost at 25 and 35 °C. Third, sharks exhibited avoidance of 31 °C *in situ*, offering support for my third hypothesis. Taken together, these data demonstrate that *C. melanopterus* exhibit minimal thermal dependence of whole-organism performance traits over a seasonal temperature range. These data also suggest that *C. melanopterus* appear to exhibit some capacity for using behaviour to avoid temperatures that affect Hb-O_2 affinity in the short-term and thermal tolerance in the long-term.

Oxygen uptake rates did not differ between acclimation temperatures in *C. melanopterus*. Sharks acclimated to 28 and 31 °C for four weeks did not exhibit different $\dot{M}O_{2Max}$, $\dot{M}O_{2Min}$, aerobic scope, EPOC, or recovery times. Maximum $\dot{M}O_2$ is predicted to have little thermal plasticity relative to $\dot{M}O_{2Min}$ (Sandblom *et al.*, 2016). Empirical data in elasmobranch fishes demonstrate no effect of temperature on $\dot{M}O_{2Max}$ in temperate skates (Di Santo, 2016; Schwieterman *et al.*, 2019b), and no effect of temperature on $\dot{M}O_{2Max}$ at comparable temperatures (28 and 32 °C) in sandbar sharks (*Carcharhinus plumbeus*; Crear et al., 2019). Minimum $\dot{M}O_2$ should exhibit a reversible thermal acclimation response in elasmobranch fishes (Lear *et al.*, 2017). Indeed, there is an apparent separation of $\dot{M}O_2$ metrics in *C. melanopterus* between 28 and 31 °C; although, a non-significant difference likely reflects a combination of the narrow temperature range that was tested and inter-individual variation in $\dot{M}O_2$. Together, the ecologically relevant, yet narrow, temperatures used and biologically relevant variation in $\dot{M}O_2$ (e.g., 2-3-fold; Metcalfe *et al.*, 2016) may have contributed to a reduced effect size of temperature. Alternatively, *C. melanopterus* could thermally compensate $\dot{M}O_{2Min}$ (Sandblom *et al.*, 2014); although, thermal compensation of oxygen uptake rates has not yet been observed in elasmobranch fishes (Tullis and Baillie, 2005), and was not tested for in this study. As such, aerobic scope was not affected in *C. melanopterus*. Similarly, aerobic scope was unaffected by temperature in two skates (*Amblyraja radiata* and *Rostraraja eglanteria*; Schwieterman et al., 2019) and in sandbar sharks (Crear *et al.*, 2019), but decreased with warming in the little skate (*Leucoraja erinacea*; Di Santo, 2016). It is possible that a thermal optimum for aerobic scope exists between 28 and 31 °C, perhaps closer to the average body temperature of sharks *in situ* (i.e., 29.6 °C) but further studies are needed to specifically investigate this. Indeed, *C. melanopterus* may exhibit broad thermal performance curves, such that whole-organism performance traits do not exhibit thermal dependence with small (i.e., 2-3 °C) changes in temperature (Lear *et al.*, 2019). Neonatal *C. melanopterus* experience daily temperatures ranging 26-34 °C, yet seasonal temperatures are considerably less variable (dry season = 28 °C, wet season = 30 °C; personal observation). Because daily thermal variation around Moorea is somewhat unpredictable and greater than

seasonal variation *C. melanopterus* could also have a modest reversible acclimation response because acclimation would be costly in such an unpredictable environment (da Silva *et al.*, 2019). Thus, a larger temperature range may be necessary to demonstrate thermal dependence of traits like oxygen uptake rate in *C. melanopterus* neonates.

Environmental tolerance traits were positively associated and increased with warming. Neonatal *C. melanopterus* were more hypoxia and temperature tolerant when acclimated to 31 °C, and sharks with higher thermal tolerance were also more hypoxia tolerant. Increasing CT_{Max} with acclimation to increasing temperatures is a well-established trend in both teleost (Di Santo and Lobel, 2017; Nyboer and Chapman, 2017; Jung *et al.*, 2019) and elasmobranch fishes (Fangue and Bennett, 2003; Dabruzzi *et al.*, 2013; Gervais *et al.*, 2018). Decreases in thermal safety margin are also well-documented with acclimation to increasing temperatures (Sandblom *et al.*, 2016; McArley *et al.*, 2017) and decreasing latitude (Deutsch *et al.*, 2008; Comte and Olden, 2017). In this study, CT_{Max} exceeded maximum habitat temperatures (36 °C) and T_b did not exceed 34 °C *in situ*. It is possible that the heating rate used to measure CT_{Max} was too rapid and caused an overestimation of CT_{Max} at temperatures above what was measured *in situ* (Mora and Maya, 2006); however, this does not preclude my finding of an effect of acclimation temperature. Whilst shallow, tropical habitats, such as those that neonatal *C. melanopterus* inhabit, routinely become supersaturated with oxygen (personal observation), CT_{Max} is generally not affected by environmental oxygen saturation (Brijs *et al.*, 2015; Ern *et al.*, 2016; McArley *et al.*, 2018). Hypoxia tolerance can be both increased (Anttila *et al.*, 2015; McBryan *et al.*, 2016; Healy *et al.*, 2018) or reduced (Nilsson *et al.*, 2010; Lapointe *et al.*, 2014) with acclimation to increasing temperatures; although, only reductions in hypoxia tolerance have been observed in elasmobranch fishes (Butler and Taylor, 1975; Crear *et al.*, 2019; Schwieterman *et al.*, 2019b). An increase in temperature can be associated with both high (e.g., supersaturation in productive, coastal environments) (McArley *et al.*, 2018; Giomi *et al.*, 2019) and low (e.g., declining oxygen saturation and respiration rates in tide pools) oxygen saturation (Richards, 2011; McArley *et al.*, 2019). Increasing hypoxia tolerance is an adaptive warm acclimation response to cope with declining oxygen availability at high temperatures in a low-oxygen scenario but may be of little relevance in supersaturated environments. Mechanisms underlying changes in thermal and hypoxia tolerance are debated (McBryan *et al.*, 2013; MacMillan, 2019), but a positive correlation between these traits suggests a common mechanism, such as cardiac or gill remodelling (Anttila *et al.*, 2015). Further, there is evidence of positive (Anttila *et al.*, 2013) and negative (Jung *et al.*, 2019) associations between thermal and hypoxia tolerance metrics that suggest that thermal tolerance is oxygen-limited (Anttila *et al.*, 2013). Interestingly, I observed that *in vitro* preparations of *C. melanopterus* haemoglobins lose their Bohr effect at 35 °C, near the maximum measured habitat temperature. These associations can have a genetic basis (Anttila *et al.*, 2013; Teague *et al.*, 2017) or hypoxia and thermal tolerance can be independent, polygenic traits (Healy *et al.*, 2018). However,

populations of *C. melanopterus* have low genetic diversity and exhibit high rates of inbreeding (Mourier and Planes, 2013; Vignaud *et al.*, 2013), and it remains to be tested whether individual variation in tolerance traits has a genetic or environmental basis. Finally, it is worth considering methodological effects, given that hypoxia tolerance and thermal tolerance were measured in the same individuals sequentially over 48 hours. Exposure to acute hypoxia results in the expression of hypoxia inducible factors (Renshaw *et al.*, 2012), but this response is short-lived upon normoxic exposure and does not affect CT_{Max} (Joyce and Perry, 2020). Conversely, hypoxia exposure also induces heat shock protein expression (Renshaw *et al.*, 2012) that does correlate with CT_{Max} (Fangue *et al.*, 2011). Indeed, given the variability in observed associations between thermal and hypoxia tolerance, additional research is warranted.

Growth performance was unaffected by temperature acclimation. Specific growth rate, body condition, and food conversion efficiency did not differ between sharks acclimated to 31 °C or 28 °C for four weeks. Growth rates in fishes typically follow thermal performance curves, where performance declines at higher temperatures (Jonsson *et al.*, 2001; Larsson and Berglund, 2005; Gräns *et al.*, 2014). As metabolic rates increase exponentially with temperature (Clarke *et al.*, 1999), the energetic costs of growth are predicted to increase, leading to a reduction in growth rate. Therefore, it follows that I did not observe an effect of acclimation temperature on growth and oxygen uptake rates. Indeed, *C. melanopterus* may have similarly broad thermal performance curves for growth as was suggested for oxygen uptake rates owing to thermal variability in their habitat (Lear *et al.*, 2019). Alternatively, it could be possible that *C. melanopterus* downregulate other physiological functions to maintain growth rates. For instance, tropical coral reef fishes upregulate genes associated with growth and metabolism when exposed to high temperatures, but downregulate genes associated with immune function and cell organisation (Veilleux *et al.*, 2018). However, this study provided no evidence to suggest downregulation of physiological function. Instead, these data demonstrate that *C. melanopterus* neonates maintain their aerobic scope over their current seasonal temperature range without adjusting energy expenditure for growth. Growth rate is a critically important trait because it can influence neonates' susceptibility to predation (DiBattista *et al.*, 2007). Therefore, variation in parturition season temperatures are unlikely to affect growth in *C. melanopterus* neonates, at least during their first month of life.

Haemoglobins in wild-caught *C. melanopterus* exhibited high temperature sensitivity but variable pH sensitivity. Whilst pH_e of blood samples was lower than what has typically been measured in other species (Morrison *et al.*, 2015), cross-study comparisons of $p50$ (and our calculation of ΔH^o at $pH_e = 7.15$) are valid because I only observed a Bohr effect at a single assay temperature. In *C. melanopterus*, Hb-O_2 affinity of whole blood exhibited higher temperature sensitivity (i.e., lower enthalpies of oxygenation) than temperate, ectothermic species (Bernal *et al.*, 2018). Haemoglobin-oxygen affinity predictably decreased across the

assay temperature range, and the reduction was greatest at 30-35 °C (i.e., the enthalpy of oxygenation was lowest). Interestingly, I only observed a Bohr effect at 30 °C. Indeed, the measured Bohr coefficient (Φ = -0.74) is quite large relative to other elasmobranch fishes (Morrison *et al.*, 2015), and the only other comparable Bohr coefficients were calculated for stripped haemoglobins with adenosine triphosphate (ATP) at 10 °C in the porbeagle shark (*Lamna nasus*; Φ = -0.76; Larsen et al., 2003), and in whole blood at 15 °C in the mako shark (*Isurus oxyrinchus*; Φ = -0.74; Bernal et al., 2018). Large Bohr effects are characteristic of high-performance fishes, including mackerels (Clark *et al.*, 2010), tunas (Bushnell and Brill, 1991; Brill and Bushnell, 2006), and lamnid sharks (Larsen *et al.*, 2003; Bernal *et al.*, 2018); yet, these species exhibit regional endothermy and very low enthalpies of oxygenation. Whilst we measured a large Bohr coefficient in whole blood of *C. melanopterus* at 30 °C, the Bohr coefficient measured in stripped haemoglobins of *C. melanopterus* at 25 °C was a modest -0.35 (Wells *et al.*, 1992). The difference could be related to erythrocyte nucleotides (e.g., guanosine triphosphate or ATP) in whole blood as they have been shown to affect Hb-O_2 affinity (i.e., *p*50) in *C. melanopterus* (Wells *et al.*, 1992). Regardless, our study is one of the few to estimate *p*50 at temperatures exceeding 30 °C in elasmobranch fishes (Morrison *et al.*, 2015). Neonatal *C. melanopterus* could exhibit a strong Bohr effect at 30 °C (i.e., the average body temperature recorded in neonatal *C. melanopterus*), an optimal temperature for oxygen binding, to confer high performance in nursery areas, where successful foraging and predator evasion are critically important. Despite this, *p*50 values were within the range measured across elasmobranch fishes (Morrison *et al.*, 2015), providing further evidence that Hb-O_2 affinity is highly conserved among cartilaginous fishes.

Biologging data for neonatal *C. melanopterus* suggest thermal preference behaviours *in situ*. Sharks appeared to exhibit avoidance of 31 °C water; although, data could only be collected for six sharks and the observed trend might have been driven by an influential individual observation. Data loggers on sharks did record temperatures at or above 31 °C, but only during 11-32% of deployments. Thermal preference has been suggested for adult *C. melanopterus* from other populations based only on *in situ* observation using temperature data-loggers (Speed *et al.*, 2012; Papastamatiou *et al.*, 2015). If temperature was indeed influencing shark behaviour, neonatal *C. melanopterus* may have been avoiding temperatures that reduce Hb-O_2 affinity with acute exposure and reduce TSM if sharks remained in waters long enough for acclimation to occur. Similarly, tropical epaulette sharks (*Hemiscyllium ocellatum*) exhibit some capacity for using behaviour to avoid high temperatures that are associated with reductions in growth rate if acclimation occurs (Gervais *et al.*, 2018). However, it is important to consider that neonatal *C. melanopterus* around Moorea use very small core habitat (0.02 km^2; personal observation) relative to other *C. melanopterus* neonate populations that could restrict their access to a sufficient diversity of habitats to seek thermal refuge (Papastamatiou *et al.*, 2009b; Chin *et al.*, 2016; Oh *et al.*, 2017b). Furthermore, it is necessary to consider the possible confounding effects of other environmental factors (Schlaff *et al.*, 2014)

and predator-prey dynamics (George *et al.*, 2019) on the apparent observation of thermal preference in *C. melanopterus* neonates. Therefore, the finding of *in situ* thermal preference behaviours in neonatal *C. melanopterus* should be interpreted with caution and warrant further investigation.

Mortality occurred in sharks during recovery from exercise in respirometry chambers above 28 °C. Whilst mortality was 0% at 28 °C, there was 30% mortality (3 of 10 sharks) at 31 °C and 80% mortality (4 of 5 sharks) at 33 °C. Fishes are known to experience high rates of mortality at temperatures nearing their upper thermal limits (Eliason *et al.*, 2013). Whilst noteworthy, however, it could not be determined whether our observation represented a genuine effect of acclimation temperature or a confounding effect of experimental protocols and acclimation temperature. Exhaustive exercise and static respirometry induce a stress response in rainbow trout (*Oncorhynchys mykiss*; Murray *et al.*, 2017). In my study, neonatal *C. melanopterus* may have also experienced stress that was exacerbated at high temperatures (i.e., > 31 °C), thus resulting in mortality. Previous work demonstrates that *C. melanopterus* in respirometry chambers exhibit higher Hct relative to free-swimming *C. melanopterus* (Schwieterman *et al.*, 2019a), suggesting a possible stress response. Indeed, I cannot know if mortality estimates reflect genuine temperature effects without having recovered free-swimming *C. melanopterus* in holding tanks for comparison. As such, my study demonstrates the difficulties of experimentally studying ram-ventilating shark species at high temperatures (Crear *et al.*, 2019). Mortality following exercise at only 1-3 °C above average wet season temperatures would have significant implications regarding the effects of ocean warming on *C. melanopterus* neonate populations. This is a critical area for further research.

In conclusion, neonatal *C. melanopterus* exhibit little thermal dependence of various physiological performance traits over a narrow, ecologically relevant temperature range but may exhibit the capacity to use behaviour to avoid unfavourable temperatures. Further, these results highlight that individuals with greater thermal tolerance have greater hypoxia tolerance, which suggests the possibility of an oxygen-dependent mechanism underlying thermal tolerance in this species (Pörtner *et al.*, 2017); although, this mechanism was not directly tested in this study. Regarding climate change, neonates of this population of *C. melanopterus* appear robust to temperature change and possibly to ocean warming. However, this species' use of shallow, nearshore habitats as nursery areas with small home ranges suggests that ocean warming may render these habitats to be ecological traps as warming throughout the rest of the century pushes this population closer to its upper thermal limits. Moving forward, there is a need for more studies to define the magnitude of the climate change threat to sharks and rays (Rosa *et al.*, 2017).

Chapter 6: Testing physiological and behavioural responses to temperature and carbon dioxide in a reef shark

6.1 Summary

Ocean warming and acidification act concurrently on marine ectotherms with the potential for detrimental, synergistic effects. Global change effects on large predatory fishes, including sharks, remain understudied. Here, I quantified the combined effects of ocean warming and acidification in neonates of a large predatory elasmobranch fish, the blacktip reef shark (*Carcharhinus melanopterus*). Sharks were acclimated to combinations of temperature (28, 31 °C) and CO_2 partial pressures (pCO_2; 650, 1,050 µatm) in a fully-factorial design. Behaviour (lateralisation, activity level) was quantified during 7-13 days of exposure and physiology (hypoxia tolerance, oxygen uptake rates, acid-base and haematological status) was quantified during 14-17 days of exposure. There was a synergistic interaction effect of high temperature and pCO_2 on minimum oxygen uptake rates (i.e., maintenance metabolism costs were increased) relative to ambient conditions. Alone, high pCO_2 had no effect on any metric, and high temperature only increased haematocrit. This species' exposure to daily pCO_2 and temperature fluctuations *in situ* may reflect the observation of little-to-no capacity for reversible acclimation in the short-term. Similar species that rely on shallow, nearshore habitats, such as those that utilise nursery areas for fitness benefits, could be at risk under climate change if these habitats transition to ecological traps.

Associated publication

Bouyoucos IA, Watson SA, Planes S, Simpfendorfer CA, Schwieterman GD, Whitney NM, Rummer JL (*In Review*) The power struggle: Assessing interacting global change stressors *via* experimental studies on sharks. *Scientific Reports*, Manuscript ID: 8dee7442-464f-4376-8a08-450a9e63d059

Data availability

Data presented in this manuscript are available from the Research Data Repository (Tropical Data Hub) at James Cook University: http://dx.doi.org/10.25903/5da407f2406f5

6.2 Introduction

Climate change threatens marine ectotherms *via* myriad global change phenomena (Lefevre, 2016). Owing to anthropogenic carbon emissions, the oceans are experiencing changes in physicochemical properties at an unprecedented rate. Unabated climate change projections for the year 2100 suggest that sea surface temperatures will increase by 3-5 °C (ocean warming), and carbon dioxide partial pressures (pCO_2) will increase by ~ 600 μatm (ocean acidification; OA) in pelagic environments; projections are more extreme and variable in coastal environments (Field *et al.*, 2014). These global change phenomena are predicted to affect the fitness and survival of marine ectotherms through reductions in an organism's physiological oxygen supply capacity (Pörtner *et al.*, 2017). However, alternative hypotheses argue for characterising physiological performance across multiple levels of biological organisation (Clark *et al.*, 2013).

Multiple physiological systems are predicted to be affected by global change stressors, including those associated with behaviours. Changes in behaviour in temperate and tropical marine ectotherms are generally associated with simulated OA conditions in a laboratory setting (Tresguerres and Hamilton, 2017), such as changes in the strength and direction of lateralisation (Domenici *et al.*, 2012), predator avoidance (Munday *et al.*, 2016), and activity levels (Laubenstein *et al.*, 2018). Beyond behavioural performance, well-documented physiological effects of simulated OA conditions include respiratory acidosis (e.g., via reduced extracellular pH) and altered acid-base homeostasis (e.g., via bicarbonate buffering), production of lactate (e.g., via reliance on anaerobic metabolism), and reduced survival (Heuer and Grosell, 2014). The physiological and behavioural responses to simulated ocean warming conditions have also been well-studied under laboratory conditions; indeed, several thermal tolerance mechanisms are under consideration (Clark *et al.*, 2013; Pörtner *et al.*, 2017).

Ocean warming and acidification can affect the physiology of marine ectotherms interactively. There is inherent value in quantifying the effects of isolated stressors, but marine environments will be exposed to multiple global change stressors (Boyd *et al.*, 2018). Interactive effects can be synergistic (i.e., both stressors disproportionately influence effect size), additive (i.e., both stressors contribute individually to effect size), or antagonistic (i.e., exposure to one stressor negates or "masks" the effect of another) (Harvey *et al.*, 2013). A recent meta-analysis demonstrated that ocean warming and acidification act additively on aerobic scope (i.e., the difference between maximum and standard metabolic rate) in marine ectotherms; yet, mechanisms underlying interactive effects of ocean warming and acidification are unknown (Lefevre, 2016). Deleterious, negative interaction effects are, therefore, unpredictable. Despite the complexity of responses observed to date for an impressive diversity of marine taxa, ecological roles, and habitat types, pervasive knowledge gaps remain.

Global change effects on the physiology of large predatory fishes are a general knowledge gap (Nagelkerken and Munday, 2016). As mesopredators and apex predators, these species can exert top-down control in ecosystems (Hammerschlag *et al.*, 2019). Work with larger specimens is restricted by equipment used to measure physiological performance traits, such as swim flumes and respirometry chambers, and the ability to treat enough replicate individuals; however, studying early life stages can be amenable to available equipment and adequate replication. Evidence suggests that early-life stages of teleost fishes (e.g., embryos and larvae) are more sensitive to elevated pCO_2 and temperatures than their adult counterparts (Pörtner and Peck, 2010; Baumann, 2019), thus emphasizing the importance of studying this life-stage to understand a populations' or species' vulnerability. Early-life stages of elasmobranch fishes (e.g., sharks) are fully developed at birth/hatch and, therefore, differ considerably from teleost fishes that undergo metamorphosis; yet, the biological ramifications of multiple global change stressors have not been investigated for large predatory elasmobranch fishes at any life stage (Rosa *et al.*, 2017).

Some shark species rely on shallow, nearshore habitats as nursery areas during early life. These habitats are thought to improve fitness relative to individuals or populations that do not use nursery areas (Heupel *et al.*, 2018). Neonates can exhibit strong site fidelity to nursery areas (Heupel *et al.*, 2018), such that these habitats can become ecological traps during extreme conditions such as heatwaves (Vinagre *et al.*, 2018). Indeed, recent studies on thermal reaction norms of volitional activity found that juvenile sharks and rays within nursery areas routinely live at or above their optimal temperature for activity (Lear *et al.*, 2019). However, shark species that use such habitats during early ontogeny (e.g., shark/egg nursery areas) have demonstrated resilience to OA-relevant conditions and capacity for reversible acclimation to ocean warming conditions (Rosa *et al.*, 2017). Conversely, some elasmobranch fishes exhibit unpredictable, deleterious responses to interacting global change stressors (Rosa *et al.*, 2014; Di Santo, 2016; Pistevos *et al.*, 2017). Shark species that derive fitness benefits from shark nursery areas could therefore be at risk if these habitats transition under climate change from nursery areas to ecological traps.

The purpose of this study was to identify *ex situ* physiological and behavioural responses of blacktip reef shark (*Carcharhinus melanopterus*) neonates to global change stressors. This species is a large-bodied mesopredator that relies on shallow, nearshore habitats in the tropics as nursery areas (Mourier and Planes, 2013). Performance was quantified at two temperatures (28 and 31 °C) and pCO_2 values (650 and 1050 µatm) *via* behavioural (lateralisation and activity) and physiological (hypoxia tolerance, oxygen uptake rates, and acid-base and haematological status) metrics. These metrics encompass the broad range of possible responses observed for elasmobranch fishes and previously documented in the literature (Rosa *et al.*, 2017). Here, I offer insight into the effects of global change on a species from a data-deficient class and predator guild that serves a functionally important role in marine ecosystems (Hammerschlag *et al.*, 2019).

These data are significant toward gauging the climate change threat to classically 'hard-to-study' species whose slow life-history traits may disproportionately put species at risk of population declines and extirpation (Dulvy *et al.*, 2014). Furthermore, as shallow, nearshore habitats are predicted to become ecological traps under global change, studying species that rely on these habitats as fitness-enhancing nursery areas will be vital to understanding the climate change threat to these species.

6.3 Methods and materials

Ethics

Permission to collect, possess, and transport sharks and shark tissues was obtained from the French Polynesian Ministère de la Promotion des Langues, de la Culture, de la Communication, et de l'Environnement (Arrêté N°5129). Experiments were approved by the James Cook University (JCU) Animal Ethics Committee (A2394).

Animal collection

Neonatal blacktip reef sharks (n = 37, total length = 569.2 ± 31.9 mm, mass = 1.0 ± 0.2 kg; data presented are means ± standard deviation unless noted otherwise) were collected from putative shark nursery areas around the island of Moorea, French Polynesia from October 2018 through January 2019. Sharks were fished at dusk using monofilament gill-nets (50 m by 1.5 m, 5 cm mesh size), and were transported in 200 L coolers of aerated seawater to the Centre de Recherches Insulaires et Observatoire de l'Environnement (CRIOBE). At the CRIOBE, sharks were marked for identification with uniquely coloured spaghetti tags (Hallprint, Hindmarsh Valley, SA, Australia) and passive integrated transponders (Biolog-id SAS, Paris, France). Animals were held under natural photoperiod in flow-through, 1250 L circular tanks (3-4 sharks per tank) that received seawater directly from Opunohu Bay. Sharks were fed *ad libitum* every second day with fresh tuna (*Thunnus* spp.) except for 24-48 hours of fasting prior to testing. Following experimentation, after 21-34 days in captivity, sharks were released at their original capture site.

Experimental design

Sharks were acclimated to combinations of temperature (28 and 31 °C) and pCO_2 (650 and 1,050 µatm) that are representative of ambient conditions of Moorea's lagoon and projected end-of-century pCO_2 in a fully factorial design (Table 6.1). Test temperatures above 31 °C (i.e., summer heatwave temperatures) are associated with mortality and were avoided. Three replicate groups of 3-4 sharks were tested at each temperature and pCO_2 combination. Behavioural assays were conducted at seven days (lateralisation) and 8-13 days of acclimation (activity levels); behavioural responses are apparent after several days at high pCO_2 (Rosa *et al.*, 2017; Tresguerres and Hamilton, 2017). Physiological assays were conducted at 14 days

(hypoxia tolerance), 16 days (oxygen uptake rates), and 17 days (acid-base and haematological status) of acclimation.

Table 6.1 Experimental treatment seawater chemistry. Values are presented as means ± standard deviation. Temperature, pH on the National Bureau of Standards scale (pH_{NBS}), salinity, and total alkalinity were measured directly and used to calculate carbon dioxide partial pressures (pCO_2) in CO2SYS (Pierrot *et al.*, 2006).

Target pCO_2	**Target temperature**	**Temperature (°C)**	**pH_{NBS}**	**Salinity**	**Total Alkalinity (µmol kg SW^{-1})**	**pCO_2 (µatm)**
650 µatm	28 °C	28.1 ± 0.2	8.01 ± 0.02	37 ± 1	2354 ± 14	657 ± 50
650 µatm	31 °C	30.7 ± 0.3	8.01 ± 0.01	38 ± 1	2351 ± 9	637 ± 5
1,050 µatm	28 °C	28.0 ± 0.1	7.86 ± 0.01	38 ± 1	2337 ± 7	1015 ± 11
1,050 µatm	31 °C	30.8 ± 0.2	7.81 ± 0.07	37 ± 0	2358 ± 32	1150 ± 76

Seawater chemistry

After habituating to the CRIOBE, temperature conditions were achieved in 0.5 C d^{-1} increments using aquarium heaters (Jager 300w, EHEIM GmbH & Co KG, Deizisau, Germany) or chillers (TK-1000/2000, TECO S.r.l., Ravenna, Italy) (Habary *et al.*, 2017). Elevated pCO_2 conditions were achieved once target temperatures were reached. Unique header tanks (288 L) for each pCO_2 treatment tank were dosed with CO_2 using a pH controller system (AT Control System, AB Aqua Medic GmbH, Bissendorf, Germany) set to pH values on the National Bureau of Standards scale (pH_{NBS}).

Four physicochemical parameters were measured to calculate seawater pCO_2: pH_{NBS}, total alkalinity, temperature, and salinity (Watson *et al.*, 2017). Holding tank pH_{NBS} was measured daily with a handheld meter (Seven2Go Pro, Mettler-Toledo GmbH, Greifensee, Switzerland) and was calibrated as needed with pH_{NBS} 4 and 7 buffer solutions. Data-loggers (DS1922L, Maxim Integrated Products, Inc., San Jose, CA, USA) recorded temperatures hourly. Salinity was measured daily with a handheld refractometer. Total alkalinity (A_T, µmol kg $seawater^{-1}$) of holding tank water was measured *via* open-cell Gran titration

following standard operating procedure 3b (Dickson *et al.*, 2007). Seawater samples (50 mL) were dosed with 0.1 M HCl in 0.1 mL increments, and A_T was calculated using custom R script (F. Gazeau, unpublished data). The titrator system (Metrohm 888 Titrando, Metrohm AG, Herisau, Switzerland) was calibrated against certified reference materials (Professor A.G. Dickson, Scripps Institution of Oceanography, San Diego, CA, USA, batch number 171). Water samples were collected three times for each replicate tank: once target temperature and pH_{NBS} conditions were achieved, and after one and two weeks of acclimation. Finally, pCO_2 was calculated by inputting pH_{NBS}, temperature, salinity, and A_T into CO2SYS(Pierrot *et al.*, 2006) alongside K1 and K2 constants by Mehrbach and colleagues refit by Dickson and Millero (Mehrbach *et al.*, 1973; Dickson and Millero, 1987) and $KHSO_4$ by Dickson.

Behavioural assays

Lateralisation was tested using a detour test in a two-way T-maze (69 cm long by 21 cm wide). Sharks were tested under ambient conditions because behavioural responses to high pCO_2 persist during acute exposure to ambient conditions (Munday *et al.*, 2016). After a five-minute habituation, turning direction was scored as sharks exited the maze. Ten turns were recorded at either side of the maze – to account for potential asymmetry of the maze – totalling 20 turning decisions per shark. The relative lateralisation index (L_R; turning preference scored from -100 to 100, where positive L_R indicates a right turning bias) and absolute lateralisation index (L_A; strength of lateralisation from 0-100) were calculated as L_R = [(right turns – left turns)/sum of turns]·100, and $L_A = |L_R|$(Domenici *et al.*, 2012).

Activity levels were quantified using acceleration data-loggers. Acceleration data-loggers (ADLs; G6A+, Cefas Technology Limited, Suffolk, UK) were mounted on the dorsal fin following standardized methods (Bouyoucos *et al.*, 2017a). Sharks were tagged by 0900 each day of testing, and were then isolated in individual holding tanks under treatment conditions. Prior to deployment, ADLs were rotated through each axis for calibration (Gleiss *et al.*, 2010). Tags recorded acceleration at 25 Hz (Brownscombe *et al.*, 2018). Dynamic acceleration was separated from raw acceleration data using a two-second running mean in Igor Pro (WaveMetrics Inc., Lake Oswego, OR, USA) (Bouyoucos *et al.*, 2017a). Overall dynamic body acceleration (ODBA) was calculated as the sum of absolute values of dynamic acceleration in each axis (Wilson *et al.*, 2006). Activity level was quantified as the mean ODBA recorded from 1100-1500.

Physiological assays

Hypoxia tolerance was quantified using critical limit methodology. Sharks were tested individually in a circular pool (100 L, 1 m diameter) under treatment conditions. After five minutes of habituation, oxygen saturation was lowered (8.9 ± 2.4% air saturation min^{-1}) by bubbling nitrogen gas into the water. Oxygen saturation was monitored continuously with a Firesting Optical Oxygen Meter (PyroScience GmbH,

Aachen, Germany). The onset of muscle spasms (OS) was used as a non-lethal experimental endpoint (Lutterschmidt and Hutchison, 1997a); the oxygen saturation at OS was recorded to quantify hypoxia tolerance. Sharks were immediately returned to their treatment tank at the conclusion of the test.

Oxygen uptake rates ($\dot{M}O_2$, mg O_2 kg^{-1} h^{-1}) were quantified using intermittent-flow respirometry(Svendsen *et al.*, 2016). Sharks underwent a single respirometry trial to measure their minimum $\dot{M}O_2$ ($\dot{M}O_{2Min}$) and maximum $\dot{M}O_2$ ($\dot{M}O_{2Max}$). To accomplish this, sharks were first exercised (three minutes of chasing and one minute of air exposure) in a pool (100 L, 1 m diameter) under treatment conditions to achieve $\dot{M}O_{2Max}$ immediately post-exercise (Norin and Clark, 2016). Sharks were then transferred to the same respirometry system described by Bouyoucos et al. (Bouyoucos *et al.*, 2018) for 24 hours of $\dot{M}O_2$ determinations (n = 96) to achieve $\dot{M}O_{2Min}$ (Chabot *et al.*, 2016b). Following respirometry, sharks were weighed and returned to their treatment tank. Background $\dot{M}O_2$ ($\dot{M}O_{2Background}$) was measured in empty chambers immediately before and after respirometry with sharks.

Briefly, $\dot{M}O_2$ was calculated as the absolute value of the slope of the linear decline in dissolved oxygen (mg O_2 L^{-1} s^{-1}) with a coefficient of determination greater than 0.95, extracted using custom R code; A. Merciere & T. Norin, unpublished data) during each determination and corrected by the volume of water in respirometry chambers. Because of variation in shark mass (range = 0.7-1.4 kg), $\dot{M}O_2$ was allometrically scaled to 1 kg using a mass-scaling coefficient of 0.89 (Lefevre *et al.*, 2017; Jerde *et al.*, 2019) following Norin et al. (Norin *et al.*, 2019). Oxygen uptake rates are, therefore, presented in units of mg O_2 h^{-1}. Shark $\dot{M}O_2$ was corrected for background respiration by fitting a line to the two $\dot{M}O_{2Background}$ measurements and subtracting the interpolated value from each $\dot{M}O_2$ determination (Rummer *et al.*, 2016). Six $\dot{M}O_2$ metrics were then calculated. First, $\dot{M}O_{2Min}$ (1) was calculated with the Mean of the Lowest Normal Distribution method using the "mclust" R package (Fraley and Raftery, 2002; Chabot *et al.*, 2016b; Scrucca *et al.*, 2016). Next, $\dot{M}O_{2Max}$ (2) was calculated from the highest slope measured over consecutive 30 s intervals during the first hour of respirometry (Bouyoucos *et al.*, 2018; Zhang *et al.*, 2019). Both absolute aerobic scope (AAS = $\dot{M}O_{2Max}$ - $\dot{M}O_{2Min}$; 3) and factorial aerobic scope (FAS = $\dot{M}O_{2Max} \cdot \dot{M}O_{2Min}^{-1}$; 4) were calculated. Excess post-exercise oxygen consumption (EPOC, mg O_2 kg^{-1}; 5) was calculated as the area bound by an exponential decay curve fit to $\dot{M}O_2$, $\dot{M}O_{2Min}$, and the intersection of these curves (Bouyoucos *et al.*, 2018); this intersection was recorded as sharks' recovery time (6) following exercise.

Blood samples (1 mL) were collected immediately after removing sharks from respirometry (Bouyoucos *et al.*, 2018). Samples were collected using caudal puncture with 23-gauge, heparinised needles. Whole blood pH (1) was measured with a temperature-correcting pH meter (HI98165, Hanna Instruments, Victoria, Australia) and correction equations for subtropical sharks (Talwar *et al.*, 2017). Whole blood lactate concentration (mmol L^{-1}; 2) was measured with an Accutrend Plus (Roche Diagnostics Ltd., Rotkreuz,

Switzerland) (Bouyoucos *et al.*, 2018). Blood samples were centrifuged (10,000 *g* for three minutes) in duplicate to measure haematocrit (Hct, %; 3). Haemoglobin (Hb) concentration ([Hb], mmol L^{-1}; 4) was measured by incubating 5 µl of whole blood in 1 mL of Drabkin's reagent (potassium cyanide – potassium ferricyanide; Sigma-Aldrich, St. Louis, MO, USA) for at least 15 minutes and measuring absorbance of 200 µl aliquots in triplicate in a 96 well plate. Absorbance was read at 540 nm and [Hb] was calculated with an extinction coefficient of 11 $mmol^{-1}$ cm^{-1} (Völkel and Berenbrink, 2000). Finally, mean corpuscular [Hb] (MCHC, mmol L^{-1}; 5) was calculated as $[Hb] \cdot Hct^{-1}$.

Statistical analyses

Behavioural and physiological assays yielded 15 metrics: L_R, L_A, ODBA, CS_{Min}, $\dot{M}O_{2Min}$, $\dot{M}O_{2Max}$, AAS, FAS, EPOC, recovery time, whole blood pH, whole blood lactate concentration, Hct, [Hb], and MCHC. All metrics were fit with linear mixed effects models assuming Gaussian distributions using the R package 'lme4' (Bates *et al.*, 2015; R Core Team, 2018), with temperature and pCO_2 as interacting nominal fixed effects and replicate group as a random effect. Significance of fixed effects was determined by generating 95% confidence intervals (CI) of fixed effect estimate distributions from 1000 posterior simulations that were run using the R package 'arm' (Gelman and Su, 2018). Finally, L_R was examined further by comparing frequency distributions and variances between treatments with Kolmogorov-Smirnov tests and Bartlett tests of homogeneity of variances, respectively (Domenici *et al.*, 2012).

6.4 Results

Behavioural assays

Behavioural metrics were quantified at 7-13 days of exposure to temperature (28 or 31 °C) and pCO_2 (650 or 1050 µatm) conditions in a fully-factorial design (n = 3 replicate groups per treatment, 9-10 sharks per treatment). Lateralisation was quantified at seven days of exposure using a two-way T-maze, and activity levels were quantified over 8-13 days of exposure using acceleration data-loggers (ADLs). The relative lateralisation index (L_R; turning preference scored from -100 to 100, where positive L_R indicates a right turning bias) did not differ between treatments (Figure 6.1A; Table 6.2). Further, there were no significant differences between the distributions of L_R (Table 6.3), or between the variance of L_R (Bartlett test, K^2 = 3.86, p = 0.276). The absolute lateralisation index (L_A; strength of lateralisation from 0-100) also did not differ between treatments (Figure 6.1B; Table 6.2). Finally, activity levels, as defined by overall dynamic body acceleration (ODBA, in *g*), did not differ between treatments (Figure 6.1C; Table 6.2).

Table 6.2 Effects of temperature and pCO_2 on behavioural and physiological metrics in blacktip reef sharks (*Carcharhinus melanopterus*). Linear mixed effects model outputs are presented as 95% confidence intervals (CI) of effect size of fixed effects terms. Bolded terms represent statistically significant parameters whose confidence intervals do not contain zero.

Response	Parameter	CI
Relative lateralisation index	Ambient (28 °C, 650 µatm pCO_2)	-10.57, 83.35
	High pCO_2 (31 °C, 650 µatm pCO_2)	-116.41, 5.17
	High temperature (28 °C, 1,050 µatm pCO_2)	-92.94, 33.85
	High temperature and pCO_2 (31 °C, 1,050 µatm pCO_2)	-13.16, 163.22
Absolute lateralisation index	**Ambient**	**29.85, 69.47**
	High pCO_2	-8.08, 50.47
	High temperature	-14.54, 44.54
	High temperature and pCO_2	-65.51, 15.01
Overall dynamic body acceleration	**Ambient**	**0.08, 0.10**
	High pCO_2	-0.03, 0.01
	High temperature	-0.01, 0.02
	High temperature and pCO_2	-0.01, 0.03
Hypoxia tolerance	**Ambient**	**21.78, 26.41**
	High pCO_2	-2.50, 3.93
	High temperature	-2.89, 3.42
	High temperature and pCO_2	-6.27, 2.39
Minimum oxygen uptake rate	**Ambient**	**119.94, 165.51**
	High pCO_2	-31.46, 32.36
	High temperature	-47.73, 21.65
	High temperature and pCO_2	**0.40, 91.84**
Maximum oxygen uptake rate	**Ambient**	**310.36, 450.35**
	High pCO_2	-89.06, 85.99
	High temperature	-115.68, 74.26
	High temperature and pCO_2	-42.71, 217.01
Absolute aerobic scope	**Ambient**	**182.79. 291.18**

	High pCO_2	-77.90, 79.13
	High temperature	-80.98, 77.04
	High temperature and pCO_2	-71.56, 148.42
Factorial aerobic scope	**Ambient**	**2.23, 3.07**
	High pCO_2	-0.59, 0.56
	High temperature	-0.45, 0.69
	High temperature and pCO_2	-1.07, 0.52
Excess post-exercise oxygen uptake	**Ambient**	**229.82, 543.27**
	High pCO_2	-3359.63, 73.78
	High temperature	-272.07, 203.79
	High temperature and pCO_2	-209.37, 413.00
Recovery time	**Ambient**	**9.6, 14.8**
	High pCO_2	-7.13, 0.28
	High temperature	-4.67, 2.45
	High temperature and pCO_2	-2.79, 7.49
Blood pH	**Ambient**	**7.96, 8.15**
	High pCO_2	-0.09, 0.18
	High temperature	-0.08, 0.19
	High temperature and pCO_2	-0.31, 0.07
Blood lactate concentration	**Ambient**	**1.30, 3.47**
	High pCO_2	-2.82, 0.35
	High temperature	-1.61, 1.75
	High temperature and pCO_2	-2.04, 2.61
Haematocrit	**Ambient**	**0.20, 0.22**
	High pCO_2	-0.02, 0.02
	High temperature	**0.01, 0.05**
	High temperature and pCO_2	-0.04, 0.01
Haemoglobin concentration	**Ambient**	**0.26, 0.66**
	High pCO_2	-0.43, 0.10
	High temperature	-0.29, 0.28
	High temperature and pCO_2	-0.30, 0.47
Mean corpuscular haemoglobin concentration	**Ambient**	**1.32, 3.09**

	pCO_2	-2.02, 0.57
	Temperature	-1.46, 0.97
	Interaction	-1.26, 2.34

Table 6.3 Two-sample Kolmogorov-Smirnov test outputs to compare distributions of the relative lateralisation index (L_R) between treatment groups. Treatment groups are ambient (28 °C and 650 µatm pCO_2), high pCO_2 (28 °C and 1,050 µatm pCO_2), high temperature (31 °C and 650 µatm pCO_2), and high temperature and pCO_2 (31 °C and 1,050 µatm pCO_2). Values above the diagonal are the *D* test statistic, and values below the diagonal are *p*-values.

	Ambient	**High pCO_2**	**High temperature**	**High temperature and pCO_2**
Ambient	-	0.556	0.444	0.300
High pCO_2	0.124	-	0.444	0.456
High temperature	0.336	0.336	-	0.256
High temperature and pCO_2	0.785	0.279	0.916	-

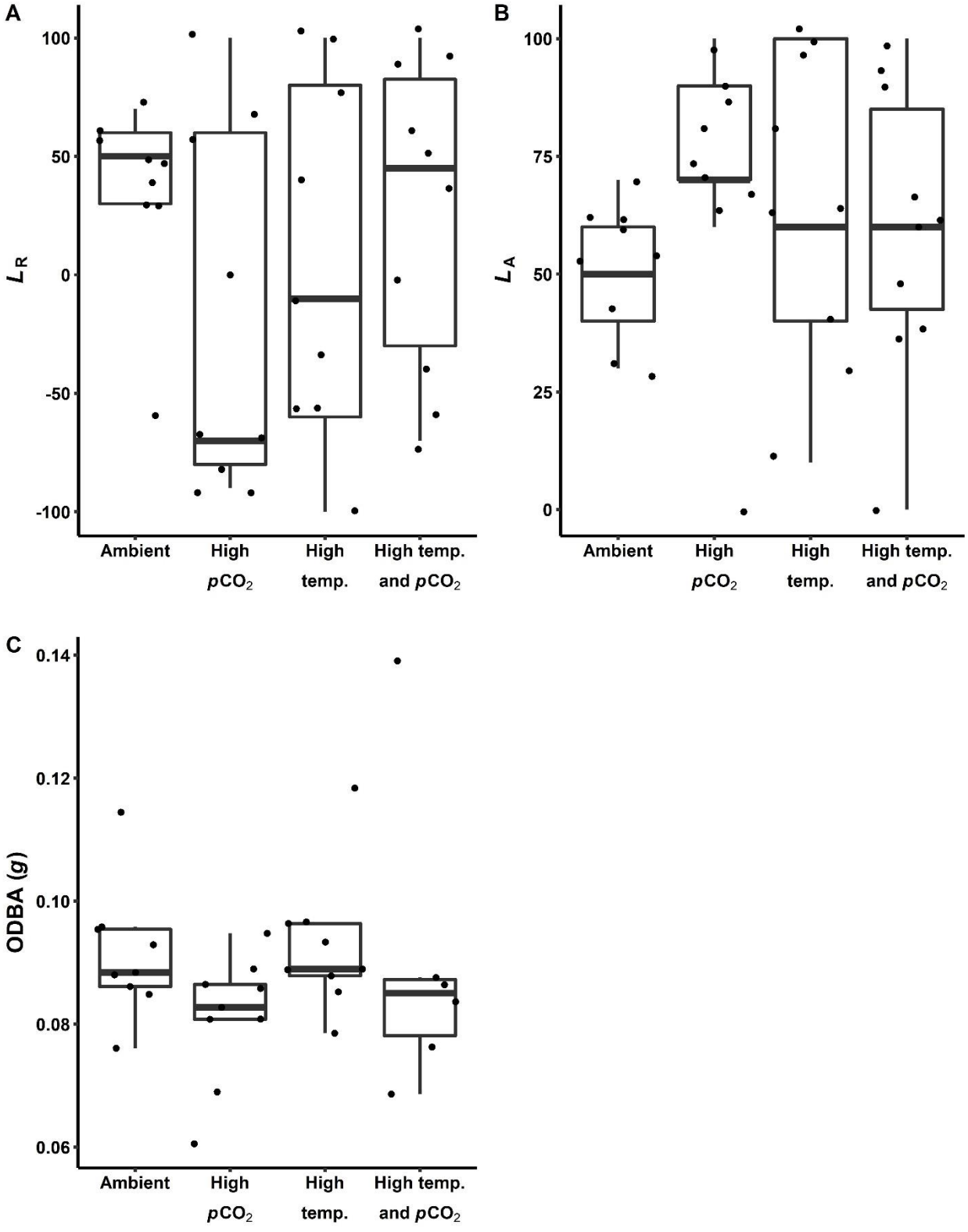
A
100
50
0
-50
-100
L_R
Ambient
High pCO_2
High temp.
High temp. and pCO_2
B
100
75
50
25
0
L_A
Ambient
High pCO_2
High temp.
High temp. and pCO_2
C
0.14
0.12
0.10
0.08
0.06
ODBA (g)
Ambient
High pCO_2
High temp.
High temp. and pCO_2

Figure 6.1 Effects of temperature and carbon dioxide partial pressure (pCO_2) on the behaviour of blacktip reef sharks (*Carcharhinus melanopterus*). Relative (L_R; A) and absolute (L_A; B) lateralisation indices, and activity levels (overall dynamic body acceleration, ODBA; C) were quantified for sharks acclimated to ambient (28 °C and 650 µatm pCO_2), high pCO_2 (28 °C and 1,050 µatm pCO_2), high temperature (31 °C and 650 µatm pCO_2), and high temperature and pCO_2 (31 °C and 1,050 µatm pCO_2) conditions for 7-13 days. Dots represent individual observations.

Physiological assays

Physiological metrics were quantified at 14-17 days of exposure in the same sharks following behavioural assays. Hypoxia tolerance (% air saturation at the onset of muscle spasms) was quantified at 14 days of exposure following critical limit methodology and did not differ between treatments (Figure 6.2; Table 6.2). Oxygen uptake rates ($\dot{M}O_2$, in mg O_2 h^{-1}) were quantified using intermittent-flow respirometry and demonstrated treatment effects (Table 6.2). Minimum oxygen uptake rate ($\dot{M}O_{2Min}$) was affected by the interaction of temperature and pCO_2, where sharks acclimated to high temperature and pCO_2 had elevated $\dot{M}O_{2Min}$ relative to ambient conditions (Figure 6.3A). Conversely, other oxygen uptake rate metrics, maximum oxygen uptake rate ($\dot{M}O_{2Max}$; Figure 6.3B), absolute aerobic scope (AAS = $\dot{M}O_{2Max}$-$\dot{M}O_{2Min}$; Figure 6.3C), factorial aerobic scope (FAS = $\dot{M}O_{2Max}$ $\dot{M}O_{2Min}^{-1}$; Figure 6.3D), excess post-exercise oxygen consumption (EPOC; the oxygen consumed during recovery, in mg O_2; Figure 6.3E), and recovery time (in hours; Figure 6.3F) were unaffected. Acid-base and haematological status was quantified at 17 days of exposure. Blood pH (Figure 6.4A) and lactate concentration (in mmol L^{-1}; Figure 6.4B) did not differ between treatments (Table 6.2). Haematocrit (Hct; the ratio of erythrocytes to plasma) was affected by temperature treatment, where Hct was higher at 31 °C relative to 28 °C (Figure 6.4C; Table 6.2). Haemoglobin concentration ([Hb], in mmol L^{-1}; Figure 6.4D) and mean corpuscular haemoglobin concentration (MCHC, in mmol L^{-1}; Figure 6.4E) were not affected by treatment (Table 6.2).

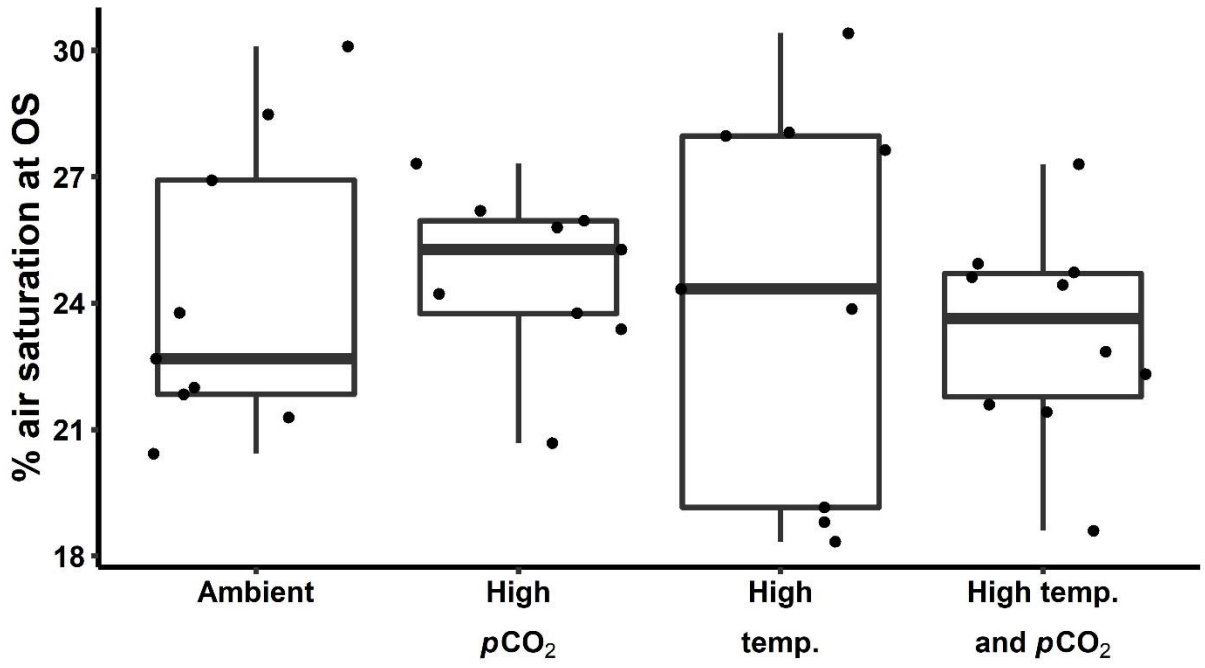

Figure 6.2 Effects of temperature and carbon dioxide partial pressure (pCO_2) on the hypoxia tolerance of blacktip reef sharks (*Carcharhinus melanopterus*). Hypoxia tolerance was quantified as the percent air saturation at which sharks exhibited the onset of muscle spasms (OS). Sharks were acclimated to ambient (28 °C and 650 µatm pCO_2), high pCO_2 (28 °C and 1,050 µatm pCO_2), high temperature (31 °C and 650 µatm pCO_2), and high temperature and pCO_2 (31 °C and 1,050 µatm pCO_2) conditions for 14 days. Dots represent individual observations.

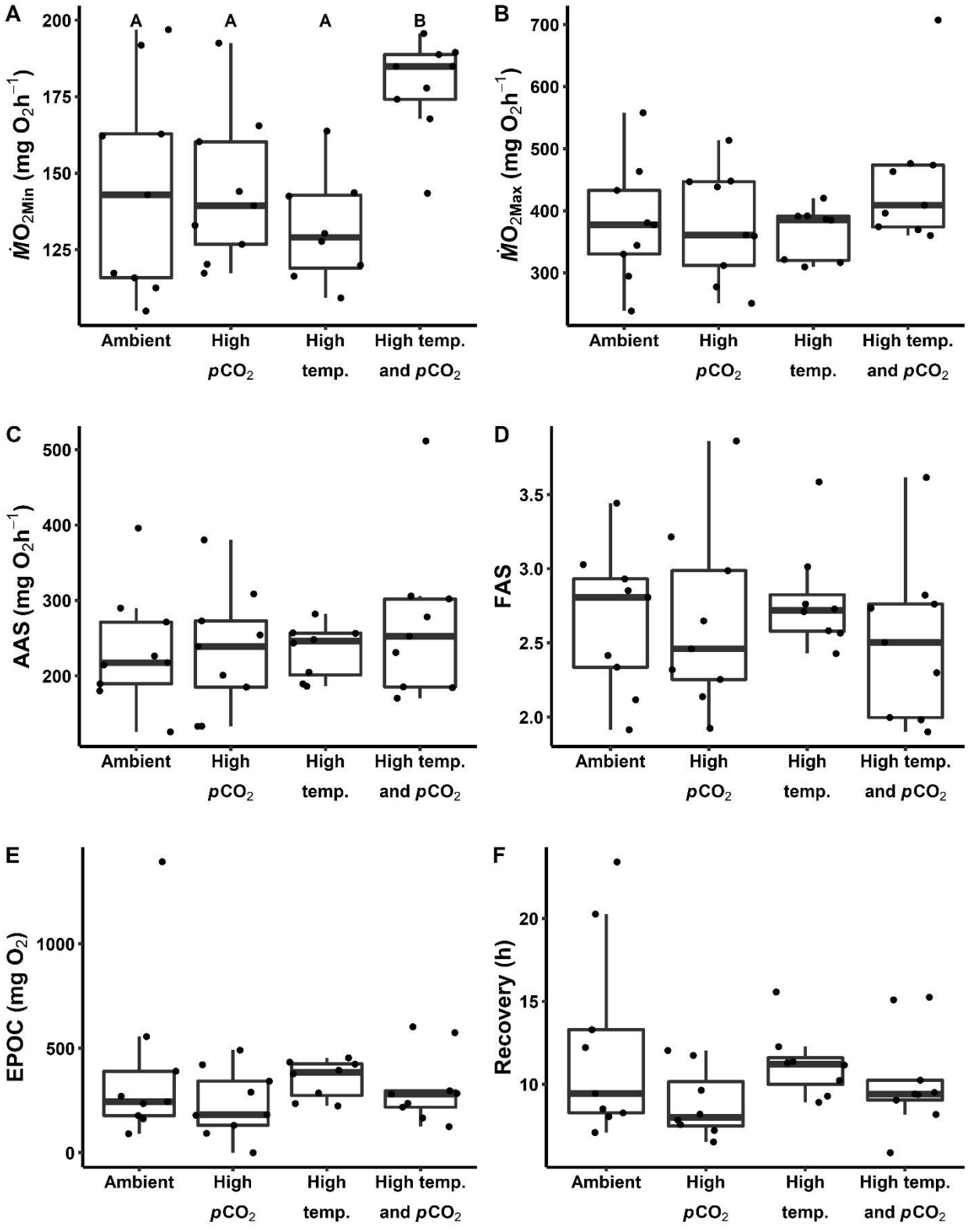
A
B
C
D
E
F
A
A
A
B
$\dot{M}O_{2Min}$ (mg O_2h^{-1})
$\dot{M}O_{2Max}$ (mg O_2h^{-1})
AAS (mg O_2h^{-1})
FAS
EPOC (mg O_2)
Recovery (h)
Ambient
High pCO_2
High temp.
High temp. and pCO_2

Figure 6.3 Effects of temperature and carbon dioxide partial pressure (pCO_2) on the oxygen uptake rates ($\dot{M}O_2$) of blacktip reef sharks (*Carcharhinus melanopterus*). Minimum ($\dot{M}O_{2Min}$; A) and maximum ($\dot{M}O_{2Max}$; B) oxygen uptake rates, absolute (AAS; C) and factorial aerobic scope (FAS; D), excess post-exercise oxygen consumption (EPOC; E), and time to recover $\dot{M}O_2$ post-exercise (F) were quantified for sharks acclimated to ambient (28 °C and 650 µatm pCO_2), high pCO_2 (28 °C and 1,050 µatm pCO_2), high temperature (31 °C and 650 µatm pCO_2), and high temperature and pCO_2 (31 °C and 1,050 µatm pCO_2) conditions for 16 days. Dots represent individual observations. Differing letters denote a statistically significant interaction effect of temperature and pCO_2 on $\dot{M}O_{2Min}$.

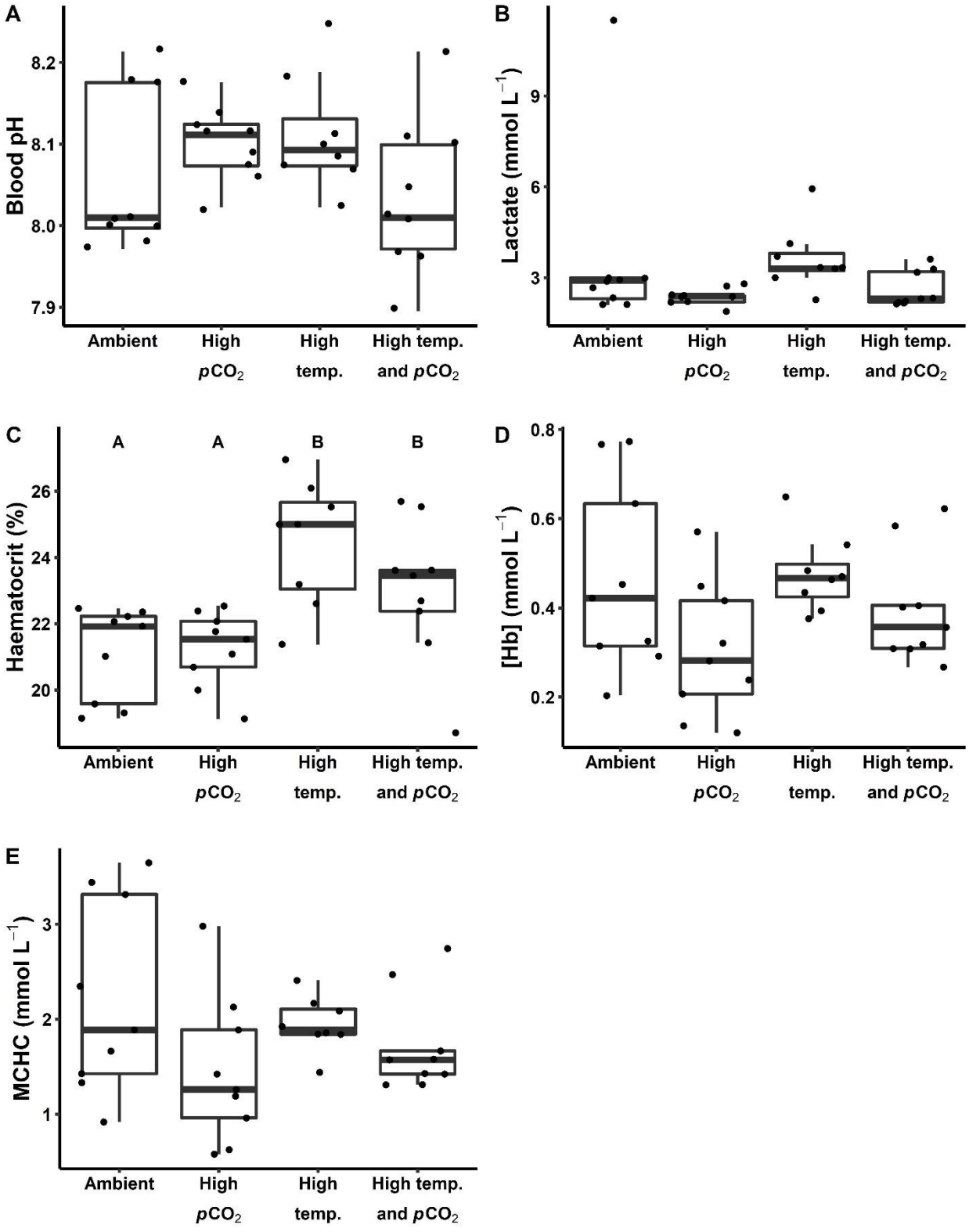
A
Blood pH
8.2
8.1
8.0
7.9
Ambient
High pCO_2
High temp.
High temp. and pCO_2
B
Lactate (mmol L^{-1})
9
6
3
C
Haematocrit (%)
26
24
22
20
A
A
B
B
D
[Hb] (mmol L^{-1})
0.8
0.6
0.4
0.2
E
MCHC (mmol L^{-1})
3
2
1

Figure 6.4 Effects of temperature and carbon dioxide partial pressure (pCO_2) on the acid-base and haematological status of blacktip reef sharks (*Carcharhinus melanopterus*). Blood pH (A) and lactate (B), haematocrit (Hct; C), haemoglobin concentration ([Hb]; D), and mean corpuscular haemoglobin concentration (MCHC; E) were quantified for sharks acclimated to ambient (28 °C and 650 µatm pCO_2), high pCO_2 (28 °C and 1,050 µatm pCO_2), high temperature (31 °C and 650 µatm pCO_2), and high temperature and pCO_2 (31 °C and 1,050 µatm pCO_2) conditions for 17 days. Dots represent individual observations. Differing letters denote a statistically significant effect of temperature on Hct.

6.5 Discussion

The purpose of this study was to gain insight into the responses of blacktip reef shark neonates upon exposure to elevated temperatures and pCO_2 levels resembling end-of-century climate change conditions. Overall, there were mostly no detectable effects of treatment on any metric. There were no detectable effects of pCO_2 at all and temperature only acted on Hct. In one instance, temperature and pCO_2 interacted synergistically, where $\dot{M}O_{2Min}$ was elevated only under high temperature and pCO_2 conditions. Indeed, further work is warranted to determine whether the presence or absence of effects represent genuine responses, or whether possible effects were masked by biological (e.g., inter-individual variability) and experimental (e.g., replication, treatment conditions, measurement error) factors. Here, I discuss the potential significance of my findings for future studies investigating global change stressors in data-deficient and hard-to-study species, such as medium-to-large-bodied sharks.

High temperature and pCO_2 had an interactive effect on aerobic performance in blacktip reef sharks. Minimum $\dot{M}O_2$ was elevated, but only under these conditions, suggesting a synergistic effect. Only several other studies have documented synergistic effects of warming and acidification on $\dot{M}O_{2Min}$ in teleost fishes (Munday *et al.*, 2009; Enzor *et al.*, 2013). Temperature and pCO_2 interact antagonistically on $\dot{M}O_{2Min}$ when looking across teleost and elasmobranch fishes (Lefevre, 2016). Conversely, these results demonstrate that the combination of high temperature and pCO_2 is a loading stress on $\dot{M}O_{2Min}$ in blacktip reef sharks. This increase in $\dot{M}O_{2Min}$ could represent higher costs associated with acid-base regulation at high temperatures. *In situ*, high temperature is associated with low pCO_2 because primary productivity increases during the daytime when the water is warmest (Gobler and Baumann, 2016). Neonatal blacktip reef sharks would then experience high temperatures at different times than they would experience high pCO_2, thereby experiencing the stressors in isolation. Exposure to fluctuating conditions is thought to confer increased tolerance to changes in temperature (Morash *et al.*, 2018) and CO_2 (Baumann, 2019) when tested in isolation. Thus, neonatal blacktip reef sharks likely exhibit little sensitivity to temperature or pCO_2 in

isolation because of the opposing nature of temperature and pCO_2 fluctuations in shallow, nearshore habitats in the tropics. However, blacktip reef sharks are likely not adapted to concomitantly high temperatures and pCO_2, as such conditions do not occur *in situ*, simultaneously. Therefore, blacktip reef sharks would not necessarily possess physiological mechanisms for metabolic compensation under both high temperature and pCO_2; although, this species' capacity for reversible acclimation over longer exposure periods is unclear. Regardless of the mechanism, exposure to high temperature and pCO_2 – below end-of-century levels – comes with an increased energetic cost to maintenance metabolism in blacktip reef sharks.

Temperature acclimation only affected the haematological status of blacktip reef sharks; Hct was the only physiological metric that was affected by temperature. Blacktip reef sharks did not exhibit variation in Hct or [Hb] over a 28-31 °C range in the wild (Bouyoucos *et al.*, 2018), which suggests a seasonal thermal acclimation response. The observed elevation in Hct could compensate for decreasing Hb-O_2 affinity with warming. This response might partially explain why hypoxia tolerance was unaffected, because Hb-O_2 affinity was documented to be associated with hypoxia tolerance in tropical elasmobranch fishes (Speers-Roesch *et al.*, 2012). Alternatively, elevated Hct could function to maintain $\dot{M}O_2$. No temperature effect on $\dot{M}O_{2Min}$ among sharks and rays is somewhat unprecedented (Di Santo, 2016; Crear *et al.*, 2019; Schwieterman *et al.*, 2019b). However, little-to-no capacity for thermal acclimation of $\dot{M}O_{2Min}$ is supported by theory for a species like the blacktip reef shark that experience large daily variation and little seasonal variation in temperature (da Silva *et al.*, 2019). Furthermore, studies on skates documented no effect of temperature on $\dot{M}O_{2Max}$ (Di Santo, 2016; Schwieterman *et al.*, 2019b), and the only other study on a shark (*Carcharhinus plumbeus*) documented no effect of temperature near this species' upper thermal limits (Crear *et al.*, 2019). It is possible that blacktip reef sharks have an optimal temperature for thermal performance where AAS and FAS are maximised between 28 and 31 °C, although broad thermal performance curves (i.e., temperature variation has little effect on performance) would be adaptive in environments characterised by large thermal variability (da Silva *et al.*, 2019). Furthermore, thermal performance curves can plateau near a species' upper thermal limits (Payne *et al.*, 2016). Finally, it is important to consider that blacktip reef sharks do experience mean water temperatures that are around 31 °C for several weeks during the summer, and that limited thermal acclimation capacity is an adaptive response to the narrow range of temperatures these sharks experience on a seasonal scale. If current temperatures already border blacktip reef sharks' upper thermal limits, then this lack of acclimation capacity could become maladaptive with warming above 31 °C.

Acidification did not have a measurable effect on the physiology of blacktip reef sharks. Hypoxia tolerance, oxygen uptake rates, and haematological status were not affected by pCO_2 level. Blood pH of blacktip reef sharks did not differ between pCO_2 treatments. Because blacktip reef sharks' haemoglobins have little pH

sensitivity (Wells *et al.*, 1992), Hb-O_2 affinity and, by association, hypoxia tolerance, should not be expected to change (Speers-Roesch *et al.*, 2012). Maintaining blood pH with increasing pCO_2 did not measurably affect maintenance costs, including those associated with acid-base regulation, because an increase in $\dot{M}O_{2Min}$ was not observed. Similarly, costs associated with acid-base regulation do not appear to be met through anaerobic metabolic pathways because blood lactate concentrations did not increase. Instead, blacktip reef sharks may have buffered increased blood pCO_2 by accumulating bicarbonate (Green and Jutfelt, 2014) and with their pH-insensitive haemoglobins, but without increasing [Hb] (Wells *et al.*, 1992). Blacktip reef sharks' capacity for acid-based regulation likely explains the finding of no effect of pCO_2 on other oxygen uptake metrics (i.e., $\dot{M}O_{2Max}$, EPOC, recovery), and low *in situ* rates of post-exercise mortality (Bouyoucos *et al.*, 2018). All other studies on sharks suggest that pCO_2 has no effect on $\dot{M}O_{2Min}$ (Green and Jutfelt, 2014; Heinrich *et al.*, 2014) or an effect on $\dot{M}O_{2Min}$ but only in combination with high temperatures (Rosa *et al.*, 2014). These studies also suggest that the effects of pCO_2 on Hct and [Hb] are variable. Unlike sharks, skates appear to exhibit greater sensitivity to pCO_2. All measured aspects of the aerobic performance (e.g., AAS, recovery) of little skates (*Leucoraja erinacea*) were affected (Di Santo, 2015, 2016), and clearnose (*Raja eglanteria*) and thorny skates (*Amblyraja radiata*) exhibit elevations in $\dot{M}O_{2Min}$ and reductions in hypoxia tolerance (Schwieterman *et al.*, 2019b). Overall, no measurable effect of pCO_2 in blacktip reef sharks can be interpreted in the context of the ocean variability hypothesis (OVH). This hypothesis suggests that a marine organism with a nearshore/coastal distribution that experiences moderate-to-large diel pCO_2 fluctuations and has no larval stage should have little sensitivity to pCO_2 (Baumann, 2019). Therefore, blacktip reef sharks, and possibly other coastal reef sharks, should be expected to be robust to high pCO_2 as an isolated stressor.

There were no effects of temperature and pCO_2 on the behaviour of blacktip reef sharks. Only one study has tested and documented an effect of temperature on L_R and L_A in sharks (*Heterodontus portusjacksoni*), but these animals were incubated throughout embryonic development, suggesting a developmental, rather than reversible, plasticity response (Vila Pouca *et al.*, 2018). Studies on juvenile coral reef fishes have not documented effects of temperature on L_R or L_A (Jarrold and Munday, 2018). Conversely, acidification has been shown to affect lateralisation in teleost fishes (Domenici *et al.*, 2012) and sharks (Green and Jutfelt, 2014). Activity levels in teleost fishes have been shown to increase with warming (Laubenstein *et al.*, 2018), and acidification has affected the activity levels of sharks in some studies. Dogfish (*Scyliorhinus canicula*) were reported to exhibit fewer, longer bouts of swimming under ~1,000 µatm CO_2 (Green and Jutfelt, 2014), but the length of swimming bouts in Port Jackson and epaulette sharks (*Hemiscyllium ocellatum*) presented with food was unaffected by acidification (Heinrich *et al.*, 2016; Pistevos *et al.*, 2017). Blacktip reef sharks in this study did not change activity levels, suggesting that energetic costs of activity might not been affected *in situ*; yet, increased maintenance costs under high temperature and high pCO_2 conditions suggests that

sharks would invest more energy into foraging. Furthermore, the links between lateralisation and foraging suggest that traits related to predator-prey interactions in wild blacktip reef sharks may not be affected under high temperature and high pCO_2 conditions.

In conclusion, high temperature and high pCO_2 acted synergistically on the maintenance metabolism of a large predatory elasmobranch fish. These data suggest that brief exposure to mild acidification (ΔpCO_2 = +400 μatm) and summer heatwave conditions (~ 31 °C) is enough to increase energetic costs associated with using shallow, coastal habitats in this tropical species. Increases in the energetic costs associated with using shallow, nearshore habitats could have population-level consequences for blacktip reef sharks if supposed energetic costs outweigh theoretical fitness benefits that are associated with fish nursery areas. Further, blacktip reef sharks' and similar species' reliance on shallow, nearshore habitats as nursery areas could render these habitats ecological traps under climate change. However, blacktip reef sharks did not exhibit responses to temperature and pCO_2 for any other physiological and behavioural metrics; indeed, additional work is required to determine whether the presence or absence of responses reflect genuine treatment effects, or reflect biological variability and measurement error associated with working with such 'hard-to-study' species. In addition, research is needed to define blacktip reef sharks' and similar species' long-term capacity for reversible acclimation and capacity for transgenerational acclimation. Large predatory fishes and species characterised by *K*-selected life-history traits may be disproportionately affected by global change if the performance and survival of early life-history stages is reduced. Therefore, research is critical to provide unequivocal, empirical evidence that yields consensus toward a physiologically-informed framework to inform responsible management for these classically 'hard-to-study' species that are already – or will be – threatened by global change.

Chapter 7: General discussion

Myriad anthropogenic impacts drive declines in global shark populations; yet, the consequences of a newly recognised and possibly very serious threat, global climate change, are poorly understood in sharks. Preliminary knowledge (*c*. 2014) exists primarily for temperate species, all of which are oviparous and characterised by benthic lifestyles (Rosa *et al.*, 2017). Thus, responses observed in this group are likely not representative of most other elasmobranch fishes. Indeed, climate change may pose a more serious threat to tropical species than temperate ones because tropical species live closer to their upper thermal limits than temperate species (Comte and Olden, 2017; Payne and Smith, 2017). However, only a limited knowledge base exists to date (*c*. 2020) on which to test these hypotheses. Three species of tropical carpet sharks (Hemiscylliidae), all of which are benthic and oviparous, have been investigated to address this knowledge gap. Data reveal mixed effects of acclimation to simulated ocean warming and acidification conditions. Epaulette sharks (*Hemiscyllium ocellatum*) are resilient to simulated ocean acidification (Heinrich *et al.*, 2014, 2016; Johnson *et al.*, 2016) but not ocean warming conditions (Gervais *et al.*, 2018). Brown-banded bamboo sharks (*Chiloscyllium punctatum*) exhibit physiological responses to simulated ocean warming conditions that are exacerbated by ocean acidification (Rosa *et al.*, 2014, 2016b, 2016a), and white-spotted bamboo sharks (*C. plagiosum*) exhibit resilience to ocean acidification (Lopes *et al.*, 2018; Pegado *et al.*, 2018). An understanding of the predicted effects of climate change conditions on sharks, including tropical species, would be improved by investigating species from different ecological niches, such as active, viviparous species (Rosa *et al.*, 2017). Research conducted in this thesis endeavoured to test mechanisms of vulnerability to climate change in active, tropical, viviparous species, the blacktip reef shark (*Carcharhinus melanopterus*) and sicklefin lemon shark (*Negaprion acutidens*), found in Moorea, French Polynesia. These data demonstrate differential sensitivity of sympatric reef shark populations to temperature change. Further, these data suggest consequences of ocean warming alone in *N. acutidens*, and consequences of ocean warming and acidification in *C. melanopterus*. Together, tropical sharks (*C. melanopterus*, *C. plagiosum*, *C. punctatum*, *H. ocellatum*, and *N. acutidens*) exhibit differential responses to simulated climate change stressors, but all share in common restricted habitat use in early life (e.g., nursery area use).

7.1 Effects of climate change in tropical sharks

Ocean warming

Ocean warming could become a significant global climate change stressor for reef sharks. Under current ocean heatwave scenarios and future ocean warming conditions (i.e., 3-5 °C above average summer temperatures), survival is reduced in embryos and neonates of *C. punctatum* and *H. ocellatum* (Rosa *et al.*,

2014; Gervais *et al.*, 2016, 2018). In *C. melanopterus*, survival after exhaustive exercise is reduced at temperatures 3-4 °C above average summer temperatures (**Chapters 4-6**). Maintenance metabolism (i.e., minimum oxygen uptake rates, $\dot{M}O_{2Min}$) is not strongly affected by temperature in *C. melanopterus* and *N. acutidens* (**Chapters 2, 4, and 5**); yet, this lack of thermal dependence may reflect the narrow range of temperatures at which these species occur and were tested under. However, $\dot{M}O_{2Min}$ and digestive enzyme activity (trypsin and alkaline phosphatase) increased with warming in *C. punctatum* (Rosa *et al.*, 2014, 2016a). Further, *C. punctatum* exhibited signs of oxidative stress when acclimated to 4 °C of warming (Rosa *et al.*, 2016b). Growth was not affected by seasonal acclimatisation in *C. melanopterus* and *N. acutidens* (**Chapter 2**), but growth rate was reduced with thermal acclimation in *H. ocellatum* (Gervais *et al.*, 2018). Growth was also not affected by thermal acclimation in *C. melanopterus* (**Chapter 5**), but this finding might reflect a combination of the narrow temperature and experimental feeding regime. In addition, body condition was reduced with thermal acclimation in *C. punctatum* (Rosa *et al.*, 2014). Conversely, *C. melanopterus* were able to improve their ability to tolerate hypoxia and thermal stress following acclimation to elevated temperatures (**Chapter 5**); although, this reduced sharks' thermal safety margin. Thus, available data suggest a potential for ocean warming to affect survival, metabolism, growth, and abundance in tropical reef shark neonate populations.

Ocean acidification

Ocean acidification could affect reef shark neonates when acting in combination with ocean warming. On its own, simulated ocean acidification conditions have little effect on the physiology of reef sharks. Neonatal *C. melanopterus* (**Chapters 4 and 6**), *C. plagiosum* (Lopes *et al.*, 2018; Pegado *et al.*, 2018), *C. punctatum* (Rosa *et al.*, 2014, 2016b, 2016a), *H. ocellatum* (Heinrich *et al.*, 2014, 2016; Johnson *et al.*, 2016), and *N acutidens* (**Chapter 4**) are competent acid-base regulators and appear fully capable of managing an acidosis associated with increases in pCO_2 of 300-600 μatm that are predicted to occur with ocean acidification. Competency in acid-base regulation is exemplified in *C. melanopterus* with no measured effects of simulated ocean acidification on oxygen uptake rates (including maintenance metabolism costs, $\dot{M}O_{2Min}$), acid-base status (i.e., blood pH), or haematology (i.e., haematocrit, haemoglobin concentration; **Chapter 6**). Similarly, $\dot{M}O_{2Min}$ in *C. punctatum* and *H. ocellatum* were not affected by ocean acidification (Heinrich *et al.*, 2014; Rosa *et al.*, 2014). Like in *H. ocellatum*, increases in plasma bicarbonate concentrations probably contributed to the maintenance of blood pH in *C. melanopterus* (Heinrich *et al.*, 2014). In addition, hypoxia tolerance in both *C. melanopterus* and *H. ocellatum* was unaffected by acidification (Heinrich *et al.*, 2014). Conversely, both *C. plagiosum* and *C. punctatum* experienced reductions in growth performance with acidification (Rosa *et al.*, 2014; Pegado *et al.*, 2018). Further, acidification was associated with oxidative damage in *C. punctatum* (Rosa *et al.*, 2016b), but not

in *C. plagiosum* (Lopes *et al.*, 2018). Behavioural impairment is predicted in some marine ectotherms as a consequence of reduced sensitivity of the olfactory epithelium (Porteus *et al.*, 2018) and hyperpolarisation of the $GABA_A$ inhibitory neurotransmitter (Nilsson *et al.*, 2012; Heuer *et al.*, 2016). No behavioural impairments have been detected in *C. melanopterus* (**Chapter 6**) or in *H. ocellatum* (Heinrich *et al.*, 2016). Only $\dot{M}O_{2Min}$ was affected in *C. melanopterus* when ocean warming and acidification interacted to produce a synergistic effect, possibly relating to the increased cost of acid-base regulation at higher temperatures (**Chapter 6**). In addition, *C. punctatum* experienced several antagonistic interactions between ocean warming and acidification that affected growth, metabolism, and survival (Rosa *et al.*, 2014, 2016b, 2016a). Thus, reef sharks appear to be resilient to ocean acidification as an isolated stressor (e.g., *C. plagiosum*; Lopes *et al.*, 2018; Pegado *et al.*, 2018), but these global climate change stressors have the potential to interact with consequences for neonates.

Ocean deoxygenation

Ocean deoxygenation is likely not an immediate threat to reef sharks. Coral reef teleost fishes (Nilsson and Östlund-Nilsson, 2004; Nilsson *et al.*, 2007) and the benthic, reef associated *H. ocellatum* (Speers-Roesch *et al.*, 2012; Devaux *et al.*, 2019) tolerate severe hypoxia, conditions that routinely occur on coral reefs. Ocean warming is predicted to reduce hypoxia tolerance in coral reef fishes (Nilsson *et al.*, 2010); however, ocean acidification is not predicted to affect hypoxia tolerance in *H. ocellatum* (Heinrich *et al.*, 2014) or *C. melanopterus* (**Chapter 6**). Neonatal *C. melanopterus* can tolerate severe hypoxia (20-30% air saturation) for short periods of time (i.e., minutes) before achieving 'ecological death.' Interestingly, their hypoxia tolerance improves under ocean warming conditions despite reductions in Hb-O_2 affinity (**Chapter 5**). Improved hypoxia tolerance at elevated temperatures is an adaptive response to declining oxygen saturation in warmer water (i.e., ocean deoxygenation) and possibly more severe hypoxic events under ocean warming conditions (Breitburg *et al.*, 2018). Further, hypoxia tolerance in *C. melanopterus* was associated with thermal tolerance (**Chapter 5**), which suggests a common mechanism. For instance, remodelling of the cardiovascular system has been associated with increased hypoxia and thermal tolerance in teleost fishes (Anttila *et al.*, 2013; Nyboer and Chapman, 2018). Because temperature and hypoxia are often considered interacting stressors for fishes (McBryan *et al.*, 2013), neonatal *C. melanopterus*, therefore, appear well-suited to tolerate ocean deoxygenation conditions induced by ocean warming relative to other tropical reef sharks.

7.2 Effects of climate change in shark nursery areas

Global climate change stressors have the potential to affect the fitness benefits that are associated with nursery areas in tropical species. For many fish and invertebrate species, nursery areas play important

functional roles as habitats yielding greater contributions to the reproductive adult populations, thereby enhancing fitness (Beck *et al.*, 2001). The nursery area concept has been applied to sharks (Heupel *et al.*, 2007), and three benefits of using nursery areas have been suggested for sharks: 1) prey availability, 2) refuge from predators, and 3) social learning (Heupel *et al.*, 2018). Temperature effects on shark performance in nursery areas have been suggested (Heithaus, 2007; Lear *et al.*, 2019), and I suggest that thermal dependence of growth and metabolic rate in *C. melanopterus* and *N. acutidens* is in line with the hypothesis that these populations use nursery areas (**Chapter 2**). Here, I suggest that climate change has the capacity to act on all four of the assumed benefits (i.e., including thermal dependence) of nursery area use in sharks with the potential for deleterious outcomes.

Prey availability

A shark species' ability to exploit ample prey resources in shark nursery areas may be affected by ocean warming and acidification. Ocean acidification is not predicted to affect activity levels (i.e., prey encounter rates) in *C. melanopterus* (**Chapter 6**). Prey detection was not affected under acidification conditions in *H. ocellatum* (Heinrich *et al.*, 2016), unlike what has been revealed in temperate sharks under acidification conditions (e.g., reductions in foraging ability; Dixson *et al.*, 2015; Pistevos *et al.*, 2015). This could be because neonatal *C. melanopterus* and *H. ocellatum* both occupy shallow, coastal habitats that are prone to diel CO_2 fluctuations that may confer greater resilience to acidification relative to fishes from stable environments with respect to CO_2 (Baumann, 2019). Behavioural lateralisation, which has been linked to predator detection in teleost fishes (Cantalupo *et al.*, 1995; De Santi *et al.*, 2001), was unaffected by acidification in *C. melanopterus*, providing further evidence that traits associated with predator-prey interactions in tropical sharks may not be sensitive to climate change relevant CO_2 levels (**Chapter 6**). Instead of behavioural impairments, increases in routine metabolic demands with warming and acidification observed in *C. melanopterus* (**Chapters 6**) and *C. punctatum* (Rosa *et al.*, 2014) would necessitate greater prey consumption rates and more time devoted toward foraging that could affect sharks' net rate of energy gain. Further, increased metabolic demands are also likely to deplete endogenous energy reserves at a faster rate (Rosa *et al.*, 2014). Thus, whilst deleterious behavioural responses to elevated CO_2 (i.e., ocean acidification) do not appear to occur in the two tropical sharks that have been tested to date (*C. melanopterus* and *H. ocellatum*), further research is needed to suggest impacts of simulated ocean warming and acidification conditions on predator-prey dynamics.

Refuge from predators

The ability to use nursery areas as refuge from predators may be affected if global climate change renders nursery areas uninhabitable. Exercise performance in *C. melanopterus*, collectively quantified as maximum

metabolic rate, scope for aerobic metabolism, anaerobic capacity (*via* post-exercise oxygen uptake), and time to recover from exhaustive activity, was not affected by elevated temperatures *in situ* (**Chapter 4**), acclimation to an ecologically relevant temperature range (**Chapter 5**), or exposure to elevated temperatures and CO_2 in combination (**Chapter 6**), which, together, suggests that the ability of *C. melanopterus* to escape predators should not be compromised. In addition, swimming performance in *C. plagiosum* was not affected by acidification (Pegado *et al.*, 2018). Behavioural lateralisation was not affected by exposure to elevated temperatures and CO_2 in combination, which provides further evidence that the capacity of *C. melanopterus* neonates to avoid predators should not be compromised under ocean warming and acidification scenarios should predators enter nursery areas. However, the shallow, coastal habitats that reef shark neonates exploit as nursery areas may become uninhabitable both under heatwave and ocean warming conditions. Indeed, tropical fishes live within degrees of their upper thermal limits and are predicted to be more vulnerable to warming than temperate species (Rummer *et al.*, 2014; Comte and Olden, 2017; Payne and Smith, 2017). Whilst neonatal *C. melanopterus* (**Chapter 5**) and *H ocellatum* (Gervais *et al.*, 2018) increase thermal tolerance with acclimation to elevated temperatures, their thermal safety margins, defined as the difference between body temperature and upper thermal limit decrease, even with short-term exposure to heatwave conditions (**Chapter 5**). Thus, predicted warming of 3-5 °C during heatwaves or as ocean warming progresses would push sharks nearer to their thermal limits and render these habitats uninhabitable. Expansion of nursery areas may be required as these populations seek thermal refuge (Bangley *et al.*, 2018). However, attempts to seek thermal refuge in deeper, adjacent habitats would be met with increased exposure to predators, thereby nullifying the anti-predator benefits of nursery habitat.

Social learning

The benefits of social interactions may be affected by global climate change if shifting energy requirements affect the carrying capacity of nursery areas. Several neonatal sharks have demonstrated the capacity for social learning (Guttridge *et al.*, 2013; Thonhauser *et al.*, 2013; Vila Pouca *et al.*, 2020) or learning by example from a conspecific. The ability to learn tasks related to foraging or anti-predator behaviours is a supposed benefit of the high neonatal shark densities that are characteristic of shark nursery areas (Heupel *et al.*, 2018). Behavioural impairments in aquatic ectotherms have been demonstrated in response to ocean acidification (Schunter *et al.*, 2019), including learning ability (Tresguerres and Hamilton, 2017). Learning (*via* quantity discrimination) has only been tested in the context of one global climate change stressor (ocean warming) in a temperate species (Vila Pouca *et al.*, 2019). Behaviour was not affected by acidification in *H. ocellatum* (Heinrich *et al.*, 2016) or by warming and acidification in *C. melanopterus* (**Chapter 6**). Lateralisation and quantity discrimination were both affected in Port Jackson sharks (*Heterodondtus portusjacksoni*) in response to ocean warming (Vila Pouca *et al.*, 2018, 2019), suggesting that these

behavioural responses may be linked. Following this logic, it is probable that learning ability in *C. melanopterus* might not be affected, as lateralisation was not affected (**Chapter 6**). Thus, the ability of neonatal *C. melanopterus* to reap benefits from social learning in nursery areas may not be directly affected by the neurological responses associated with ocean warming and acidification. Reductions in shark abundance could indirectly affect social learning in nursery areas. Indeed, higher energetic costs associated with using nursery areas under ocean warming and acidification conditions could affect these habitats' carrying capacity (**Chapter 6**), especially in food-limited nursery areas like those found around Moorea (Weideli *et al.*, 2019a). Reduced shark density (i.e., reduced carrying capacity and abundance) within nursery areas would reduce the likelihood of social interactions occurring between sharks (Mourier *et al.*, 2012), particularly if individuals do not simply interact with any conspecific (Wilson *et al.*, 2015; Finger *et al.*, 2018). Therefore, whilst the innate ability of social learning in tropical sharks does not appear to be affected by global climate change stressors (Heinrich *et al.*, 2016; Schunter *et al.*, 2019), the nature of social interactions (i.e., shark density, group size) would be affected by decreases in shark abundance in shark nursery areas.

Thermal dependence

Finally, global climate change stressors have the potential to act on the thermal dependence of physiological traits that are associated with shark nursery areas. In this thesis, I provide support for the hypothesis that fitness related physiological traits should exhibit minimal thermal dependence in shark populations that are confined to nursery areas (**Chapter 2**). Indeed, some reef shark neonates that are restricted to nursery areas may exhibit little thermal dependence of physiological traits if habitat temperatures routinely exceed optimal temperatures for performance (Lear *et al.*, 2019). Conversely, thermal dependence of physiological traits in fishes can explain thermal dependence of abundance, including activity level (Gannon *et al.*, 2014; Payne *et al.*, 2018), growth (Payne *et al.*, 2016), and aerobic scope (Del Raye and Weng, 2015). If there is a thermal suitability basis for shark nursery areas, then temperature-dependent physiological performance would affect the chance of neonates contributing to the adult reproductive population. Thus, possible thermal benefits of nursery areas warrant further investigation and represent an additional pathway for climate change to act on the fitness of reef sharks.

7.3 Concluding remarks

Here in this thesis and in studies by others, I corroborate the capacity for climate change to impact early life-history stages of tropical sharks with physiological and behavioural consequences. However, this thesis marks the first study to examine the effects of multiple global climate change stressors in active, tropical, viviparous sharks. At a glance, the applied conservation potential of this thesis may seem limited, as the

target populations have been protected from fishing in French Polynesia since 2006. The ecophysiology of elasmobranch fishes is so understudied that the research presented herein represents a series of unique cases (i.e., active, tropical, viviparous sharks), rather than data contributing to well-established, or even developing, trends. Perhaps more importantly, however, this thesis advances basic nursery area theory in sharks to include thermal dependence of physiological performance traits and, in so doing, suggests a potential mechanism that climate change can act on to affect fitness in shark populations. As such, this thesis suggests an avenue for future research to further develop an understanding of the benefits associated with shark nursery areas or, more broadly, nursery areas for aquatic ectotherms, the importance of nursery areas for survival and lifetime reproductive success of individuals (i.e., Darwinian fitness), and the extent of the climate change threat to sharks *via* reductions in physiological performance. Incorporating physiological practices to advance ecological theory (e.g., the metabolic theory of ecology, oxygen- and capacity-limited thermal tolerance, multiple performances multiple optima, gill-oxygen limitation, etc.) is a fundamental approach that is required to enact appropriate and effective conservation measures, by targeting those physiological traits with unequivocally demonstrated links to fitness. The fishing threat to sharks is of paramount concern, but it will be essential for future work to prepare us tackle the growing threat of climate change to elasmobranch fishes.

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