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RESEARCH ARTICLE

Thermal tolerance and hypoxia tolerance are associated in blacktip reef shark (*Carcharhinus melanopterus*) neonates

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ABSTRACT

Thermal dependence of growth and metabolism can influence thermal preference and tolerance in marine ectotherms, including threatened and data-deficient species. Here, we quantified the thermal dependence of physiological performance in neonates of a tropical shark species (blacktip reef shark, *Carcharhinus melanopterus*) from shallow, nearshore habitats. We measured minimum and maximum oxygen uptake rates ($\dot{M}_{O_2}$), calculated aerobic scope, excess post-exercise oxygen consumption and recovery from exercise, and measured critical thermal maxima (CT_{max}), thermal safety margins, hypoxia tolerance, specific growth rates, body condition and food conversion efficiencies at two ecologically relevant acclimation temperatures (28 and 31°C). Owing to high post-exercise mortality, a third acclimation temperature (33°C) was not investigated further. Acclimation temperature did not affect $\dot{M}_{O_2}$ or growth, but CT_{max} and hypoxia tolerance were greatest at 31°C and positively associated. We also quantified *in vitro* temperature (25, 30 and 35°C) and pH effects on haemoglobin–oxygen (Hb–O_2) affinity of wild-caught, non-acclimated sharks. As expected, Hb–O_2 affinity decreased with increasing temperatures, but pH effects observed at 30°C were absent at 25 and 35°C. Finally, we logged body temperatures of free-ranging sharks and determined that *C. melanopterus* neonates avoided 31°C *in situ*. We conclude that *C. melanopterus* neonates demonstrate minimal thermal dependence of whole-organism physiological performance across a seasonal temperature range and may use behaviour to avoid unfavourable environmental temperatures. The association between thermal tolerance and hypoxia tolerance suggests a common mechanism warranting further investigation. Future research should explore the consequences of ocean warming, especially in nearshore, tropical species.

KEY WORDS: Aerobic scope, Critical thermal maximum, Growth, Haemoglobin–oxygen affinity, Temperature dependence, Thermal preference

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INTRODUCTION

Shallow, nearshore environments are dynamic and important habitats for neonate and juvenile shark populations (Knip et al., 2010), but compared with pelagic habitats, nearshore habitats experience short-term fluctuations in environmental conditions over diel and tidal cycles that act as a form of environmental stress (Schulte, 2014). Because shark neonates may be more capable of tolerating environmental stress than sharks at other ontogenetic stages (Baumann, 2019; Pörtner and Farrell, 2008), neonates can exploit shallow, nearshore habitats as nursery areas, which should theoretically increase fitness (Beck et al., 2001; Heupel et al., 2018). In these shallow, nearshore habitats, including ecologically important nursery areas (McLean et al., 2019), temperature is an important environmental stressor that can affect survival (Giomi et al., 2019; Vinagre et al., 2018). For instance, temperature is predicted to affect nursery area use in sharks (Heithaus, 2007), and juvenile sharks that are confined to nursery areas may exhibit a greater capacity to tolerate increasing temperatures (i.e. heating tolerance) than sub-adults and adults that occur outside of nursery areas (Lear et al., 2019). Whilst fishes from hotter environments (e.g. the tropics) tend to exhibit lower heating tolerance than fishes from more temperate environments (Payne and Smith, 2017; Payne et al., 2016), heating tolerance in shark neonate populations from shallow, nearshore areas (i.e. the ontogenetic stage associated with high heating tolerance) that are also from the tropics (i.e. the habitat associated with low heating tolerance) is not well understood. Heating tolerance is commonly investigated by assessing the thermal dependence of an organism's physiological performance in the laboratory (Schulte et al., 2011). It has been established that tropical shark neonates exposed to increasing temperatures exhibit changes in survival, growth, metabolic rate and upper thermal limits (Gervais et al., 2018; Rosa et al., 2014; Tullis and Baillie, 2005). However, the mechanisms underpinning thermal dependence of physiological performance, tolerance and preference need to be defined to understand adaptations that allow shark neonates to tolerate such dynamic thermal environments and predict how these populations will respond to environmental warming.

Temperature is a powerful controlling factor of physiological performance in aquatic ectotherms. In fishes, oxygen uptake rates ($\dot{M}_{O_2}$) are some of the most commonly measured whole-organism performance traits (Clark et al., 2013). Studies generally measure minimum $\dot{M}_{O_2}$ ($\dot{M}_{O_2\mathrm{min}}$, the oxygen cost of maintaining homeostasis and a proxy for standard metabolic rate; Chabot et al., 2016) and maximum $\dot{M}_{O_2}$ ($\dot{M}_{O_2\mathrm{max}}$, the upper limit to oxygen uptake and a proxy for maximum metabolic rate; Norin and Clark, 2016) to calculate aerobic scope (AS, an organism's ability to match oxygen uptake rate 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

List of symbols and abbreviations

AAS	absolute aerobic scope
AS	aerobic scope
ATP	adenosine triphosphate
CRIOBE	Centre de Recherches Insulaires et Observatoire de l'Environnement
$\mathrm{CS}_{\mathrm{min}}$	critical saturation minimum
$\mathrm{CT}_{\mathrm{max}}$	critical thermal maximum
d_b	mean deviation in body temperature from set temperature
DO	dissolved oxygen concentration
EPOC	excess post-exercise oxygen consumption
FAS	factorial aerobic scope
Hb	haemoglobin
$\mathrm{Hb{-}O_2}$	haemoglobin–oxygen
Hct	haematocrit
MCHC	mean corpuscular haemoglobin concentration
$\dot{M}_{\mathrm{O_2}}$	oxygen uptake rate
$\dot{M}_{\mathrm{O_2max}}$	maximum oxygen uptake rate
$\dot{M}_{\mathrm{O_2min}}$	minimum oxygen uptake rate
n_{50}	Hill coefficient
OD	optical density
OEC	oxygen equilibrium curve
OS	onset of spasms
P_{50}	partial pressure of oxygen at 50% haemoglobin saturation
$P_{\mathrm{CO_2}}$	partial pressure of carbon dioxide
P_{crit}	critical oxygen partial pressure
$\mathrm{pH_e}$	extracellular pH
$P_{\mathrm{O_2}}$	partial pressure of oxygen
SGR	specific growth rate
T_b	body temperature
$\mathrm{TCO_2}$	total carbon dioxide content
T_e	environmental temperature
T_{set}	set temperature
TSI	thermal selection index
TSM	thermal safety margin
ΔH^o	enthalpy of oxygenation
Φ	Bohr coefficient

of recovery from anaerobic exercise; Svendsen et al., 2010) and hypoxia tolerance. The metabolic phenotype is predictably affected by temperature, where $\dot{M}_{\mathrm{O_2min}}$ increases with temperature at a higher rate than $\dot{M}_{\mathrm{O_2max}}$ (Sandblom et al., 2016), such that AS should plateau or even decrease with increasing temperature (Schulte, 2015); however, AS can increase across an ecologically relevant temperature range (Lefevre, 2016). Where AS is documented to decrease with increasing temperatures, studies have sought to predict upper thermal limits for AS (i.e. where AS is reduced to zero at the critical thermal maximum, $\mathrm{CT}_{\mathrm{max}}$), assuming that thermal tolerance is oxygen dependent (Payne et al., 2016; Pörtner et al., 2017). Further, thermal dependence of $\dot{M}_{\mathrm{O_2}}$ is hypothesised to explain thermal dependence of growth (Gräns et al., 2014; Pörtner, 2014). Indeed, multiple physiological traits should be considered to adequately predict the effects of temperature on fitness (Clark et al., 2013; Gangloff and Telemeco, 2018; Kellermann et al., 2019).

Properties of the oxygen transport cascade suggest several mechanisms that underpin thermal dependence of whole-organism traits, including oxygen uptake rates and environmental tolerance traits. Haemoglobin–oxygen ($\mathrm{Hb{-}O_2}$) affinity is predictably affected by temperature in ectothermic fishes (Bernal et al., 2018; Nelson et al., 2019), where an increase in temperature is associated with a decrease in oxygen affinity (via P_{50}, the blood–oxygen partial pressure required to achieve 50% Hb saturation). Teleost fishes have a remarkable capacity to enhance oxygen unloading from Hbs under acidotic conditions (Rummer and Brauner, 2015), and this mechanism has been suggested to explain increases in $\dot{M}_{\mathrm{O_2max}}$ and AS in tropical damselfishes faced with mild aquatic acidification (Couturier et al., 2013; Rummer et al., 2013). Hypoxia tolerance, another trait of the metabolic phenotype, is also associated with $\mathrm{Hb{-}O_2}$ affinity, where higher $\mathrm{Hb{-}O_2}$ affinity (i.e. lower P_{50}) 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 $\mathrm{CT}_{\mathrm{max}}$, a thermal tolerance trait, although evidence is mixed. For instance, studies have found an association between $\mathrm{CT}_{\mathrm{max}}$ and haematocrit (Hct), which is the ratio of erythrocytes to whole blood volume (Beers and Sidell, 2011; Wang et al., 2014). In addition, cardiac myoglobin (Anttila et al., 2013) and Hb concentration (Rodgers and De Boeck, 2019) exhibit associations with $\mathrm{CT}_{\mathrm{max}}$. 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); however, 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 their 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 AS has been suggested to predict thermal preference (via habitat suitability; Del Raye and Weng, 2015) and upper thermal limits (via $\mathrm{CT}_{\mathrm{max}}$; 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 habitat temperature and $\mathrm{CT}_{\mathrm{max}}$), as has been documented by the progressive poleward displacement of marine ectotherm populations with increasing sea surface temperatures (Sunday et al., 2012). TSMs predictably decrease 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, characterising thermal dependence of physiological performance and thermal tolerance can contribute to a better 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. Like teleosts, sharks' capacity for maximal oxygen uptake is limited by cardiovascular function (Hillman et al., 2013), but unlike teleosts, sharks generally do not increase their heart rate substantially (Lai et al., 1989; Piiper et al., 1977; Scharold and Gruber, 1991), their Hbs exhibit reduced sensitivity to changes in pH than in teleosts (Bushnell et al., 1989; Wells et al., 1992), they do not exhibit adrenergically sensitive red blood cells (Berenbrink et al., 2005; Randall et al., 2014), and they do not alter Hct to a great degree (Brill et al., 2008; Lowe et al., 1995). 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=2–12), 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 for sharks; yet, of the few studies that have investigated thermal dependence of AS, all documented no effect of temperature on $\dot{M}_{\mathrm{O_2max}}$ (Bouyoucos et al., 2018; Crear et al., 2019; Di Santo, 2016; 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 under simulated ocean warming conditions (Gervais et al., 2018; Rosa et al., 2014). 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 responses to increasing temperature in a tropical reef shark neonate population. Using the blacktip reef shark [*Carcharhinus melanopterus* (Quoy and Gaimard 1824)] as a model reef shark, we tested for physiological responses to temperature over various time scales (acute exposure and thermal acclimation) to explain temperature preference *in situ*. We 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 ($\dot{M}_{\mathrm{O_2min}}$, $\dot{M}_{\mathrm{O_2max}}$, AS, EPOC and recovery), tolerance traits ($\mathrm{CT_{max}}$, TSM and hypoxia tolerance), and growth performance (growth rate, body condition and food conversion efficiency) to test hypothesis 1, and to define temperature and pH sensitivity of whole-blood Hb–$\mathrm{O_2}$ affinity (P_{50}) of wild, non-acclimated sharks *in vitro* to test hypothesis 2. Specific field objectives were to identify preference or avoidance of acclimation temperatures of free-ranging sharks *in situ* to test hypothesis 3. Taken together, this study represents a comprehensive investigation into the thermal biology of an elasmobranch model species that improves our understanding of the associations between temperature, physiology and behaviour in fishes. These data are also significant toward understanding and predicting population-level consequences of ocean warming in a protected species.

MATERIALS AND METHODS

Ethical approval

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 (*ca*. 2006) within a so-called 'shark sanctuary' (Ward-Paige, 2017). We aimed to test the smallest reasonable sample of sharks because of ethical considerations of working with protected species and restrictions on the number of sharks that could be sampled under our research permit.

Animal collection

Sharks were collected from shore around the island of Moorea, French Polynesia (17°30′S, 149°50′W), from October 2017 to March 2018. Gillnets (50.0×1.5 m, 5.0 cm mesh) were deployed at dusk and capture durations were brief (3±3 min; values presented are means±s.d. unless noted otherwise). Animals were transported to a holding facility (Centre de Recherches Insulaires et Observatoire de l'Environnement; CRIOBE) in 200 litre coolers of aerated seawater. Sharks were habituated under natural photoperiod in 1250 litre, flow-through circular tanks for 1 week and fed 5% of their body mass in fresh tuna (*Thunnus* spp.) every other day. A 48-h fast was used prior to experimentation. Following experimentation, sharks were released at their site of capture.

Thermal dependence of performance

We quantified thermal dependence of oxygen uptake rates (i.e. $\dot{M}_{\mathrm{O_2min}}$, $\dot{M}_{\mathrm{O_2max}}$, AS, EPOC and recovery), hypoxia tolerance, thermal tolerance (i.e. $\mathrm{CT_{max}}$ 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, target acclimation temperatures were achieved in sharks' holding tanks by 0.5°C $\mathrm{day^{-1}}$ (Rummer et al., 2014) changes using commercially available heaters (Jager, Eheim GmbH & Co. KG, Deizisau, Germany) and chillers (TK-1000, Teco S.r.l., Ravenna, Italy). Sharks were maintained at acclimation temperatures for 4 weeks and tested to quantify performance metrics over the following week (Table 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; four females and five 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

Table 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±s.d.

higher acclimation temperature (33°C; an ocean warming scenario) was abandoned owing to high post-exercise mortality (4 of 5 sharks) during measurement of oxygen uptake rates, although mortality did also occur at 31°C (3 of 10 sharks).

Oxygen uptake

Sharks were subjected to an exhaustive exercise protocol to generate a range of oxygen uptake rates that are characteristic of the full aerobic range. Immediately prior to respirometry, sharks were chased for 3 min in a 100 litre, 1 m diameter pool at acclimation temperatures followed by 1 min of air exposure. A chase of 3 to 5 min is sufficiently long to fully exhaust many fishes in warm water (Clark et al., 2013), including *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 $\dot{M}_{O_2\max}$ (Norin and Clark, 2016); however, $\dot{M}_{O_2\max}$ estimated using this method could still be an underestimate (Killen et al., 2017; 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, 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 litres), 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 5×10 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 litres 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 5 min long and flush phases (i.e. when flush pumps are enabled) were 7 min 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 h. 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, $\dot{M}_{O_2}$ (mg O_2 kg^{-1} h^{-1}) was calculated using the equation:

$$\dot{M}_{O_2} = SV_{\mathrm{resp}}M^{-1}, \qquad (1)$$

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 litres), and M is the mass of the shark (in kg). Slopes were calculated in LabChart (7.3.8, ADInstruments, Dunedin, New Zealand). We 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 the $\dot{M}_{O_2}$ determination for each shark (Rummer et al., 2016). Because sharks exhibited approximately two-fold 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 (Jerde et al., 2019; Lefevre et al., 2017) and the following equation:

$$\dot{M}_{O_2\mathrm{scaled}} = \dot{M}_{O_2\mathrm{measured}}\,(M_{\mathrm{measured}}\,{M_{\mathrm{scaled}}}^{-1})^{(1-b)}, \qquad (2)$$

where $\dot{M}_{O_2\mathrm{scaled}}$ is the mass-adjusted $\dot{M}_{O_2}$, $\dot{M}_{O_2\mathrm{measured}}$ is the value calculated using Eqn 1, 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 h^{-1}. Six $\dot{M}_{O_2}$ metrics were then estimated. (1) Minimum $\dot{M}_{O_2}$ ($\dot{M}_{O_2\min}$) was estimated with the mean of the lowest normal distribution method with the 'mclust' R package (Chabot et al., 2016; Fraley and Raftery, 2002; Scrucca et al., 2016). (2) Maximum $\dot{M}_{O_2}$ ($\dot{M}_{O_2\max}$) was estimated as the highest $\dot{M}_{O_2}$ calculated in sequential 30-s intervals during the first five $\dot{M}_{O_2}$ determinations after exhaustive exercise (Zhang et al., 2019). (3) Absolute aerobic scope (AAS) was calculated as the difference between $\dot{M}_{O_2\max}$ and $\dot{M}_{O_2\min}$, and (4) factorial aerobic scope (FAS) was calculated as $\dot{M}_{O_2\max}$ divided by $\dot{M}_{O_2\min}$. (5) Excess post-exercise oxygen consumption (EPOC, in mg O_2) was estimated for each shark by first fitting an exponential decay curve to $\dot{M}_{O_2\max}$ and mean hourly $\dot{M}_{O_2}$. The value of EPOC was calculated as the area bound by the exponential decay curve, $\dot{M}_{O_2\min}$, the time of the first measurement, and the time when the curve intersected the upper 95% confidence interval limit for $\dot{M}_{O_2\min}$ (Bouyoucos et al., 2018). (6) The curve intersection was recorded as each sharks' recovery time (in h).

Tolerance traits

Following respirometry, sharks were fed (i.e. 5% of their body mass in fresh tuna, *Thunnus* spp., every other day) over 4 days before undergoing a hypoxia tolerance test. Hypoxia tolerance was assessed using a modified loss-of-equilibrium test (Wood, 2018). Individual sharks were transferred from holding tanks to a 100 litre, 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 5 min prior to testing. Oxygen saturation (%) of the water 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 reduced using 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 h because previous data suggest that fish acclimated to normoxia and then acutely exposed to hypoxia (i.e. 1–12 h at ∼10% air saturation) require approximately 5 h to recover (Borowiec et al., 2018; Svendsen et al., 2012). Further, data from our group suggests that *C. melanopterus* require up to 9 h to recover from gill-net capture – a technique that impedes ventilation – supplemented with air exposure (Bouyoucos et al., 2018). Sharks accepted food within hours of the hypoxia tolerance test.

Thermal tolerance was assessed 48 h 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 5-min 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 that used in other studies of sharks and rays (Dabruzzi et al., 2013; Fangue and Bennett, 2003; 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 $\mathrm{CT_{max}}$ (Lutterschmidt and Hutchison, 1997a,b). TSM was calculated as $\mathrm{CT_{max}}$ minus acclimation temperature (Sandblom et al., 2016). Sharks were immediately transferred to their holding tanks after reaching $\mathrm{CT_{max}}$ without any apparent effects.

Growth

Growth performance was quantified after sharks spent approximately 4 weeks at their acclimation temperatures. Sharks were weighed (i.e. wet mass in kg) and measured (i.e. total length in cm) following 2 weeks of acclimation and again at 4 weeks. Three growth performance metrics were calculated. First, specific growth rate (SGR, in % day^{-1}) was calculated as follows:

$$\mathrm{SGR} = ((\ln M_\mathrm{F} - \ln M_\mathrm{I})t^{-1})100, \quad (3)$$

where M_F and M_I are wet mass measured following 4 weeks (final) and 2 weeks (initial) 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) as follows:

$$K = (M \cdot \mathrm{TL}^{-3})100, \quad (4)$$

where M is wet mass (in g) measured after 4 weeks of acclimation and TL is the animal's total length (in cm) measured after 4 weeks of acclimation. Third, conversion efficiency (in %) was estimated by dividing the difference in wet mass between 2 and 4 weeks of acclimation and the approximate wet mass of food consumed for each shark (Norin and Clark, 2017).

Oxygen transport properties

We characterised *in vitro* temperature and pH sensitivity of whole-blood Hb–$\mathrm{O_2}$ affinity of a subset of wild-caught, non-acclimated sharks (n=6, five females and one male; 1.10±0.14 kg) to estimate thermal dependence of oxygen binding *in situ*. Whole-blood Hb–$\mathrm{O_2}$ affinity was estimated *in vitro* at ecologically relevant assay temperatures (25, 30 and 35°C; Bouyoucos et al., 2018) and physiologically relevant $\mathrm{CO_2}$ tensions (0.25 and 1.00%; Harter et al., 2015). Water temperatures at capture sites of these sharks during the week prior to their capture was 27.1±0.2°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 min 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 h of collection. Blood was then refrigerated and subsequently analysed at the University of British Columbia 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–$\mathrm{O_2}$ 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% $\mathrm{N_2}$ for at least 30 min to fully deoxygenate Hb. After reaching a stable OD, the $\mathrm{CO_2}$ tension was set to either 0.25 or 1.00%, and the oxygen tension was increased by 11 increments of $\mathrm{O_2}$ balanced with $\mathrm{N_2}$. Desired gas mixtures of $\mathrm{O_2}$, $\mathrm{CO_2}$ and $\mathrm{N_2}$ 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% $\mathrm{O_2}$. A final OD was recorded at 30% $\mathrm{O_2}$ and 0% $\mathrm{CO_2}$, where it was assumed that Hb–$\mathrm{O_2}$ saturation was 100%. For each equilibration, step the OD difference (ΔOD) between 430 and 390 nm was used to determine Hb–$\mathrm{O_2}$ fractional saturations by relating the change in ΔOD between deoxygenated 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 $\mathrm{CO_2}$ combination; however, in some cases, only one reliable OEC could be generated for each individual at each temperature and $\mathrm{CO_2}$ combination.

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

$$y = d(1 + 2.718^{b(\log_{10} P_{\mathrm{O_2}} - \log_{10} e)})^{-1}, \quad (5)$$

where y is Hb–$\mathrm{O_2}$ saturation, and d, b and e are the fitted parameters. The $P_{\mathrm{O_2}}$ at 50% Hb–$\mathrm{O_2}$ saturation (i.e. P_{50}) was estimated for each OEC by inserting the fitted parameters into a rearranged form of Eqn 5. The cooperativity of Hb–$\mathrm{O_2}$ subunit binding (i.e. the Hill coefficient, n_{50}) was estimated by differentiating the Hill equation at P_{50}.

Whole blood pH

Extracellular blood pH ($\mathrm{pH_e}$) and total $\mathrm{CO_2}$ content ($\mathrm{TCO_2}$) were measured in approximately 500 µl of blood equilibrated for 60 min with 21% $\mathrm{O_2}$ and either 0.25 or 1.00% $\mathrm{CO_2}$ (balanced with $\mathrm{N_2}$) in rotating Eschweiler glass tonometers thermostatted to either 25, 30 or 35°C. Blood was then drawn into a gas-tight syringe, and $\mathrm{pH_e}$ was measured by drawing approximately 200 µl of blood through a Microelectrodes 16-705 flow-through pH electrode in combination with a 16-702 flow-through reference electrode (Microelectrodes Inc., Bedford, NH, USA) thermostatted to the experimental temperature. The remaining 300 µl of blood was then centrifuged, and $\mathrm{TCO_2}$ (mmol l^{-1}) 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, MA, 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 (13,000 ***g***) for 5 min. Haemoglobin concentration ([Hb], in mmol l^{-1}) 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 $\mathrm{mmol^{-1}\ cm^{-1}}$ at a wavelength of 540 nm (Völkel and Berenbrink, 2000). Both Hct and [Hb] were measured in triplicate. Mean corpuscular haemoglobin concentration (MCHC, in mmol l^{-1}) was calculated as [Hb] divided by Hct.

Thermal preference

We deployed temperature data-loggers on a subset of sharks (*n*=6 sharks, 1 female and five 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 were high relative to other locations around Moorea (Fig. 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 (October 2017–March 2018). These animals were not used in any other part of the study.

Body temperature

Temperature data-loggers (iButton, DS1922L, Maxim Integrated Products, Inc., San Jose, CA, USA) were programmed to record temperatures every 10 min 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 3.0×2.0×0.8 cm (frontal cross-sectional area=1.6 $\mathrm{cm^2}$) and weighed 4.2 g in water. As such, the package was approximately 4.9% of the frontal area of sharks (calculated 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 taut across a second foam backing plate on the other side of the fin with multiple surgeon's knots. We considered external attachment to be a far less invasive technique than surgically implanting tags inside the body cavity. Attachment took under 5 min 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 (Fig. 1). Loggers were synchronised using a delayed start feature and logged temperatures every 10 min. Because neonatal *C. melanopterus* have small home ranges (0.04 $\mathrm{km^2}$; I. A. Bouyoucos, unpublished data) and were not caught at adjacent fishing sites, it was assumed that sharks stayed within the area of our data-loggers, such that the loggers recorded temperatures that were representative of the sharks' core habitat.

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 ($\dot{M}_{\mathrm{O_2max}}$, $\dot{M}_{\mathrm{O_2min}}$, AAS, FAS, EPOC

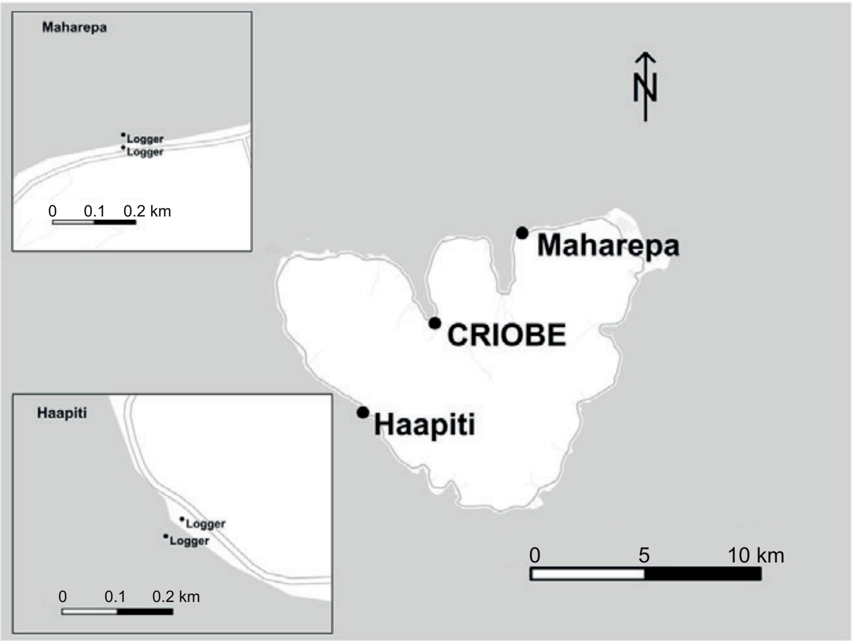

Fig. 1. The study site, Moorea, French Polynesia (17°30′S, 149°50′W). Neonatal blacktip reef sharks (*Carcharhinus melanopterus*) were collected from all around the island and brought to the Centre de Recherches Insulaires et Observatoire de l'Environnement (CRIOBE) for experimentation. Additionally, at two sites, Maharepa and Haapiti, neonates were tagged with temperature data-loggers and recaptured to retrieve data-loggers and body temperature data. Within these sites, two temperature data-loggers were deployed to characterise environmental temperatures (insets). Note that loggers deployed very close to shore (e.g. within mangrove or rock microhabitat) appear to be on land but are not.

and recovery time), tolerance traits (CT_{max}, TSM and CS_{min}) and growth (SGR, Fulton's *K* and conversion efficiency) were fitted 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 (CIs). 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 (https://CRAN.R-project.org/package=arm). 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) as per Zuur et al. (2007). Analyses were conducted using the 'Stats' R package (https://www.r-project.org/).

Oxygen transport properties

The pH sensitivity of Hb–O_2 affinity of whole blood from *in vitro* treatments was assessed first using general linear models. We tested for differences in the slope of the linear relationship between log P_{50} and pH_e at each assay temperature (25, 30 and 35°C). Log P_{50} was fitted 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 P_{50} and pH_e at each assay temperature following:

$$\Phi = \Delta \log P_{50} \cdot \Delta \, pH_e^{-1}. \qquad (6)$$

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 P_{50} was first estimated at a pH of 7.15 – the average pH of plasma by the time it was measured at the University of British Columbia – 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 P_{50} and pH_e; an absent Bohr effect), the average P_{50} was used. Enthalpies were calculated according to the van't Hoff isochore:

$$\Delta H^o = 2.303 \cdot R \cdot \Delta \log P_{50} \cdot \Delta \frac{1}{T}, \qquad (7)$$

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 set temperature, T_{set} (i.e. $d_b=|T_b–T_{set}|$), using simple linear regression; a negative linear relationship suggests preference, in that a larger TSI is associated with a smaller d_b (Dubois et al., 2009; Hertz et al., 1993). We used 28 and 31°C as possible T_{set} 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 dependence study.

RESULTS

Thermal dependence of performance

There was no effect of acclimation temperature on any measured or calculated oxygen uptake rate metric (Fig. 2, Table 2). However, all tolerance traits were affected by temperature acclimation (Table 2). Sharks had a higher CT_{max} and lower TSM at 31°C relative to 28°C (Fig. 3A,B). Further, *C. melanopterus* had a lower CS_{min} at 31°C relative to 28°C (Fig. 3C). Across treatments, CT_{max} and CS_{min} were significantly, negatively associated (mean effect size=−1.85, 95% CI=−3.02 to −0.59; Fig. 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 (Fig. 4, Table 2).

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 P_{50} increased (i.e. Hb–O_2 affinity decreased) from 25 to 30°C (mean effect size=6.89, 95% CI=1.66 to 11.82), but was not different between 30 and 35°C (mean effect size=0.42, 95% CI=−5.52 to 6.53). There was evidence of a strong Bohr effect, but only at 30°C (mean effect size=−0.95, 95% CI=−1.63 to −0.21). Oxygen equilibrium parameters, temperature and CO_2 conditions, Bohr coefficients, enthalpies and haematological metrics are presented in Table 3.

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, $P<0.001$; Fig. 5). Sharks did not exhibit preference or avoidance of 28°C *in situ* (mean effect size=−7.04, 95% CI=−16.65 to 1.72; Fig. 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 to 6.79; Fig. 6B).

DISCUSSION

The purpose of this study was to investigate the thermal dependence of performance, thermal preference and thermal tolerance in neonates of a tropical reef shark species to understand population-level responses to increasing temperatures. We 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 temperature range, (2) whole blood–oxygen affinity is reduced with warming *in vitro*, and (3) sharks exhibit thermal preference and avoidance behaviour *in situ*. Our data offer varying support of our first hypothesis. Oxygen uptake rates and growth performance were not affected by acclimation temperature; however, thermal tolerance and hypoxia tolerance both increased with acclimation and were associated across individuals. In support of our 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 to 35°C. Further, Hbs exhibited pH sensitivity (i.e. a Bohr effect) at 30°C, but this pH sensitivity was not observed at 25 and 35°C. In support of our third hypothesis, sharks

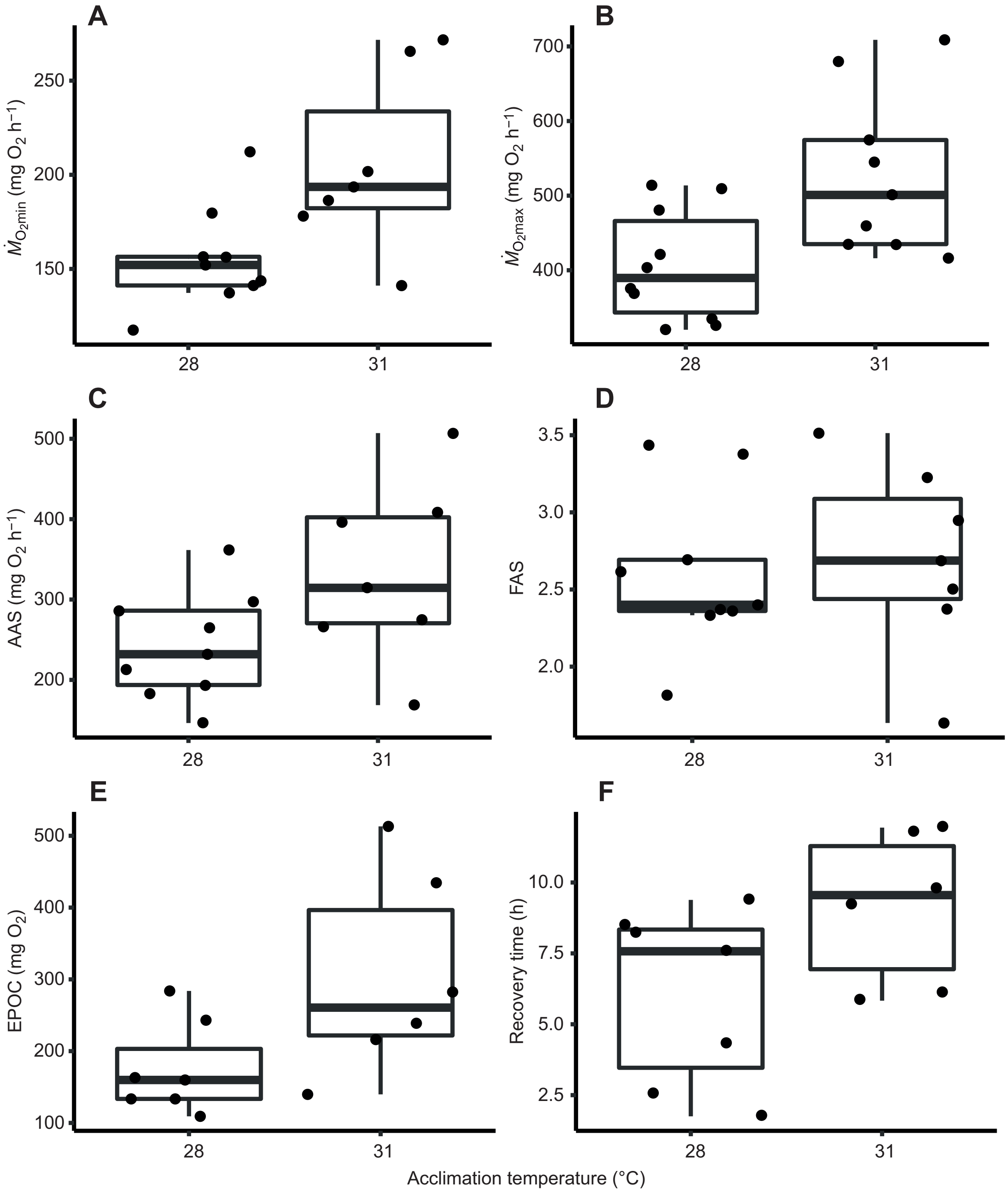

Fig. 2. Effects of acclimation temperature on oxygen uptake rates and recovery in neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Minimum oxygen uptake rate ($\dot{M}_{O_2\mathrm{min}}$; A), maximum oxygen uptake rate ($\dot{M}_{O_2\mathrm{max}}$; B), absolute aerobic scope (AAS; C) and factorial aerobic scope (FAS; D) were not affected by acclimation temperature (28°C, *n*=9; 31°C, *n*=7). Similarly, excess post-exercise oxygen consumption (EPOC; E) and recovery time (F) were unaffected by acclimation temperature (28°C, *n*=7; 31°C, *n*=6). Individual points represent observations for individual sharks.

exhibited avoidance of 31°C *in situ*. 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* have 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 in *C. melanopterus* did not differ between acclimation temperatures. Sharks acclimated to 28 and 31°C for 4 weeks did not exhibit different $\dot{M}_{O_2\mathrm{max}}$, $\dot{M}_{O_2\mathrm{min}}$, AS, EPOC or recovery times. Maximum $\dot{M}_{O_2}$ is predicted to have little thermal plasticity relative to $\dot{M}_{O_2\mathrm{min}}$ (Sandblom et al., 2016). Empirical data in elasmobranch fishes demonstrate no effect of temperature on $\dot{M}_{O_2\mathrm{max}}$ in temperate skates (Di Santo, 2016; Schwieterman et al., 2019b) and no effect of temperature on $\dot{M}_{O_2\mathrm{max}}$ at comparable temperatures (28 and 32°C) in sandbar sharks (*Carcharhinus plumbeus*; Crear et al., 2019). Although minimum $\dot{M}_{O_2}$ should exhibit a reversible thermal acclimation response in

Table 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% CI
Minimum oxygen uptake rate (mg O_2 h^{-1})	Intercept	153.71	110.79 to 193.59
28°C acclimation (*n*=9)	31°C acclimation	42.21	−22.71 to 104.66
31°C acclimation (*n*=7)	31°C replicate group 1	40.12	−35.62 to 113.23
	28°C replicate group 2	−19.11	−98.13 to 57.52
	31°C replicate group 2	−10.67	−90.05 to 64.19
	28°C replicate group 3	17.09	−48.49 to 79.54
Maximum oxygen uptake rate (mg O_2 h^{-1})	Intercept	361.19	291.74 to 432.21
28°C acclimation (*n*=10)	31°C acclimation	88.98	−16.93 to 191.91
31°C acclimation (*n*=9)	31°C replicate group 1	244.20	121.98 to 374.84
	28°C replicate group 2	96.66	−25.34 to 224.63
	31°C replicate group 2	65.25	−56.95 to 196.24
	28°C replicate group 3	43.77	−71.68 to 143.33
Absolute aerobic scope (mg O_2 h^{-1})	Intercept	207.44	135.22 to 283.23
28°C acclimation (*n*=9)	31°C acclimation	46.02	−70.42 to 147.78
31°C acclimation (*n*=7)	31°C replicate group 1	204.00	71.00 to 332.05
	28°C replicate group 2	115.98	−9.69 to 240.99
	31°C replicate group 2	78.83	−40.52 to −198.77
	28°C replicate group 3	26.10	−83.87 to 137.32
Factorial aerobic scope	Intercept	2.36	1.83 to 2.86
28°C acclimation (*n*=9)	31°C acclimation	0.05	−0.81 to 0.85
31°C acclimation (*n*=7)	31°C replicate group 1	0.59	−0.38 to 1.62
	28°C replicate group 2	1.03	0.17 to 1.93
	31°C replicate group 2	0.38	−0.56 to 1.36
	28°C replicate group 3	0.03	−0.76 to 0.82
Excess post-exercise oxygen consumption (mg O_2)	Intercept	166.82	36.38 to 301.34
28°C acclimation (*n*=7)	31°C acclimation	91.63	−164.89 to 320.28
31°C acclimation (*n*=6)	31°C replicate group 1	66.49	−205.21 to 351.72
	31°C replicate group 2	69.36	−186.25 to 373.09
	28°C replicate group 3	15.34	−191.81 to 231.24
Recovery time (h)	Intercept	7.17	3.56 to 11.04
28°C acclimation (*n*=7)	31°C acclimation	2.37	−4.06 to 8.47
31°C acclimation (*n*=6)	31°C replicate group 1	−0.58	−7.65 to 6.31
	31°C replicate group 2	−0.64	−7.88 to 6.57
	28°C replicate group 3	−2.65	−7.81 to 3.08
Critical thermal maximum (°C)	Intercept	37.00	36.46 to 37.55
28°C acclimation (*n*=9)	31°C acclimation	1.97	1.14 to 2.78
31°C acclimation (*n*=7)	31°C replicate group 1	−0.06	−1.05 to 0.91
	28°C replicate group 2	−0.18	−1.15 to 0.80
	31°C replicate group 2	−0.28	−1.32 to 0.71
	28°C replicate group 3	0.06	−0.82 to 0.89
Thermal safety margin (°C)	Intercept	8.98	8.42 to 9.53
28°C acclimation (*n*=9)	31°C acclimation	−1.03	−1.90 to −0.19
31°C acclimation (*n*=7)	31°C replicate group 1	−0.05	−1.08 to 0.98
	28°C replicate group 2	−0.23	−1.23 to 0.76
	31°C replicate group 2	−0.23	−1.26 to 0.70
	28°C replicate group 3	0.09	−0.70 to 0.90
Critical saturation minimum (% air saturation)	Intercept	25.79	23.05 to 28.48
28°C acclimation (*n*=9)	31°C acclimation	−5.23	−9.26 to −1.22
31°C acclimation (*n*=7)	31°C replicate group 1	1.05	−3.84 to 5.94
	28°C replicate group 2	−1.95	−6.32 to 2.91
	31°C replicate group 2	−0.37	−4.99 to 4.85
	28°C replicate group 3	−3.21	−7.23 to 0.74
Specific growth rate (% day^{-1})	Intercept	0.82	0.32 to 1.30
28°C acclimation (*n*=10)	31°C acclimation	−0.03	−0.77 to 0.75
31°C acclimation (*n*=9)	31°C replicate group 1	−0.29	−1.12 to 0.43
	28°C replicate group 2	−0.49	−1.25 to 0.28
	31°C replicate group 2	−0.20	−1.03 to 0.65
	28°C replicate group 3	−0.25	−1.02 to 0.48
Fulton's condition index (*K*)	Intercept	0.51	0.44 to 0.55
28°C acclimation (*n*=10)	31°C acclimation	−0.01	−0.08 to 0.04
31°C acclimation (*n*=9)	31°C replicate group 1	−0.05	−0.02 to 0.11
	28°C replicate group 2	0.02	−0.03 to 0.08
	31°C replicate group 2	0.03	−0.04 to 0.10
	28°C replicate group 3	−0.02	−0.0 to 0.04

Continued

Table 2. Continued

Response	Parameter	Mean effect size	95% CI
Conversion efficiency (%)	Intercept	38.53	14.56 to 61.88
28°C acclimation (*n*=10)	31°C acclimation	0.79	−34.58 to 35.85
31°C acclimation (*n*=9)	31°C replicate group 1	−19.05	−57.58 to 16.84
	28°C replicate group 2	−23.92	−62.29 to 11.89
	31°C replicate group 2	−11.51	−49.33 to 26.88
	28°C replicate group 3	−10.34	−49.17 to 25.79

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

elasmobranch fishes (Lear et al., 2017), that was not the case in neonate *C. melanopterus* between 28 and 31°C. Alternatively, *C. melanopterus* could be thermally compensating $\dot{M}_{O_2\mathrm{min}}$ (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 directly in this study. As such, AS was not affected by temperatures between 28 and 31°C in *C. melanopterus* neonates. Similarly, AS was also unaffected by temperature in two skates (*Amblyraja radiata* and *Raja eglanteria*; Schwieterman et al., 2019b) 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 *C. melanopterus* neonates exhibit a thermal optimum for AS 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* neonates 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). This population of neonatal *C. melanopterus* around the island of Moorea experience daily temperatures ranging from 26 to 34°C; yet, seasonal temperatures here are considerably less variable (dry season=28°C, wet season=30°C; I. A. Bouyoucos, unpublished data). Because daily thermal variation around Moorea is somewhat unpredictable and greater than seasonal variation, *C. melanopterus* could have a modest, reversible acclimation response to temperature change (da Silva et al., 2019); acclimation would otherwise be costly in such an

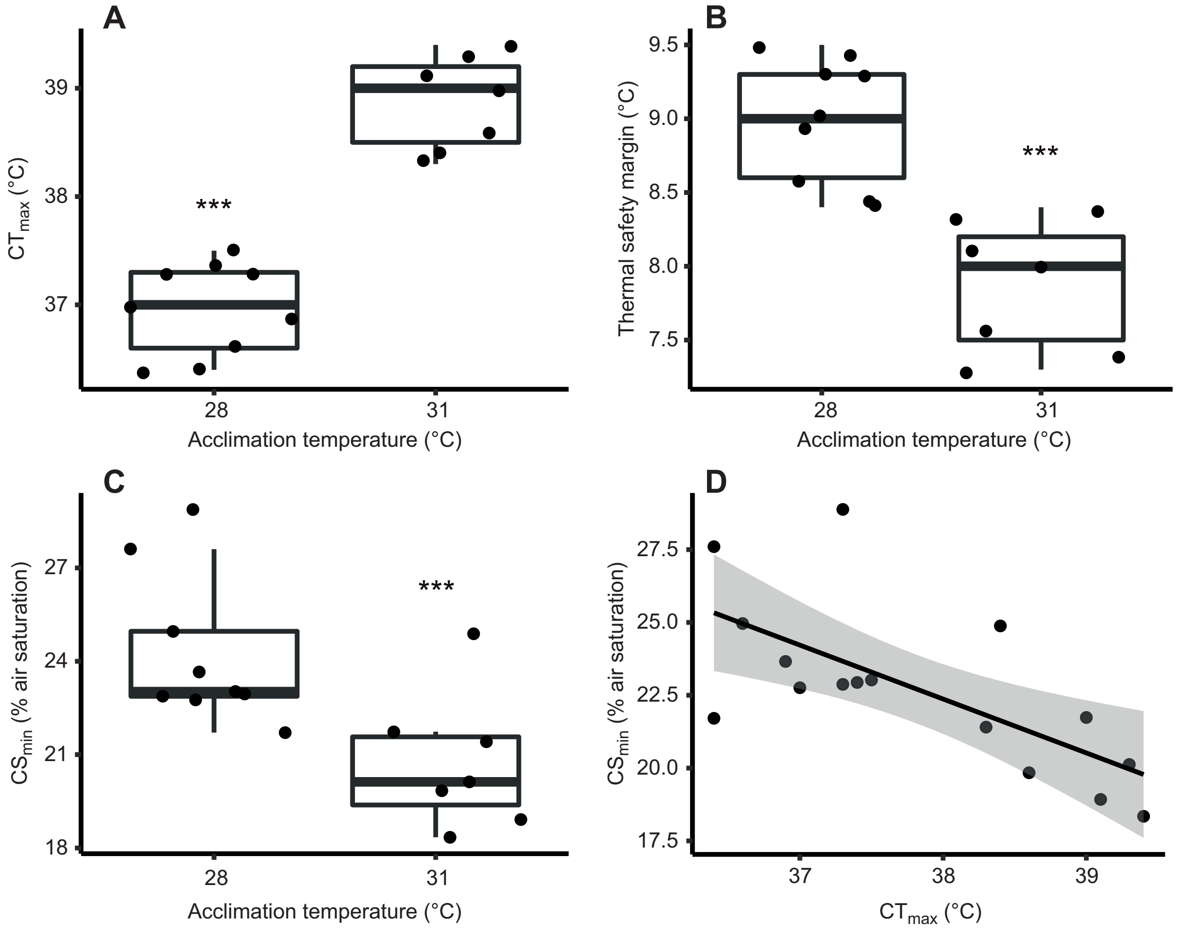

Fig. 3. Effects of acclimation temperature on tolerance traits of neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Asterisks denote statistically significant effects of acclimation temperature (28°C, *n*=9; 31°C, *n*=7) on (A) critical thermal maximum (CT$_{max}$), (B) thermal safety margin (CT$_{max}$ minus acclimation temperature) and (C) critical saturation minimum (CS$_{min}$), a hypoxia tolerance metric. (D) Further, CT$_{max}$ and CS$_{min}$ were significantly correlated (*n*=16); 95% error bars are represented by the shaded area. Individual points represent observations for individual sharks.

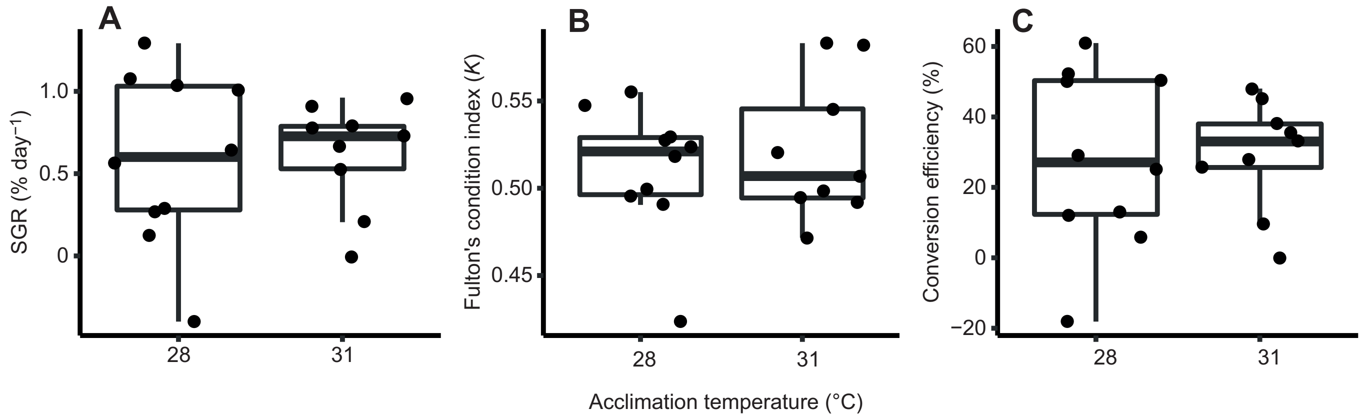

Fig. 4. Effect of acclimation temperature on growth performance in neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Specific growth rate (SGR; A), Fulton's condition index (*K*; B) and conversion efficiency (C) were not affected by temperature acclimation (28°C, *n*=10; 31°C, *n*=9). Individual points represent observations for individual sharks.

unpredictable environment. Thus, a larger temperature range may be necessary to demonstrate thermal dependence of traits such as oxygen uptake rates in *C. melanopterus* neonates.

Environmental tolerance traits were positively associated and increased with acclimation. Neonatal *C. melanopterus* were more hypoxia and temperature tolerant when acclimated to 31°C as opposed to 28°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; Jung et al., 2019; Nyboer and Chapman, 2017) and elasmobranch fishes (Dabruzzi et al., 2013; Fangue and Bennett, 2003; Gervais et al., 2018). Decreases in TSM are also well documented with acclimation to increasing temperatures (McArley et al., 2017; Sandblom et al., 2016) and decreasing latitudes (Comte and Olden, 2017; Deutsch et al., 2008). In this study, CT_{max} (roughly 37 and 39°C for 28 and 31°C acclimated sharks, respectively) 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 our finding of an effect of acclimation temperature on CT_{max}. Whilst shallow, tropical habitats can routinely become supersaturated with oxygen, as do waters inhabited by neonatal *C. melanopterus* during daytime hours (I. A. Bouyoucos, unpublished data), 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 either increase (Anttila et al., 2015; Healy et al., 2018; McBryan et al., 2016) or decrease (Lapointe et al., 2014; Nilsson et al., 2010) 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). However, increasing temperatures can be associated with both high (e.g. supersaturation in productive, coastal environments; Giomi et al., 2019; McArley et al., 2018) and low oxygen saturation (e.g. declining oxygen saturation with increasing respiration rates in tide pools; McArley et al., 2019; Richards, 2011). 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 benefit in supersaturated environments. Mechanisms underlying changes in thermal and hypoxia tolerance have been debated (MacMillan, 2019; McBryan et al., 2013), but a positive correlation between these traits suggests

Table 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
P_{CO_2} (mm Hg)	1.9	7.6	1.9	7.6	1.9	7.6
TCO_2 (mmol l^{-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
P_{50} (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 ($\Delta\log P_{50}\cdot\Delta\log pH_e^{-1}$)	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] (mmol l^{-1})	0.64±0.06					
MCHC (mmol l^{-1})	2.99±0.19					

Values are presented as means±s.d. Φ, Bohr coefficient; P_{CO_2}, CO_2 partial pressure; ΔH^o, enthalpy of oxygenation; pH_e, extracellular pH; [Hb], haemoglobin concentration; n_{50}, Hill coefficient; MCHC, mean corpuscular haemoglobin concentration; P_{50}, oxygen partial pressure at 50% haemoglobin saturation; TCO_2, total CO_2 content. Bohr coefficients are presented unless the slope of the linear relationship between pH_e and log p_{50} was not statistically significant (n.s.). 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.

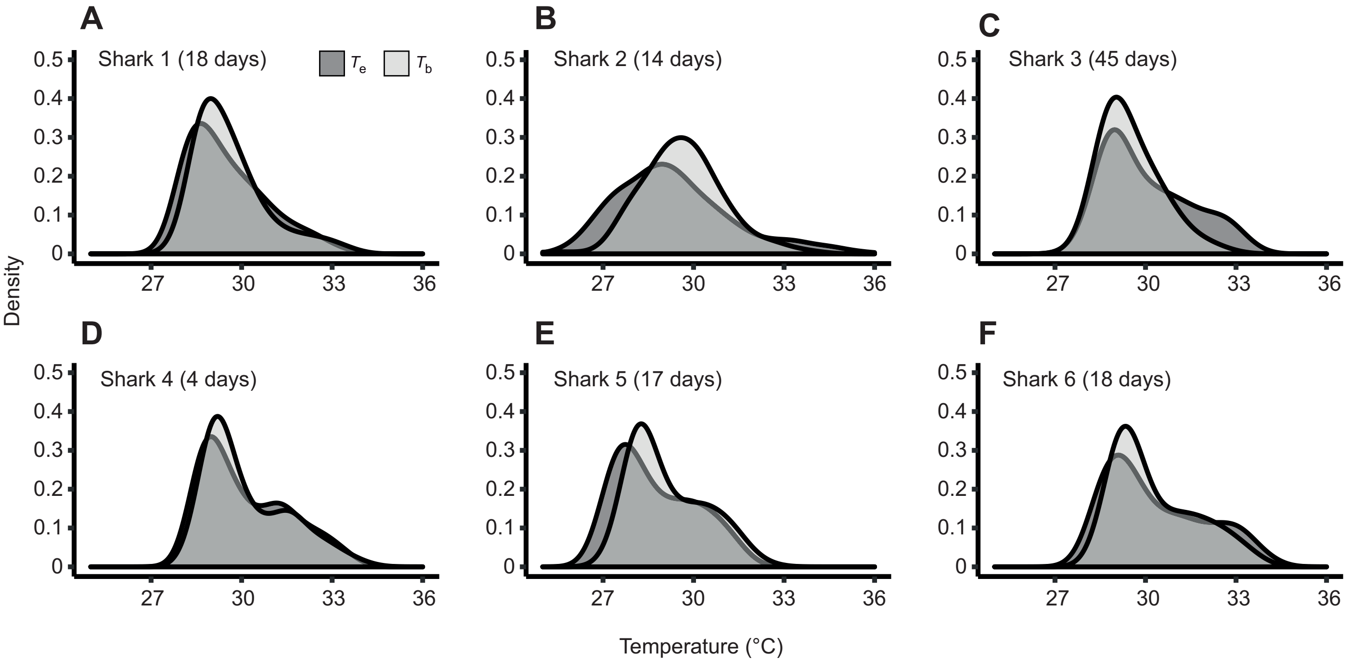

Fig. 5. Density plots of body temperatures (T_b) of neonatal blacktip reef sharks (*Carcharhinus melanopterus*) and environmental temperatures (T_e). Density was calculated as the relative time a logger recorded a temperature within 0.5°C bins. All loggers recorded temperatures every 10 min. Each panel represents an individual shark (*n*=6) and the temperature of its habitat during measurement. The duration of measurement is indicated in parentheses beside shark ID.

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). 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). Populations of *C. melanopterus* have low genetic diversity and exhibit high rates of inbreeding (Mourier and Planes, 2013; Vignaud et al., 2013). Indeed, it remains to be tested whether individual variation in tolerance traits has a genetic or environmental basis in *C. melanopterus*.

Growth performance was unaffected by temperature acclimation. Specific growth rates, body condition and food conversion efficiency did not differ between sharks acclimated to 28 or 31°C for 4 weeks. Growth rates in fishes typically follow thermal performance curves, where growth declines at higher temperatures (Gräns et al., 2014; Jonsson et al., 2001; Larsson and Berglund, 2005). 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 rates. Therefore, it follows that we did not observe an effect of acclimation temperature on growth and oxygen uptake rates. Indeed, *C. melanopterus* neonates may have similarly broad thermal performance curves for growth as was suggested for oxygen uptake rates, owing to the 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

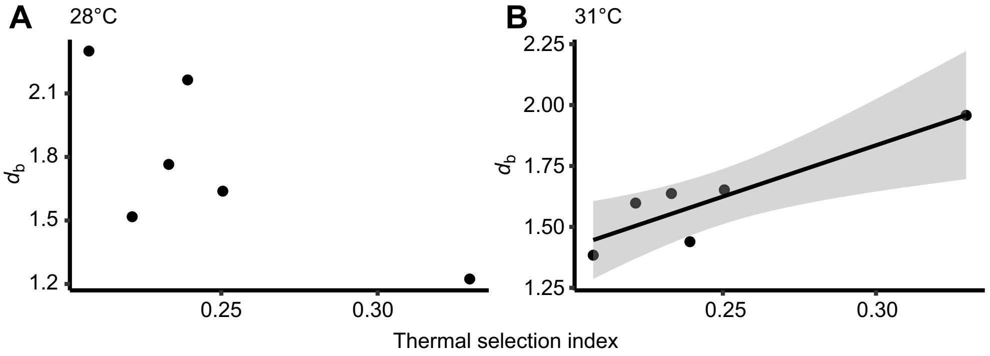

Fig. 6. Evidence of *in situ* thermal preference in neonatal blacktip reef sharks (*Carcharhinus melanopterus*). Sharks with a large thermal selection index had body temperatures that differed greatly from environmental temperatures. Deviation from body temperature (d_b) represents the difference in mean body temperature from 28°C (A) and 31°C (B). Data from the same six sharks were used to calculate thermal selection index and d_b at 28 and 31°C. 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.

associated with immune function and cell organisation (Bernal et al., 2020; Veilleux et al., 2018). However, the present study provided no evidence to suggest downregulation of physiological function. Further, food availability and temperature exhibit interactive effects on growth in fishes (Cominassi et al., 2020; McLeod et al., 2013), such that temperature may have a different effect on growth in wild *C. melanopterus* neonates that do not feed as often as in captivity (Weideli et al., 2019). It is also possible that growth was not measured over long enough durations to detect meaningful differences between acclimation temperatures. Instead, these data demonstrate that *C. melanopterus* neonates maintain their AS over their current seasonal temperature range without adjusting energy expenditure for growth. As a neonate, growth rate is a critically important trait because it can influence 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 P_{50} (and our calculation of ΔH° at pH_e=7.15) are valid because we 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 that in temperate, ectothermic species (Bernal et al., 2018). Hb–O_2 affinity predictably decreased across the assay temperature range, and the reduction was greatest between 30 and 35°C (i.e. the enthalpy of oxygenation was lowest). Interestingly, we only observed a Bohr effect at 30°C. Indeed, the measured Bohr coefficient (Φ=−0.74) is quite large relative to that of other elasmobranch fishes (Morrison et al., 2015), and the only other comparable Bohr coefficients were calculated for stripped Hbs with adenosine triphosphate (ATP) at 10°C in the porbeagle shark (*Lamna nasus*; Φ=−0.76; Larsen et al., 2003) and in whole blood of the mako shark (*Isurus oxyrinchus*) at 15°C (Φ=−0.74; Bernal et al., 2018). Large Bohr effects are characteristic of high-performance fishes, including mackerels (Clark et al., 2010), tunas (Brill and Bushnell, 2006; Bushnell and Brill, 1991) and lamnid sharks (Bernal et al., 2018; Larsen et al., 2003); yet, these species exhibit regional endothermy and very low enthalpies of oxygenation. Although we measured a large Bohr coefficient in whole blood of *C. melanopterus* at 30°C, the Bohr coefficient measured in stripped Hbs 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 exist *in situ*. Sharks appeared to avoid 31°C water; however, data could only be collected for six sharks, and thus the observed trend might have been driven by a single individual. 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 also based on *in situ* observations using temperature data-loggers (Papastamatiou et al., 2015; Speed et al., 2012). 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 have very small home ranges (0.04 km^2; I. A. Bouyoucos, unpublished data) relative to other *C. melanopterus* populations, which could restrict their access to a sufficient diversity of habitats to seek thermal refuge (Chin et al., 2016; Oh et al., 2017; Papastamatiou et al., 2009). Thermal preference could be verified *ex situ* using conventional methods, such as an annular chamber (Cocherell et al., 2014) or shuttle box (Habary et al., 2017), in future studies. 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. Indeed, thermal preference behaviours in neonatal *C. melanopterus* warrant further investigation.

Mortality occurred during recovery from exercise in respirometry chambers above 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 the present study, neonatal *C. melanopterus* may have also experienced stress that was exacerbated at high temperatures (i.e. >31°C), resulting in mortality. Previous work by our group demonstrates that *C. melanopterus* in respirometry chambers exhibit higher Hct relative to free-swimming *C. melanopterus* (Bouyoucos et al., 2018; Schwieterman et al., 2019a), suggesting a possible stress response. Indeed, we cannot know whether mortality estimates reflect genuine temperature effects without having recovered free-swimming *C. melanopterus* in holding tanks for comparison. As such, our 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 for understanding the effects of ocean warming on *C. melanopterus* neonate populations. This is a critical area for further research.

In conclusion, neonatal *C. melanopterus* appear to be thermal generalists with the capacity to avoid unfavourable temperatures. Further, our 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); however, this mechanism was not directly tested in this study. Regarding climate change, neonates of this population of *C. melanopterus* appear robust to temperature changes within their current seasonal range, and possibly to ocean warming as well. However, this species' use of shallow, nearshore habitats as nursery areas with small home ranges suggests that ocean warming may turn these habitats into 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).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: I.B., S.P., C.S., C.B., J.R.; Methodology: I.B., P.M., O.W., S.P., C.B., J.R.; Formal analysis: I.B.; Investigation: I.B., P.M., O.W., E.J., J.R.; Resources: S.P., C.B., J.R.; Writing - original draft: I.B.; Writing - review & editing: I.B., P.M., O.W., E.J., S.P., C.S., C.B., J.R.; Supervision: S.P., C.S., J.R.; Funding acquisition: I.B., S.P., C.B., J.R.

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Data availability

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

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