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Diving in a warm world : thermal constraints on the diving capacity of estuarine crocodiles (**Crocodylus porosus**): thesis

DIVING IN A WARMING WORLD:

Thermal constraints on the diving capacity of estuarine crocodiles (*Crocodylus porosus*)

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ABSTRACT

A central challenge in conserving biodiversity is predicting the consequences of anthropogenic climate change on species' distributions and persistence. Forced climate change has severely altered thermal regimes in marine and freshwater habitats. Rapid escalations in environmental temperatures may be particularly threatening to ectothermic species (almost all plants, invertebrates, fish, amphibians and reptiles), where body temperature and concomitant functional performance are strongly tied to the thermal environment. The threat of overheating is salient for air-breathing, ectothermic divers, such as the estuarine crocodile (*Crocodylus porosus*, Schneider, 1801), because submergence times are inversely related to water temperature. It is unknown how *C. porosus* will fare in warming waters but diving oxygen stores are hypothesised to be consumed more rapidly at elevated temperatures leading to a reduction of aerobic dive limits (i.e. maximum submergence time before lactate is accumulated). Shorter dive durations may force animals to spend more time at the water surface, leaving less time available for obligate underwater activities (e.g. predator avoidance and hunting for aquatic prey).

This thesis assessed the effect of elevated water temperatures (emulating climate change scenarios) on the diving physiology and behaviour of *C. porosus*. The thermal sensitivity of predator avoidance dives (i.e. minutes submerged) was assessed in juveniles at three water temperatures reflecting climate change scenarios (Chapter 2). Diving performance was thermally sensitive with dive durations halving between the 'no warming' and 'moderate warming' scenarios. Ectotherms are however revered for their thermal acclimation/acclimatisation capacity following long term exposure to novel temperatures; whereby an animal's underlying physiology is responsively altered to maintain or optimise performance. For this reason, the acclimation capacity of *C. porosus* was assessed by exposing crocodiles to thermal acclimation treatments for a minimum of 30 days. Thermal acclimation treatments had no effect on dive durations - a result indicative of absent thermal acclimation capacity at elevated temperatures within 30 days.

The physiological mechanisms underlying compromised diving performance at elevated temperatures were subsequently examined (Chapter 3). Reduced diving performance was hypothesised to be linked to increased oxygen demands and a reduced capacity for metabolic depression at elevated temperatures. Diving oxygen uptake, diving heart rate and post-dive plasma-lactate concentrations were assessed at two test temperatures (i.e. 28∘C and 34∘C). Diving metabolic rate increased threefold between 28∘C and 34∘C and the capacity to depress metabolic demands (from surface levels) was inhibited by 46%. Post-dive plasma-lactate accumulation was independent of water temperature. Collectively, these results show the aerobic dive limit of *C. porosus* was significantly reduced at elevated temperatures and animals behaviourally terminated dives earlier rather than increasing reliance on anaerobic metabolism.

The thermal sensitivity and plasticity of aerobic capacity in juvenile *C. porosus* was examined and compared to diving oxygen uptake rate ($\dot{V}O_{2DIVE}$), to assess if diving performance is constrained by aerobic capacity (Chapter 4). Resting ($\dot{V}O_{2STANDARD}$) and maximum ($\dot{V}O_{2MAX}$) rates of oxygen consumption were measured and absolute aerobic scope ($AAS = \dot{V}O_{2MAX} - \dot{V}O_{2STANDARD}$) was calculated. Resting rates of oxygen consumption increased markedly between 28 - 36∘C and did not differ between thermal acclimation treatments. In contrast, $\dot{V}O_{2MAX}$ was thermally insensitive but phenotypically plastic; warm-acclimated animals exhibited a twofold increase in $\dot{V}O_{2MAX}$ compared to animals acclimated to 28∘C. Absolute aerobic scope maintained a broad plateau of thermal independence between 28 - 36∘C and increased in warm-acclimated animals due to elevated $\dot{V}O_{2MAX}$. The thermal sensitivity of $\dot{V}O_{2DIVE}$ (Q_{10} = 7.4) was threefold greater than $\dot{V}O_{2STANDARD}$ (Q_{10} = 2.28), and thermal thresholds marking decrements in diving performance did not align with reductions in aerobic capacity. Together, these findings suggest assessing the vulnerability of diving ectotherms based solely on aerobic scope measurements underestimates susceptibility.

Lastly, diving behaviour of adult, free-ranging *C. porosus* inhabiting the Wenlock River in Cape York (Queensland, Australia) was examined to gauge the influence of seasonal thermal fluctuations (Chapter 5). Satellite and acoustic transmitters with wet-dry and pressure sensors were attached/surgically implanted in 24 animals, and diving behaviour was logged throughout August – December 2015. Aerobic dive limits were estimated (cADL) for animals in the coldest and warmest month, and compared to

observed submergence times. Dive durations were inversely related to water temperature, with mean and maximum dive durations reducing by 40% and 18%, respectively. A greater percentage of maximum dives exceeded cADLs in December (27.4%) compared to August (18.7%). These results suggest the aerobic dive capacity of *C. porosus* is influenced by present-day seasonal thermal increases.

This body of work highlights a previously overlooked threat to ectothermic divers- climate change. My findings reveal that thermal acclimation is unlikely to buffer estuarine crocodiles from the negative consequences of elevated temperatures on dive capacity. Reduced dive durations may see time available for underwater activities cut short and forced time at the water surface may increase the conspicuousness of hatchlings and juveniles to predators. If the findings here apply to other air-breathing diving ectotherms, obligate underwater activities of this group will likely experience serious disruption under climate warming.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed publications

Rodgers EM, Schwartz JJ, Franklin CE (2015) Diving in a warming world: the thermal sensitivity and plasticity of diving performance in juvenile estuarine crocodiles (*Crocodylus porosus*). *Conserv Physiol* 3: cov054.

Conference abstracts

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Contributor	Statement of contribution
Essie M. Rodgers (Candidate)	Concept and design (70%), data collection (90%), data analysis (100%), writing (100%), editing (70%)
Jonathon J. Schwartz	Concept and design (10%), data collection (10%)
Craig E. Franklin	Project support (100%), Concept and design (20%), editing (30%)

Contributions by others to the thesis

Craig Franklin, through discussion and feedback, contributed significantly to the conception and design of this research. Craig White provided assistance with respirometry system designs used in Chapters 3 and 4, and offered advice regarding 'best-practice' techniques for calculating metabolic rates. Simon Blomberg provided advice on statistical analyses used in Chapter 2. Ross Dywer, Craig Franklin and a team from Australia Zoo captured and tagged wild crocodiles, providing data for Chapter 5. Ross Dwyer downloaded data from acoustic receivers in the field annually, provided statistical advice and aided with code development for data analyses in Chapter 5. Craig Franklin critically reviewed the final draft of this thesis.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

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Keywords

climate change, diving behaviour, diving physiology, ectotherm, phenotypic plasticity, thermal acclimation, aerobic dive limit, diving metabolism, bradycardia, thermal sensitivity

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LIST OF ABBREVIATIONS

ADL: Aerobic dive limit

cADL: Calculated/estimated aerobic dive limit

TBO: Total body oxygen stores

PDSI: Post-dive surface interval

Tw: Water temperature

TL: Total length

SVL: Snout-vent length

TPC: Thermal performance curve

CT_{Min}: Critical thermal minima

CT_{Max}: Critical thermal maxima

T_{opt}: Thermal performance optima

$\dot{V}O_2$ = Oxygen uptake rate

$\dot{V}O_{2STANDARD}$: Resting oxygen uptake rate

$\dot{V}O_{2MAX}$: Maximum oxygen uptake rate

$\dot{V}O_{2DIVE}$: Diving oxygen uptake rate

$\dot{V}O_{2SURFACE}$: Surface/pre-dive oxygen uptake rate

$\dot{V}O_{2POSTDIVE}$: Post-dive oxygen uptake rate

$\dot{V}O_{2DEBT}$: Post-dive oxygen debt

$\dot{V}O_{2DEPRESSION}$: oxygen uptake depression during diving (subtracted from surface levels)

AAS: Absolute aerobic scope

FAS: Factorial aerobic scope

FR: Flow-rate

RQ: Respiratory quotient

Q_{10}: Temperature quotient

$f_{H,surface}$: Surface heart rate

$f_{H,diving}$: Diving heart rate

IPCC: Intergovernmental Panel on Climate Change

OCLTT: Oxygen- and capacity-limited thermal tolerance hypothesis

CTD: Conductivity-temperature-depth logger

Conservation Physiology in a Changing World

Human activity has placed permanent signatures on global ecosystems and their constituent microbiota, flora and fauna, triggering the biodiversity crisis (Wake and Vredenburg, 2008; Barnosky et al., 2011; Lewis and Maslin, 2015). With unprecedented species loss, understanding the threats to biodiversity remains a priority for conservation practitioners. The value of evidence based conservation solutions is becoming increasingly recognised and scientists are now at the forefront of management actions (Cooke et al., 2017). Physiological theory can provide a mechanistic (i.e. cause-and-effect) approach to understanding how stressors impact organismal functioning, fitness, and ecological success (Cooke et al., 2013).

Physiologically-informed conservation solutions can be derived by examining the interactions between anthropogenic stressors and organismal functioning at cellular, tissue and whole-organismal levels (Huey et al., 2012). Optimal functioning of physiological systems is intimately related to the cellular state (e.g. temperature and acid-base balance), which in turn, is greatly affected by the external environment (e.g. water temperature and pH). Sub-optimal environmental conditions can constrain physiological functioning and negatively impact fitness-related traits, such as growth (Howe and Marshall, 2002), fecundity (Berger et al., 2008), immune competence (Yu et al., 2009), locomotor capacity (Wilson and Franklin, 2000) and survival (Berrigan, 2000).

Organisms face an ever-increasing risk of losing functional performance as ongoing climate change drives environmental temperatures beyond physiological limits (Pörtner and Farrell, 2008). Forced climate change has severely altered thermal regimes in marine and freshwater habitats; with upper water depths (0 - 75 m) warming at a rate of $\sim 0.1 \circ$C decade^{-1} since 1971 (Collins et al., 2013). Oceanic temperatures are predicted to rise by 2 - 3∘C by 2100, with even greater increases (> 3∘C) probable if global greenhouse gas emissions are not curbed (Collins et al., 2013). Large-scale impacts of climate change are evident, with reports of geographic range shifts and contractions (Poloczanska et al., 2013; Maffucci et al., 2016; Pecl et al.,

2017), advances in phenology (Bartomeus et al., 2011) and local extinctions (Sinervo et al., 2010). This past year (i.e. 2016) was the third consecutive year with record high temperatures across the globe (NASA, 2017, NOAA, 2017). If future biodiversity losses are to be mitigated, robust representations of species' responses to climate change are urgently required to inform conservation decisions (e.g. direction of funds, reserve selection and translocation of threatened species; Guisan et al., 2013).

Ectotherm Vulnerability and Safeguards

Rapid escalations in environmental temperatures may be particularly threatening to ectothermic species (almost all invertebrates, fish, amphibians and reptiles), where body temperature and concomitant functional performance, are strongly tied to the thermal environment. The functional capacity of all organisms is optimised within a limited range of body temperatures (i.e. envelope) as a result of molecular, cellular and metabolic processes being thermally sensitive (Hochachka,1967; Hochachka and Somero, 1968). The thermal sensitivity of key traits is typically described using thermal performance curves (TPC), where an organisms' performance is modelled as a function of environmental/body temperature (Huey and Stevenson, 1979; Huey et al., 2012). Ectotherm TPCs are often bell-shaped, with reduced performance occurring at low and high environmental/body temperatures approaching critical thermal minima (CT_{Min}) and maxima (CT_{Max}), and optimal performance (T_{opt}) occurs within this range (Fig. 1.1 A).

Performance decrements at stressfully high temperatures are hypothesised to stem from oxygen demands exceeding oxygen supply capacities (i.e. cardio-respiratory system failure; oxygen- and capacity-limited thermal tolerance hypothesis; OCLTT; Pörtner, 2001; Pörtner and Knust, 2007; Eliason et al., 2011; Giomi and Pörtner, 2013). Compromised aerobic performance may occur at high temperatures when maximal rates of oxygen uptake($\dot{V}O_{2MAX}$) plateau or decrease but resting/standard rates of oxygen consumption ($\dot{V}O_{2STANDARD}$) increase exponentially, reducing absolute aerobic scope (AAS, $AAS = \dot{V}O_{2MAX} - \dot{V}O_{2STANDARD}$; Fig. 1.1 C - D). A narrowed aerobic scope is hypothesised to translate into a reduced capacity for activities including growth, movement, digestion and reproduction (Pörtner, 2002), and the thermal effects on individuals may scale up to affect population and community dynamics (Pörtner and Peck, 2010). Reduced aerobic capacity at high temperatures is apparent in many

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tropical ectotherms (Munday et al., 2009; Nilsson et al., 2009; Johansen and Jones, 2011; Rummer et al., 2014); however the OCLTT hypothesis is not universally applicable (Clark et al., 2013; Ern et al., 2014; Norin et al., 2014; Ern et al., 2015; Ern et al., 2016). Moreover, some studies supporting the OCLTT hypothesis (i.e. Munday et al., 2009; Nilsson et al., 2009) did not meet the minimum requirements for aquatic respirometry (Clark et al., 2013), and these findings may reflect methodological artefacts. Additional factors identified to play a role in a loss of performance at thermal extremes include: disrupted nervous function (Ern et al., 2015), oxidative damage (Lushchak and Bagnyukova, 2006), enzyme inactivation (Sharpe and DeMichele, 1977; Schoolfield et al., 1981) and protein denaturation (Hofmann and Somero, 1995; Tomanek, 2015).

The deleterious effects of climate change on ectotherms may, however, be counteracted by compensatory responses (Seebacher et al., 2015). Thermal stress can be buffered by both behavioural and physiological strategies. Pockets of thermally favourable habitat can be sought out or shuttled between to maintain body temperature within a preferred range (Seebacher, 2005; Seebacher and Franklin, 2005). Alternatively, long-term changes in thermal regimes (e.g. seasonal shifts in temperatures) can induce physiological changes, where the thermal effects on biochemical processes are blunted (Johnston and Dunn, 1987). The capacity to track thermal perturbations and responsively alter the sensitivity of underlying physiology allows ectotherms to maintain or optimise whole-animal function (Prosser, 1991) and is termed acclimatisation when observed in the field, or alternatively termed acclimation, when observed under experimentally controlled conditions (Wilson and Franklin, 2002). The magnitude of phenotypic plastic responses vary from complete compensation (i.e. a full shift in T_{opt} to match immediate conditions), partial compensation (i.e. improved performance in immediate conditions, but not optimal) or non-existent (Fig. 1.1 B). Thermal acclimation responses operate at every level of organismal organisation, from the up- or down-regulation of particular genes (Dietz and Somero, 1992 ; Podrabsky and Somero, 2004) to the alteration of enzyme reaction rates (Somero, 1978) and cell membrane thickness (Hazel, 1995 ; Hulbert and Else, 1999) to changes in muscle contractility and cardiovascular control (Hicks and Farrell, 2000). These alterations often manifest into a functional cascade, with improved or maintained whole-animal performance resulting (Glanville and Seebacher, 2006).

Increases in global temperatures and thermal variability are theorised to create or strengthen selection pressure favouring plastic phenotypes, particularly in long-lived species (e.g. crocodylians, sturgeon, marine turtles and iguanas; Kawecki, 2000). Genetic adaptation is also recognised as a crucial mechanism in predicting extinction risk; however, successful genetic adaptation is dependent on generation time being shorter than the rate of climate warming and on changes in environmental temperature being predictable to facilitate directional selection (Kawecki, 2000). The persistence of long-lived ectotherms is therefore likely to be determined by changes occurring within a single lifetime (i.e. thermal acclimatisation capacity).
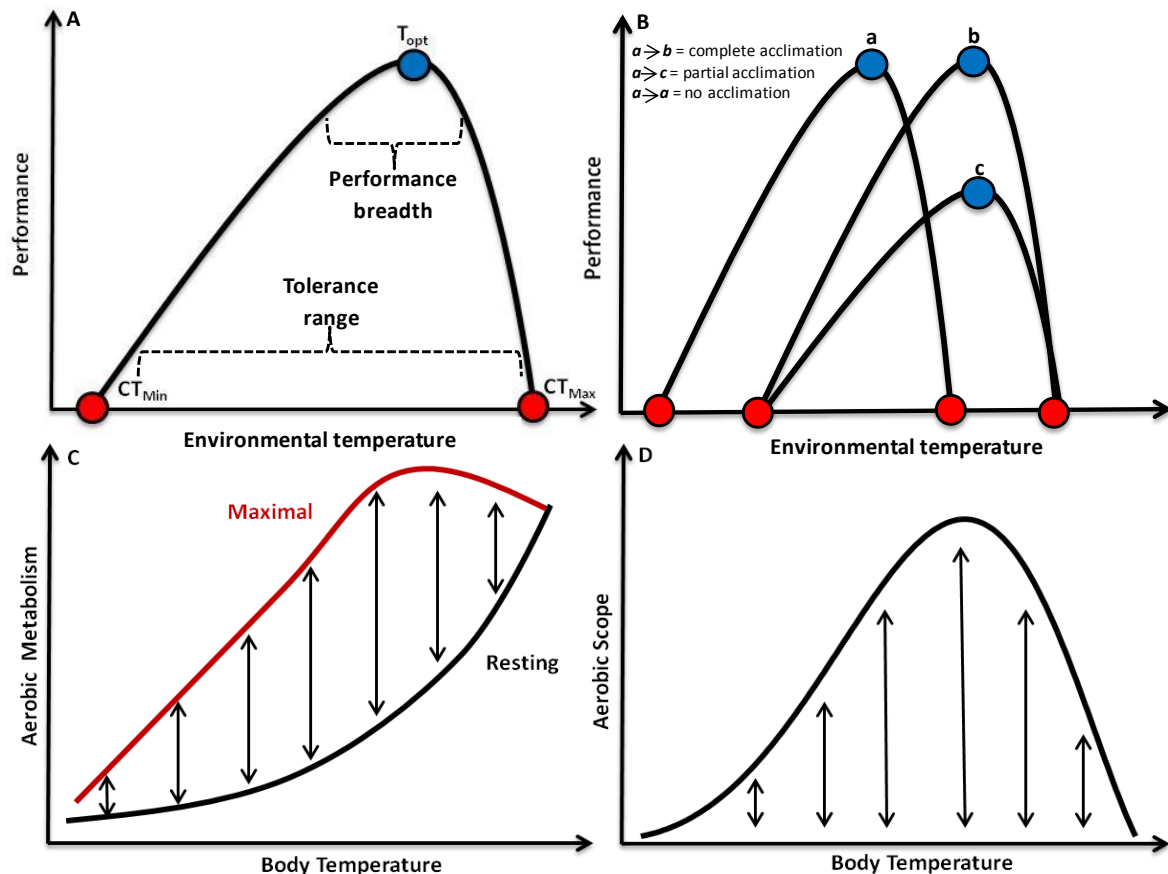
Figure 1.1: (**A**) Thermal performance curve for a hypothetical ectotherm. Performance is highest at T_{opt} (blue circle) and declines at temperatures either side of the performance breadth. Critical thermal minima (CT_{Min}) and maxima (CT_{Max}) endpoints are marked by red circles. The physiological thermal tolerance range/window represents temperatures within these endpoints (i.e. CT_{Max} - CT_{Min}). (**B**) Theoretical shifts in an initial thermal performance curve following chronic exposure to a higher temperature. Acclimatisation and acclimation responses can be complete (i.e. shift from a ⟶ b), partial (i.e. shift from a ⟶ c) or absent where the performance curve does not shift (i.e. a ⟶ a). (**C**) Hypothetical resting (black line) and maximal (red line) rates of oxygen consumption as a function of ectotherm body temperature. Resting rates of oxygen consumption typically increase exponentially with rising body temperature. In contrast, maximal rates of oxygen consumption generally decline or reach a plateau at very high temperatures. Arrows represent the magnitude of absolute aerobic scope (i.e. maximal oxygen consumption – resting oxygen consumption). (**D**) Hypothetical thermal sensitivity of ectotherm aerobic scope arising from disparities in the thermal sensitivity of resting and maximal rates of oxygen consumption. Aerobic scope is typically reduced at very low and high body temperatures. Adapted from Pörtner (2010).

Diving Vertebrates

The evolution of air-breathing organs in vertebrates made terrestrial environs habitable, but some species returned to aquatic environment, leading to the reinvasion of air-breathing species (Butler and Jones, 1982; Kooyman, 1989). Aquatic air-breathers represent a highly diverse group and include: mammals (e.g. sea lions, dolphins, seals, dugongs, otters and whales), birds (e.g. cormorants, penguins, grebes and waterfowl), amphibians (e.g. salamanders) and reptiles (e.g. crocodylians, marine turtles, freshwater turtles and iguanas). These species are primarily aquatic but optimising time underwater is constrained by the need to surface and replenish oxygen stores.

The aerobic dive limit (ADL) is a conceptual representation of the maximum submergence time an air-breathing diver can sustain prior to the use of anaerobic metabolism and associated lactate accumulation (Butler, 2006). A diver's ADL can be calculated (1) by dividing its total body oxygen stores by the rate at which oxygen is consumed:

$$(1) \text{ADL} = \frac{\text{TBO}}{\dot{V}O_{2DIVE}}$$

where ADL represents the aerobic dive limit (i.e. time submerged), TBO represents total body oxygen stores (ml) and $\dot{V}O_{2DIVE}$ represents oxygen consumption during submergence (i.e. diving metabolic rate; ml min^{-1}). Oxygen can be stored in the lungs, blood and tissue, and the percent contribution of stores varies between species (Kooyman et al., 1989).

Vertebrates enter a unique physiological state when diving, with a suite of cardiovascular alterations often occurring (Andersen, 1966). This state is termed the 'dive response' and includes a marked decrease in heart rate (i.e. diving bradycardia), a redistribution of blood stores to essential organs (i.e. peripheral vasoconstriction) and a cardiac-shunt (in some species) (Blix and Folkow, 1983; Butler and Jones, 1997). These alterations facilitate prolonged submergences by lowering oxygen demands and animals enter a hypometabolic state where oxygen demands are lower than surface levels (Davis et al., 2004; Hastie et al., 2007). The pulmonary by-pass (PBS) shunt occurs in crocodylians, for example, when oxygen poor blood is recirculated to the systemic

circuit instead of the lungs (Axelsson et al., 1997). This redirection of blood flow has been hypothesised to be extend aerobic dive limits by conserving lung oxygen stores, sequestering carbon dioxide away from the lungs and inducing hypometabolism (White, 1969; Hicks and Wang, 2004). However, several studies have rejected this hypothesis, with the view that an adaptive function of the PBS may not exist (Hicks, 2002; Eme et al., 2009; Hicks and Wang, 2012). The initiation of the 'dive response' appears to be context specific in some species (Gaunt and Gans, 1969; Noren et al., 2012); with crocodylians, for example, markedly reducing heart rate (65 ± 6% reduction) during predator avoidance dives (i.e. 'fright-dives') and only small cardiovascular changes (14 ± 6% reduction) accompanying voluntary, undisturbed dives (Wright et al., 1992).

Diving in a Warming World: Constraints and Underlying Mechanisms

Climate change presents a unique threat to ectothermic, air-breathing, vertebrate divers (e.g. turtles, sea snakes, iguanas, salamanders and crocodylians). Time directed towards dive-dependent activities (e.g. foraging, predator avoidance, sleep, rest and social interactions) may be cut short at elevated water temperatures, with animals required to surface and replenish oxygen stores more frequently. The thermal sensitivity of ectotherm diving performance is hypothesised to be linked to a reduction in the ADL as a result of diving metabolic rate (i.e. oxygen consumption) increasing exponentially with rising water/body temperature (Fig. 1.2; Hayward et al., 2016). The dive capacity of aerially respiring ectotherms may be compromised at elevated water temperatures in several forms: i) dive durations may be shortened due faster depletion of TBO stores, ii) dive durations may remain unchanged, but animals increasingly rely on anaerobic metabolism, incurring the cost of longer post-dive surface intervals (PDSI) to clear anaerobic debt, or iii) dive durations remain unchanged due to thermal acclimatisation.

Reduced dive capacity at elevated water temperatures has been experimentally demonstrated in numerous ectothermic vertebrates, including : sea snakes (spine-bellied sea snake, *Hydrophis curtus*, elegant sea snake, *Hydrophis Elegans* and the Arafura file snake, *Acrochordus arafurae*), the alpine newt (*Triturus alpestris*) and freshwater turtles (Fitzroy River turtle, *Rheodytes leukops;* Mary River turtle, *Elusor macrurus*; white-throated snapping turtle, *Elseya albagula*) (Priest and Franklin, 2002;

Clark et al., 2008; Storey et al., 2008; Samajova and Gvozdik, 2009; Pratt and Franklin, 2010; Udyawer et al., 2016). Dive durations in the Arafura file snake, for example, are reduced by 63% in response to acute water temperature increases from 20°C to 32°C (i.e. from 77 min to 28 min; Pratt and Franklin, 2010). Likewise, submergence times of freshwater crocodiles, freshwater turtles and marine turtles are reduced in summer months compared to winter months (Carr et al., 1980; Gordos et al., 2003; Hochscheid et al., 2005; Bradshaw et al., 2007; Hochscheid et al., 2007; Campbell et al., 2010a). However, these findings are limited when trying to gauge the effect of climate change on dive capacity. Experimental temperatures exceeding present-day summer thresholds are yet to be assessed, as well as, the capacity for compensatory responses (e.g. thermal acclimation/acclimatisation) to act as safeguards (c.f. Clark et al., 2008).

The physiological basis for loss of performance at thermal extremes remains unclear in many taxa but the threat of climate change has reinvigorated recent efforts (Clark et al., 2013). Ectotherm TBO stores decline with increasing temperature (Pough, 1976; Fuster et al., 1997); ectotherms diving at elevated temperatures have less oxygen reserves to begin with compared to ectotherms diving a lower temperatures. However dive durations are reduced at elevated temperatures beyond the extent expected from reduced TBO stores alone (Hayward et al., 2016). The thermal sensitivity of ectotherm diving performance is hypothesised to stem from diving metabolism increasing in line with body temperature, leading to a faster depletion of TBO stores (Hayward et al., 2016). The validity of this hypothesis was recently assessed in sea snakes, finding diving oxygen consumption increased with rising water temperature, at the cost of decreased dive durations and increased surfacing frequency (Udyawer et al., 2016). The mechanisms underlying the thermal sensitivity of dive capacity likely differ from the OCLTT hypothesis, which does not consider hypometabolic states and may be further complicated by thermal limits placed on the 'dive response'. Metabolic depression during submergence may be curtailed at upper thermal limits, or diving metabolism may increase at a faster rate than resting/standard metabolic rates. Upper thermal limits may see dives usually accompanied by metabolic depression (i.e. predator avoidance submergences) experiencing little to no metabolic adjustments. The thermal

sensitivity of diving metabolic depression remains untested but requires placement within existing theoretical frameworks.
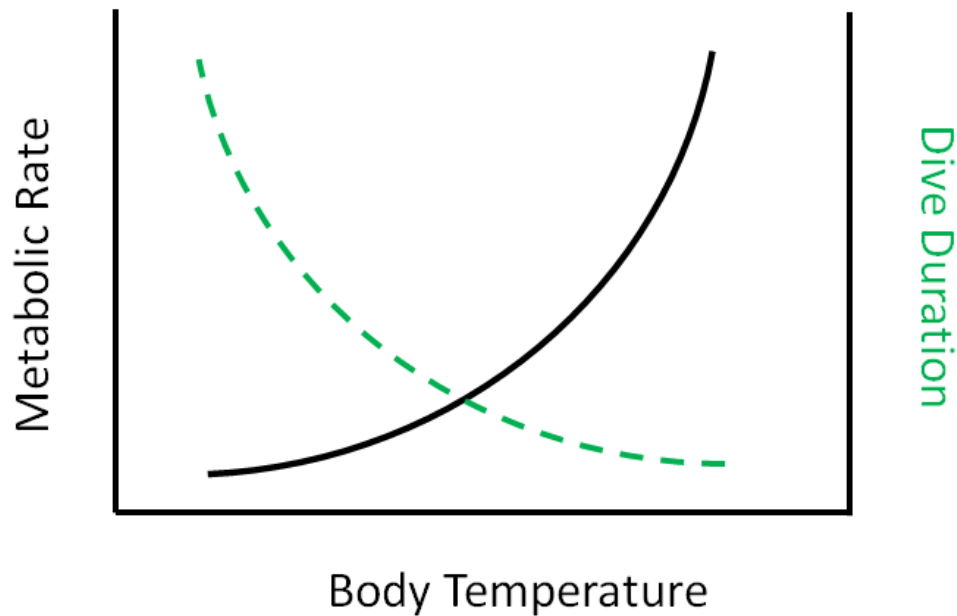
Figure 1.2: Schematic representing the relationships between ectotherm body temperature, metabolic rate (solid black line) and dive duration (dotted green line). Metabolic rate increases exponentially with rising temperature whilst dive duration decreases. The thermal sensitivity of dive duration is hypothesised to stem from faster depletion of oxygen stores at high temperatures due to increased metabolism.

Diving in Crocodylians

The water is a place of safety and refuge for crocodylians, and their survival and reproductive success are thought to be inherently linked to dive capacity. As primarily aquatic organisms, submergence behaviour facilitates a range of critical activities including: predator avoidance, foraging, sleep and recovery (Seebacher et al., 2005b; Campbell et al., 2010b). Predator escape dives are vital in hatchlings and juveniles where they may fall prey to a range of species including: monitor lizards (*Varanus mertensi, V. panoptes*), barramundi (*Lates calcarifer*), whistling kites (*Haliastur sphenurus*), olive pythons (*Liasis olivasceus*), great egrets (*Ardea alba*) and larger crocodiles (Grigg and Kirshner, 2015; Rootes and Charbeck, 1993). Foraging dives are likely important for crocodylians of all life stages as stomach content analyses show predominantly aquatic prey (e.g. fishes, crabs, crayfish and aquatic insects) are

consumed across all size classes (Taylor, 1979; Elsey et al., 1992; Wallace and Leslie, 2008). Additionally, long (i.e. 20 - 30 min), inactive dives are thought to be associated with periods of rest and avoidance of rapid surface currents (Campbell et al., 2010c).

Study Species: Estuarine Crocodile (*Crocodylus porosus*)

The estuarine crocodile (*Crocodylus porosus*, Schneider, 1801) is an air-breathing, diving ectotherm and was used as the model species for this project. This tropical species inhabits estuaries, inland lakes, swamps and coastal waters and established populations span northern Australia, East Timor, Papua New Guinea, Solomon Islands, Indonesia, Brunei, Malaysia, Singapore, Sri Lanka and Bangladesh (Webb et al., 2010). The diving repertoire of free-ranging *C. porosus* remains largely uncharacterised, despite it being the largest (total length ~ 6 m), extant, semi-aquatic, apex predator (Grigg and Kirshner, 2015). Oxygen exchange in *C. porosus* is entirely aerial and underwater excursions must be continuously interrupted to replenish oxygen stores (Wright, 1986). A prior attempt to remotely monitor diving behaviour of *C. porosus* yielded data for only a single animal due to tag dysfunctions (Grigg et al., 1985). This individual performed frequent, short dives (3 - 5 min) and two prolonged dives (22 min and 30 min). However, whether these data are representative of typical diving behaviour is unclear.

A wealth of physiological diving data exist for *C. porosus* and stems from experimental assessments. Crocodylians are intermittent breathers, making them well-suited to diving (Munns et al., 1998). Very short, routine dives fall within regular ventilatory periods but many submergences exceed these times and require breath holding (Wright, 1987). The physiological support for crocodylian diving greatly differs from mammalian divers. Crocodylians and other ectotherms have very low metabolic rates (~80% less than birds and mammals) allowing slow use of TBO stores (Grigg and Kirshner, 2015). Pulmonary (i.e. lungs) oxygen represents the majority of TBO stores (~67.0%), followed by blood oxygen (~28.9 %), and tissue oxygen contributes the least (~4.1%) due to low myoglobin stores (Wright, 1985). Crocodylians experience a pulmonary by-pass shunt during predator avoidance dives, which is hypothesised to induce metabolic depression and facilitate prolonged dives (reviewed in Grigg and Kirshner, 2015; c.f. Farmer et al., 2008; Eme et al., 2009; Hicks and Wang, 2012). Experimental assessments of diving in juvenile *C. porosus* showed voluntary,

undisturbed dives to last < 5 min and be accompanied by little to no bradycardia (Wright, 1987; Wright et al., 1992). In contrast, dives in response to an experimental threat (i.e. 'fright-dives') were accompanied by severe bradycardia (65 ± 6% of surface heart rate) and dives were extended to 15 - 20 min (Wright et al., 1992).

The estuarine crocodile is long-lived and late to mature (estimated lifespan > 70 y; Grigg and Kirshner, 2015) and persistence in a rapidly warming world is likely dependent on thermal compensation occurring within a single lifetime. Previous assessments have identified *C. porosus* to be physiologically plastic, with the capacity to fully compensate swimming performance, muscle power output and lower level physiological function (i.e. mitochondrial oxygen consumption, membrane fatty acid composition and regulatory enzyme activity) in response to cool temperatures approaching the lower end of its thermal tolerance window (Glanville and Seebacher, 2006; Seebacher and James, 2007). It is presently unclear whether estuarine crocodiles can undergo adequate physiological acclimation at elevated temperatures and potentially offset thermal effects on diving performance.

Aims and Structure of Thesis

This research endeavoured to deepen our understanding of the thermal constraints acting on the dive capacity of aerially respiring, ectothermic vertebrates, specifically at elevated water temperatures akin to forecasted climate change. The estuarine crocodile was used as a model species and research was conducted on both juveniles (in the laboratory) and adults (in the field). This project was set in motion by first conducting an assessment of the thermal sensitivity and plasticity of diving performance (Chapter 2). These baseline data provided insight into the vulnerability of juvenile *C. porosus* to projected climate change and the potential for thermal acclimation to act as a safeguard.

From this point, the research aimed to elucidate the physiological mechanisms constraining dive capacity at elevated temperatures (Chapter 3). Diving oxygen consumption, diving heart rate, post-dive plasma-lactate concentrations and blood oxygen-carrying capacity were assessed at test temperatures reflecting climate change scenarios. These measurements were used to test the hypothesis that compromised

diving performance is linked to increased oxygen demands and a reduced capacity for metabolic depression at elevated temperatures.

The third aim was to compare the thermal sensitivity and plasticity among resting, maximum and diving metabolic rates (Chapter 4). This allowed diving metabolism to be placed in existing theoretical frameworks (e.g. OCLTT) and extend our understanding of the interplay between temperature and hypometabolic states.

The final research chapter took to the field and examined the correlations between present-day thermal fluctuations (i.e. seasonal) and the diving behaviour of free-ranging, adult *C. porosus* (Chapter 5). This allowed for a concurrent characterisation of the diving repertoire of *C. porosus* and a gauge for whether experimental findings from previous chapters translate into an ecological setting in large, mature crocodiles. The culmination of this research combined key findings from experimental chapters to discuss the potential impacts of climate change on the diving ecology of *C. porosus* and the applicability of these findings to air-breathing, diving ectotherms as a group (Chapter 6).

This thesis comprises of four experimental chapters (Chapters 2 - 5), which are written as independent manuscripts containing the following sections: abstract, introduction, materials and methods, results and discussion. Chapter 2 is published in *Conservation Physiology* (Rodgers et al., 2015). All experiments complied with The University of Queensland animal ethics requirements (permit no. SBS/018/14/ARC/AUST ZOO) and animals were obtained under a Scientific Purposes Permit WISP1424321.

CHAPTER 2
THE THERMAL SENSITIVITY AND PLASTICITY OF DIVING PERFORMANCE

Abstract

Air-breathing, diving ectotherms are a crucial component of the biodiversity and functioning of aquatic ecosystems, but these organisms may be particularly vulnerable to the effects of climate warming on submergence times. Ectothermic dive capacity is thermally sensitive, with dive durations significantly reduced by acute increases in water temperature; it is unclear whether diving performance can acclimate/acclimatise in response to long-term exposure to elevated water temperatures. We assessed the thermal sensitivity and plasticity of 'fright-dive' capacity in juvenile estuarine crocodiles (*Crocodylus porosus*; N = 11). Crocodiles were exposed to one of three long-term thermal treatments, designed to emulate water temperatures under differing climate change scenarios (i.e. current summer, 28°C; 'moderate' climate warming, 31.5°C; 'high' climate warming, 35°C). Dive trials were conducted in a temperature-controlled tank across a range of water temperatures. Dive durations were independent of thermal acclimation treatment, indicating a lack of thermal acclimation response. Acute increases in water temperature resulted in significantly shorter dive durations, with mean submergence times effectively halving with every 3.5°C increase in water temperature (Q_{10} = 0.17, p < 0.001). Maximal diving performances, however, were thermally insensitive across the temperature range of 28 – 35°C. These results suggest that *C. porosus* have a limited or non-existent capacity to thermally acclimate sustained 'fright-dive' performance. If the findings here are applicable to other air-breathing, diving ectotherms, the functional capacity of these organisms will likely be compromised under climate warming.

Introduction

Ectotherms face an ever-increasing risk of losing functional performance as ongoing climate change drives environmental temperatures beyond physiological limits (Pörtner and Farrell, 2008). The functional capacity of all organisms is optimised within a limited range of body temperatures (i.e. envelope) as a result of molecular, cellular and metabolic processes being thermally sensitive (Hochachka and Somero, 1968; Hochachka, 1967). Shifts in body temperature outside this range are intrinsically linked to decrements in fitness-related traits, such as growth (Howe and Marshall, 2002), fecundity (Berger et al., 2008), immune competence (Yu et al., 2009), locomotor capacity (Wilson and Franklin, 2000) and survival (Berrigan, 2000).

The threat of overheating is particularly salient for ectothermic species (almost all fish, amphibians and reptiles), where body temperature is strongly tied to the thermal environment. Ectotherms exposed to stressfully high temperatures are hypothesised to experience compromised metabolic functioning, where maximal rates of oxygen consumption decrease but resting metabolic rates increase exponentially with increasing temperature, creating an imbalance between oxygen supply and demand (oxygen- and capacity-limited thermal tolerance hypothesis, OCLTT; Pörtner, 2002). This mismatch in oxygen supply and demand is thought to constrain aerobic metabolism, depriving tissues of oxygen and subsequently causing a collapse in whole-organism functioning and/or mortality (Pörtner, 2001, 2002, 2010), however support for this hypothesis is mixed (Ern et al., 2014; Norin et al., 2014).Tropical ectotherms living close to their upper thermal limits are therefore predicted to be vulnerable to even very slight rises in temperature (Sunday et al., 2014).

The deleterious effects of climate change on ectotherms may, however, be counteracted by compensatory responses. Thermal stress can be buffered by both behavioural and physiological strategies. Pockets of thermally favourable habitat can be sought out or shuttled between to maintain body temperature within a preferred thermal range (Seebacher, 2005; Seebacher and Franklin, 2005). Alternatively, long-term changes in thermal regimes (e.g. seasonal shifts in temperatures) can induce physiological changes, where the thermal effects on biochemical processes are blunted (Johnston and Dunn, 1987). The capacity to track thermal perturbations and responsively alter the sensitivity of underlying physiology allows ectotherms to

maintain or optimise whole-animal function (Prosser, 1991) and is termed acclimatisation when observed in the field in natural conditions, or alternatively termed acclimation, when observed in experimentally controlled conditions (Wilson and Franklin, 2002). Thermal acclimation responses operate at every level of organismal organization, from the up- or downregulation of particular genes (Dietz and Somero, 1992; Podrabsky and Somero, 2004) to the alteration of enzyme reaction rates (Somero, 1978) and cell membrane thickness (Hazel, 1995; Hulbert and Else, 1999) to changes in muscle contractility and cardiovascular control (Hicks and Farrell, 2000). These alterations often manifest into a functional cascade, with improved or maintained whole-animal performance resulting (Glanville and Seebacher, 2006).

Plasticity in functional traits is set to play a pivotal role in buffering the deleterious effects of anthropogenic climate warming on ectotherms (Chevin et al., 2010; Somero, 2010; Huey et al., 2012; Seebacher et al., 2015), but its role is often overlooked when making inferences relating to how species will fare. Increases in global temperatures and thermal variability are theorised to create or strengthen selection pressure favouring plastic phenotypes, particularly in long-lived organisms (Kawecki, 2000). Genetic adaptation is also recognised as a crucial mechanism in predicting extinction risk; however, successful genetic adaptation is dependent on generation time being shorter than the rate of climate warming and on changes in environmental temperature being predictable to facilitate directional selection (Kawecki, 2000). The persistence of long-lived ectothermic species (e.g. crocodylians, marine turtles and marine iguanas), is therefore likely determined by changes occurring within a single lifetime (i.e. thermal acclimatisation capacity).

Ectothermic, air-breathing divers (e.g. marine and freshwater turtles, iguanas, sea snakes and crocodylians) provide a good model for examining the potential buffering role of physiological acclimation, because dive capacity is optimised within a limited thermal window (Hayward et al., 2016). The maximum duration an animal can remain submerged before oxygen debt is incurred is defined as the aerobic dive limit (ADL; Butler, 2006). The ADL of ectotherms is inversely related to water temperature (Herbert and Jackson, 1985; Fuster et al., 1997, Prassack et al., 2001; Priest and Franklin, 2002). As the body temperature of a diving ectotherm equilibrates with water temperature, oxygen depletion rates become directly related to water temperature

(temperature quotient, Q_{10} effect; Pough, 1976; Jackson, 2007), with oxygen stores being consumed more rapidly at 'warm' compared with 'cool' temperatures.

Elevated temperatures in marine and freshwater habitats, set to come with climate change, pose a threat to ectothermic divers, with the duration of time performing obligate underwater activities potentially being greatly reduced (Kooyman et al., 1980; Kramer, 1988; Costa et al., 2004). The dive capacity of aerially respiring ectotherms may be compromised at elevated water temperatures in the following ways: (i) dives may be terminated sooner to maintain submergences within aerobic limits; or (ii) dive durations may remain unchanged, but animals increasingly rely on anaerobic metabolism, incurring the cost of longer post-dive surface intervals (PDSIs) to clear anaerobic debt (Kooyman et al., 1980; Costa et al., 2004). Both modifications to diving behaviour result in a reduction of total time available for underwater activities throughout a bout of continuous diving.

Although acute (i.e. short-term) increases in water temperature markedly reduce diving performance in a number of ectothermic vertebrates (Priest and Franklin, 2002; Gordos et al., 2003; Campbell et al., 2010a; Pratt and Franklin, 2010), these findings are limited when estimating an organism's vulnerability to climate warming, as the capacity for thermal acclimation to act as a safeguard is rarely assessed (cf. Clark et al., 2008; Bruton et al., 2012). To remedy this, we assessed the capacity for thermal acclimation to mitigate the impact of elevated water temperatures on the diving performance of a long-lived, aerially respiring (Wright, 1986) ectotherm, the estuarine crocodile (*Crocodylus porosus*, Schneider, 1801). Crocodylians are primarily aquatic organisms, spending up to 11 h a day submerged (Campbell et al., 2010a). Dive capacity is thought to be linked to the survival and reproductive success of crocodylians because predator avoidance, foraging, sleep/recovery and social interactions all occur underwater (Seebacher et al., 2005b; Campbell et al., 2010b).

The estuarine crocodile is a physiologically plastic organism, with the capacity to 'perfectly' compensate swimming performance, muscle power output and lower level physiological function (i.e. mitochondrial oxygen consumption, membrane fatty acid composition and regulatory enzyme activity) in response to cool temperatures approaching the lower end of its thermal tolerance window (Glanville and Seebacher,

2006; Seebacher and James, 2007). It is presently unclear whether the estuarine crocodile can undergo adequate physiological acclimation at the upper, 'hot' spectrum of its thermal tolerance window, but we predicted that the capacity to acclimate to low temperatures would translate to high temperatures. Specifically, we hypothesised diving performance of *C. porosus* to be thermally sensitive, exemplified by an inverse relationship between dive duration and water temperature, and/or a positive correlation between PDSI and water temperature (H_1). Further to this, we predicted *C. porosus* to demonstrate complete thermal acclimation in dive capacity following long-term exposure to elevated water temperatures, so that maximal diving performance shifts in parallel with treatment temperature (H_2). The assessment of thermal acclimation capacity was subsequently used to gauge how future climate warming may impact the dive capacity of estuarine crocodiles.

Materials and Methods

Animal maintenance

Estuarine crocodile (*Crocodylus porosus*, $N = 11$) eggs were obtained from a single clutch at David Fleay Wildlife Park (Burleigh Heads, Queensland, Australia). Eggs were transported to The University of Queensland (St Lucia, Queensland, Australia), where they were incubated in an *R*-com 50 egg incubator (Auto Elex Co. Ltd, GimHae, Korea) for 88 days at $32 \pm 1°C$ and 85 – 90% humidity. Upon hatching [hatchling body mass (BM) = 81.7 ± 7.9 g mean $\pm$ s.d.], animals were maintained in an environment aimed at optimising healthy growth for 8 months prior to experimentation (water temperature = 29°C; photoperiod = 12 h:12 h light:dark). Crocodiles were fed regularly (three times per week, totalling 15% of their body mass) a mixture of minced beef and chicken supplemented with powdered calcium and vitamin D (Vetafarm, Wagga Wagga, NSW, Australia), pilchards (*Clupeidae*) and freshwater prawns (*Macrobrachium australiense*). Enclosures were cleaned, with complete water changes after feeding. All experiments complied with The University of Queensland animal ethics requirements (permit no. SBS/018/14/ARC/AUST ZOO).

Experimental design and thermal acclimation treatments

Crocodiles were randomly assigned to one of three thermal acclimation treatments (N = 3 or 4 per treatment; 11 individuals in total). Thermal acclimation treatments were identical apart from water temperature. Animals were acclimated to one of three water temperatures, based on the following Intergovernmental Panel on Climate Change (IPCC, 2007) climate change scenarios for coastal regions in Northern Australia:

(i) Low rate of global warming (SRES B1 storyline; 10th percentile of IPPC global warming range)/current summer water temperature. This scenario is based on a low-emissions future, with a global shift towards clean and sustainable resource use. Summer water temperatures do not increase under this scenario and are representative of temperatures currently experienced by *C. porosus.* Experimental water temperature emulating this scenario was 28 ± 0.5°C.

(ii) Moderate rate of global warming (SRES A1B storyline; 50th percentile of IPCC global warming range). This scenario is based on a moderate rate of warming, resulting from a rapidly expanding economy and growing population. Summer water temperatures are predicted to average 32°C. Experimental water temperature emulating this scenario was 31.5 ± 0.5°C.

(iii) High rate of global warming (SRES A1F1 storyline; 90th percentile of IPCC global warming range). This scenario is centred on the intensive and continued use of fossil fuels, with unprecedented levels of carbon emissions, population growth and industrial expansion. Summer water temperatures are predicted to average 33°C, reaching highs of 35°C. Experimental water temperature emulating this scenario was 35 ± 0.5°C.

Thermal acclimation enclosures were large wooden tanks (3.35 m × 0.85 m × 0.75 m; length × width × height), designed to emulate thermally heterogeneous environments conducive to thermoregulatory behaviour. The tanks contained freshwater filled to a depth of 0.15 m (sufficient for full submersion of animals). Water temperature was maintained using 200 W submersible heaters (AquaOne; Ingleberg) attached to thermostats (HabiStat; Living Earth Electronics). Dry platforms were situated at each end of the tank, one being a relatively 'warm' platform situated underneath a ceramic heat lamp (250 W; OzWhite, Enfield, South Australia; suspended 26 cm above the platform) and a UV-B light (10%, 18 W; Sylvania ReptiStar) and the

other a relatively 'cool' platform with no lamps. Basking opportunity (i.e. time heat lamp was switched on) was 8 h day^{-1} (08.00 – 16.00 h) for all treatments, with substrate temperature underneath the heat lamp averaging 29 ± 7°C (mean ± s.d.). A summer photoperiod was used, with a constant 14 h:10 h light:dark regimen (05.00–19.00 h) for all treatments. Animals were left to acclimate to thermal treatments for 32.7 ±1.5 d (mean ± s.d.) prior to performance testing, a sufficient period of time previously shown to induce cold-acclimation responses in juvenile *C. porosus* (Glanville and Seebacher, 2006). To maximise sample size, three rounds of acclimation experiments took place, during which all crocodiles were run through each acclimation treatment [acclimation period 1, 28 May 2014 to 29 June 2014 (total length (TL), mean ± s.d. 52.9 ± 5.7 cm; BM, mean ± s.d. 479.2 ± 137.8 g); acclimation period 2, 4 September 2014 to 6 October 2014 (TL, mean ± s.d. 52.3 ± 9.9 cm; BM, mean ± s.d. 542.24 ± 210.3 g) and acclimation period 3, 9 December 2014 to 7 January 2015 (TL, mean ± s.d. 59.33 ± 9.6 cm; BM, mean ± s.d. 710.7 ± 267.5 g)]. Experimental round number was included in all statistical analyses.

'Fright-dive' trials

'Fright-dive' trials were held in a large experimental tank (1.8 m × 2.0 m × 1.9 m, length × width × height; volume = 6840 L) constructed from foam fibreglass. The dive tank was evenly partitioned into three sections with opaque plastic partitions to enable three dive trials to run concurrently. The dive tank contained filtered freshwater to a depth of 1.3 m. Water temperature was finely controlled using a spa heater (900 EVO; Elecro Engineering, Stevenage, UK). Thermal profiling of the dive tank using thermocron temperature loggers (iButtonLink Technology, Whitewater, WI, USA) confirmed uniformity of temperature throughout the water column. Each partitioned section of the dive tank contained a floating rest platform (0.6 m × 0.15 m × 0.05 m; length × width × height), where crocodiles could rest and breathe on the water surface whilst their body remained submerged. Dive trials were recorded with a video camera (JVC Everio Inc., Mississauga, CA) situated in front of the dive tank. Depth markings were made at 0.2 m intervals on the front of the dive tank. The dive tank was sectioned off in a quiet area, free from visual and auditory disturbances.

Diving performance was assessed in animals from all three thermal acclimation treatments (i.e. 'low', 'moderate' and 'high' climate warming scenarios) at three test temperatures (water temperatures = 28, 31.5 and 35°C). The order of test temperature and crocodile position in the dive tank (i.e. partition assignment) were randomised. Animals were allowed to habituate to tank conditions for 1 h before a dive trial commenced. After the habituation period, a 'fright-dive' trial commenced, in which crocodiles were continuously 'threatened' by a loud auditory disturbance, created by the experimenter banging a plastic lid over the back end of each tank partition once each minute for an hour. Dive data were extracted from the video recordings, and each dive event was characterised by depth (m), duration (min) and PDSI (i.e. time spent at the surface between dives; min). The following three measures of diving performance were extracted from the data: maximal diving performance (i.e. longest submergence); mean diving performance (average dive duration); and sustained diving performance (cumulated time spent underwater throughout the 1 h bout of continuous 'fright-diving'). A dive event was defined as an animal being entirely submerged at a depth ≥ 0.3 m for longer than 30 s.

Statistical analyses

Analyses were performed using the statistical programming package R (version 3.1.2; R Core Development Team, 2010). The effects of acute and long-term thermal treatments on maximal, mean and sustained diving performance were analysed using linear mixed-effects (lme) models (Pinheiro et al., 2012). Indicator variables were incorporated for each level of acclimation temperature and test temperature. Body mass, dive depth, dive frequency and partition number were included as fixed factors, and experimental round and crocodile identification number were included as random-effect factors. Assumptions of homoscedasticity and normality of errors were graphically assessed, and dive duration was log10-transformed to comply with model assumptions. A Wald *post hoc* test was used to discern statistical differences among thermal treatments, where necessary. The minimal adequate model was determined using maximum likelihood simplification. Statistical significance was accepted at $p \leq 0.05$. Results are presented as mean ± s.e.

Results

Thermal sensitivity of diving performance

Sustained diving performance (i.e. total time spent submerged during the 60 min continual threat treatment) was thermally sensitive, with performance inversely related to water temperature (Fig. 2.1 A). Crocodiles diving at 28°C spent an average of 36.8 ± 2.9 min underwater, whereas animals diving at 31.5 and 35°C spent an average of 23.5 ± 3.9 and 21.3 ± 2.7 min submerged, respectively (Q_{10} = 0.46, $F_{2,26}$ = 6.84, $p < 0.01$, lme). Wald *post hoc* analyses showed that sustained diving performance was significantly greater at 28°C compared with other water temperatures, but no differences were observed between 31.5 and 35°C. Likewise, increasing water temperature from 28 to 35°C resulted in a ~3.5-fold reduction in mean 'fright-dive' duration from 8.0 ± 0.8 to 2.3 ± 0.9 min (Fig. 2.1 B; Q_{10} = 0.17, *d.f.* = 10, $p < 0.0001$, $F_{1,51}$ = 50.4, lme). Wald *post hoc* analyses showed that the mean 'fright-dive' duration was significantly different between all three test temperatures, with an approximate twofold reduction in dive duration for every 3.5°C increase in water temperature. In contrast, maximal diving performance showed a plateau of thermal independence across the temperature range assessed here (i.e. 28–35°C), with all dives lasting 8.8 ± 0.4 min (Fig. 2.2 A; $p = 0.16$, $F_{2,44}$ = 1.9, lme).

Post-dive surface intervals were independent of water temperature ($p = 0.50$, $F_{2,199}$ = 0.69, lme) and dive duration ($p = 0.52$, $F_{1,199}$ = 0.42, lme), with crocodiles surfacing for an average of 3.2 ± 0.3 min between submergences at all water temperatures (Fig. 2.1 C). Dive frequency was independent of water temperature, with animals performing 5.2 ± 1, 6.7 ± 1.1 and 6.2 ± 1.3 dives h^{-1} at 28, 31.5 and 35°C, respectively ($p = 0.29$, $F_{2,48}$ = 1.27, lme, Fig. 2.1 D). Dive depth did not vary, with 99.98% of all recorded dives settling at the bottom of the dive tank (1.3 m).

Thermal plasticity of diving performance

Mean, maximal and sustained diving performances were independent of thermal acclimation treatment, with no observed differences in dive duration between *C. porosus* acclimated at 28, 31.5 and 35°C ([mean] p = 0.69, $F_{2,51}$ = 0.37, lme; [maximal] p = 0.58, $F_{2,44}$ = 0.55, lme; [sustained] p = 0.51, $F_{2,19}$ = 0.70, lme; Fig. 2.2 A - B). Thermal acclimation treatment had no effect on PDSI (p = 0.25, $F_{2,199}$ = 1.39, lme) or dive frequency (p = 0.75, $F_{2,48}$ = 0.29, lme). Covariate interactions among body mass ($p \geq$ 0.23, lme), partition number ($p \geq$ 0.33, lme) and experimental round ($p \geq$ 0.46, lme) were not significant in all analyses, and were excluded from final models.

Discussion

Thermal acclimation responses are considered to be one of the most important defences that ectotherms have against the deleterious effects of climate change (Chevin et al., 2010; Somero, 2010). In the present study, we found that estuarine crocodiles were unable to acclimate dive capacity to long-term increases in water temperature, with mean submergence times almost halving with every 3.5°C increment in temperature, regardless of thermal acclimation treatment. Together, these findings indicate that sustained diving performance is thermally sensitive but not plastic.

Dissimilar thermal sensitivities of sustained and maximal diving performance

Water temperature has long been identified as an influential factor constraining dive times in ectotherms (Herbert and Jackson, 1985; Fuster et al., 1997), but the separate effects of water temperature on maximal performance compared with continuous, sustained diving have not been previously assessed. Here, maximal diving performance was found to be thermally insensitive, with all dives lasting between 7.39 and 9.5 min, whereas sustained diving performance was markedly reduced at high temperatures. It is likely that the differences in thermal sensitivity between sustained and maximal diving performances reflect the extent to which each is supported by aerobic vs. anaerobic metabolism. Sustained diving requires continuous descents and ascents between the water surface and substratum and is likely to be supported aerobically if extended recovery times (i.e. PDSIs) are to be avoided. Optimising sustained diving

performance can, in theory, be achieved by performing a series of shorter dives within aerobic limits, thereby minimising recovery times and maximising total time underwater. Alternatively, maximal once-off dives likely exceed ADLs and rely on anaerobiosis. Both 'strategies' can explain my observed results, with sustained diving performance seeing animals terminate dives sooner at elevated temperatures to maintain dives within aerobic limits, and maximal dive times remaining unchanged, with respect to temperature, as animals may increasingly rely on anaerobic metabolism to extend dive times beyond ADLs.

Indications of anaerobic debt accumulated during a dive can be drawn from post-dive recovery times (i.e. PDSI). Recovery times remained constant for both sustained and maximal dives, suggesting that all dives were performed aerobically, but anaerobic debt incurred during maximal performances might be masked by recovery processes occurring more rapidly at elevated temperatures (Galloway and Kieffer, 2003). Direct measurements of post-dive physiology, such as blood lactate levels and post-dive oxygen consumption, are recommended for future endeavours.

The plateau of thermal independence observed for maximal dives may play an adaptive role where once-off maximal performances are integral to survival or in situations where extended recovery times incur a minimal fitness cost. Free-ranging crocodylians, however, spend the majority of their time in the water, performing between 50 and 70 dives per day (Campbell et al., 2010a), so it is likely that sustained diving performance or endurance is of greater ecological relevance than maximal performance. Estuarine crocodiles have previously demonstrated a plateau of thermal independence in prolonged swimming performance, across a similar thermal range (23 – 33°C; Elsworth et al., 2003), and this came at the cost of post-exercise recovery times tripling to clear anaerobic debt (Campbell et al., 2013b). Maintaining performance across a breadth of temperatures may come at the cost of increasingly relying on anaerobic metabolism, but my results suggest that this cost may be minimal at very high temperatures because PDSIs remained constant. Investigation into the possible trade-offs between reduced dive durations and faster recovery rates at elevated temperatures could provide a better understanding of the diving behaviour observed here.

Limitations associated with single clutch studies

The use of study animals from multiple clutches ensures genetic diversity and controls for inter-clutch differences, known as clutch effects (Bagatto et al., 2012). The crocodiles used here were from a single clutch fathered by one male, although multiple paternity is common in *C. porosus* (Lewis et al., 2013). Inter-clutch differences have been recorded in crocodylians with respect to growth, lung and liver mass and cardiovascular function during exercise (Turton et al., 1997; Bagatto et al., 2012). It is unknown if diving performance, thermal sensitivity and thermal phenotypic plasticity differ between individual clutches in *C. porosus*. The use of sibling animals is a notable limitation of this study and the findings presented here need to be confirmed in non-sibling *C. porosus* to confirm/reject the wider applicability of these findings.

Ecological implications for a lack of thermal plasticity

Aquatic and semi-aquatic ectotherms have long been revered for their capacity to acclimate to altered thermal regimens (Hazel and Prosser, 1974; Johnston and Temple, 2002), but the bulk of this research has examined organismal responses to decreases in ambient temperature resembling current habitat conditions (Aho and Vornanen, 2001; Hochscheid et al., 2004; Glanville and Seebacher, 2006; Seebacher and James, 2007; Guderley and Seebacher, 2011). My study here complements this body of work by examining thermal compensation at the other, 'hot', end of the spectrum. The results from the present study reveal that *C. porosus* have a limited capacity to acclimate sustained diving performance to elevated water temperatures, as chronic exposure to 'high' water temperatures does not elicit improved performance. This finding is unusual, because thermal acclimation in the form of altered cardiovascular control (Jayasundara and Healy, 2013), muscle contractility rates (Johnston and Temple, 2002) and locomotor performance (Johnson and Bennett, 1995) has been extensively documented in ectotherms. Further to this, estuarine crocodiles have a remarkable capacity to compensate during cold acclimation, exemplified by musculature, metabolic and biochemical alterations leading to 'perfect' maintenance of swimming performance (Glanville and Seebacher, 2006; Seebacher and James, 2007). Taken together, these findings are consistent with the idea that tropical ectotherms, such as *C. porosus*, have greater flexibility in adjusting to decreases in temperatures compared with increases, as

they are assumed to inhabit microclimates nearing their upper thermal limits (Janzen, 1967; Somero, 2010; Sunday et al., 2014). My findings therefore emphasise the need to assess acclimation capacity across a range of temperatures, because complete acclimation to 'cool' temperatures or temperatures resembling current habitat temperature may only translate into partial or non-existent acclimation capacity at elevated temperatures reflective of climate change.

Partial or non-existent acclimation capacity in diving performance at elevated temperatures appears to be a shared trait among ectothermic divers, although comparative data are scant. Diving performance in the Mary River turtle (*Elusor macrurus*), for instance, only partly acclimates to elevated water temperatures of 28°C, but this is linked to differential reliance on aquatic respiration at low temperatures (Clark et al., 2008). Likewise, submergence times of free-ranging crocodylians and turtles are reduced in summer months compared with winter months (Carr et al., 1980; Gordos et al., 2003; Hochscheid et al., 2005; Bradshaw et al., 2007; Hochscheid et al., 2007; Campbell et al., 2010a), suggesting that these ectotherms are not fully compensating for present-day seasonal changes or diving behaviour changes seasonally. My results suggest that sustained dive capacity of juvenile estuarine crocodiles is likely to be further compromised as summer water temperatures continue to rise under climate change. Reduced dive durations may see cumulated time available for underwater activities cut short, forcing animals to spend a greater amount of time at the water surface and potentially making them more conspicuous to aerial predators.

Organisms have a finite number of responses to climate change; they can change their thermal tolerance limits by genetic adaptation (requiring multiple generations in a short space of time), acclimatise to the altered thermal regimen (requiring within-lifetime physiological changes), behaviourally compensate, or shift their geographical range to thermally preferable habitat (typically towards the poles or higher altitudes). My findings reveal that thermal acclimation/acclimatisation is unlikely to buffer estuarine crocodiles from the negative consequences of elevated temperatures on dive capacity. This, together with the long-lived life-history of estuarine crocodiles, suggests that this species may be reliant on behavioural strategies to buffer extreme temperatures. Crocodiles may be able to seek refuge in deep, cool water pockets, thereby defending dive times, or shift their geographic range to cooler, southerly

waters. Before we can fully understand the constraints that elevated temperatures place on the ecological functioning of *C. porosus*, an entire suite of performance traits (e.g. swimming capacity, digestive capacity and immune function) needs to be assessed (Kearney and Porter, 2009). Nonetheless, elevated water temperatures accompanying climate change will be likely to cause serious disruption to the diving behaviour of estuarine crocodiles.

Figures
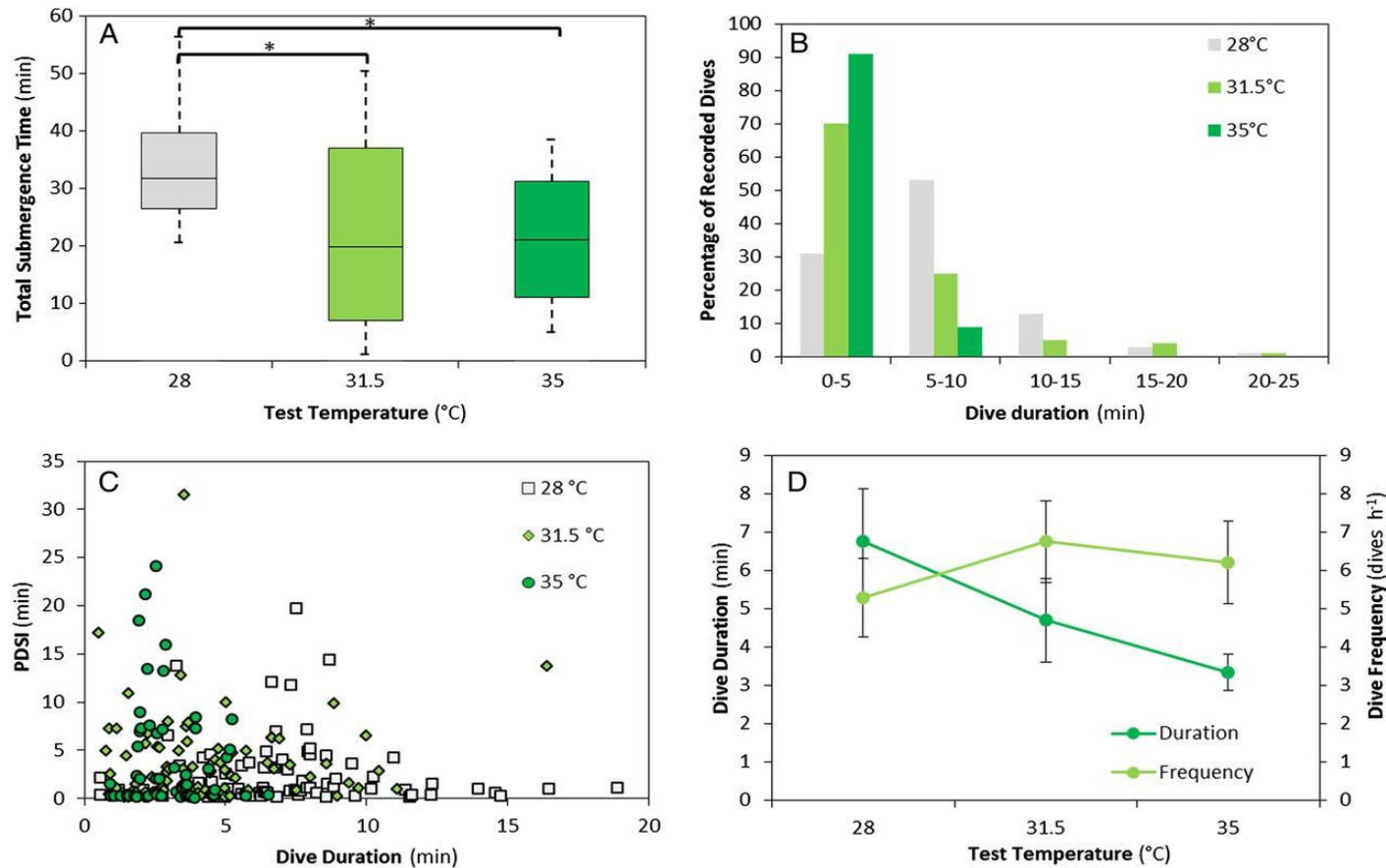
Figure 2.1: Thermal sensitivity of diving performance in juvenile estuarine crocodiles (*Crocodylus porosus*). Pooled data from the three thermal acclimation treatments showing the effect of water temperature on the following: (**A**) total submergence time (throughout the 60 min continual threat treatment); (**B**) frequency histogram of 'fright-dive' durations; (**C**) post-dive surface interval (PDSI); and (**D**) frequency and duration of 'fright-dives'. 'Fright-dive' duration was inversely related to water temperature, with 91% of 'fright-dives' at 35°C lasting < 5 min, whereas the majority (70%) of submergences performed at 28°C exceeded 5 min. Total submergence time was significantly greater at 28°C compared with the warmer water temperatures ($p < 0.001$, *d.f.* = 10, lme). Post-dive surface intervals were independent of water temperature. Dive frequency was independent of water temperature and dive duration.
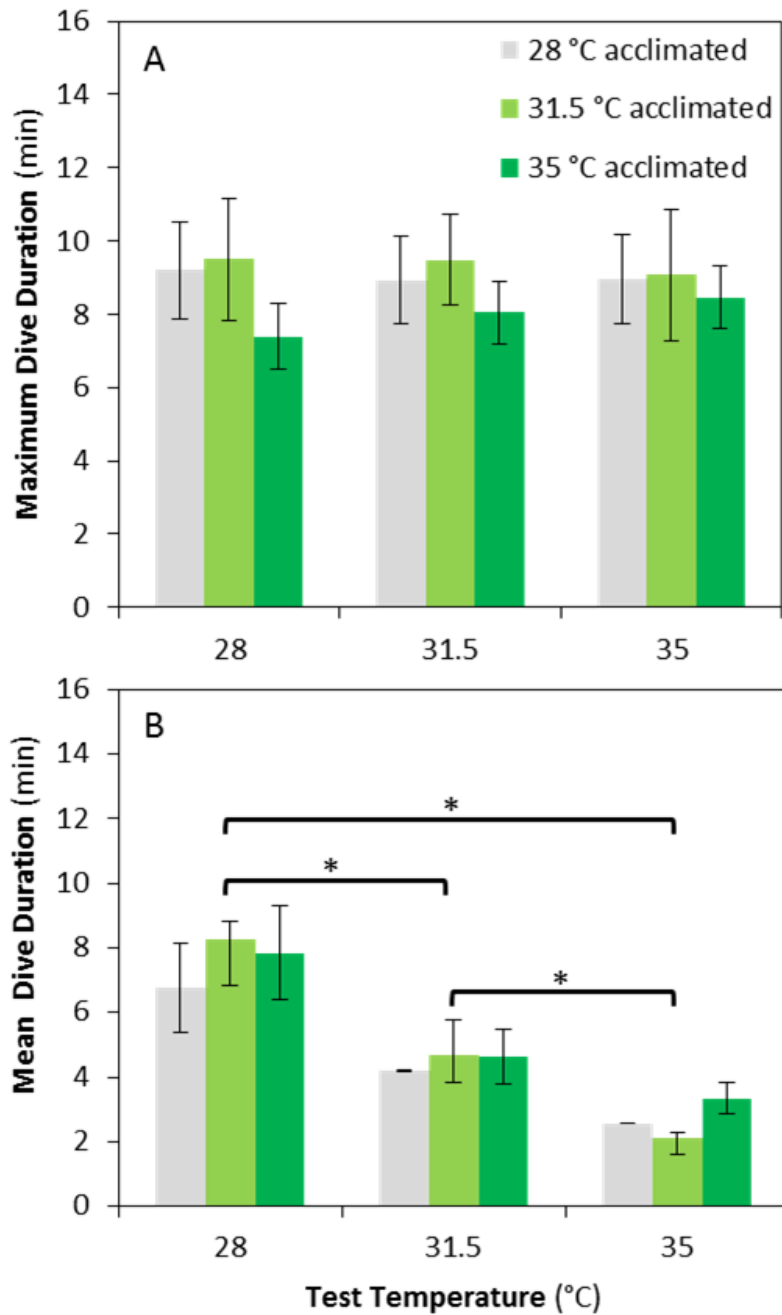
Figure 2.2: Thermal plasticity of diving performance in juvenile estuarine crocodiles (*C. porosus*). Effect of test water temperature on 'fright-dive' performance (i.e. dive duration, expressed as minutes submerged) in *C. porosus* acclimated to 28, 31.5 or 35°C (*N* = 11). (**A**) Maximal submergence times (mean ± s.e.) were independent of both test temperature ($p \geq 0.37$, *d.f.* = 10, lme) and thermal acclimation treatment ($P \geq 0.51$, *d.f.* = 10, lme). (**B**) Mean (± s.e.) submergence times were inversely related to water temperature, with shorter dives performed at 35°C compared with 28°C (*d.f.* = 10, $p < 0.0001$, lme), regardless of thermal acclimation treatment.

CHAPTER 3

THE PHYSIOLOGICAL MECHANISMS CONSTRAINING DIVING PERFORMANCE AT ELEVATED TEMPERATURES

Abstract

Survival of air-breathing, diving ectotherms is dependent on their capacity to optimise time available for obligate underwater activities. Submergence times are thermally sensitive, with dive durations significantly reduced by increases in water temperature, deeming these animals particularly vulnerable to the effects of climate change. The physiological mechanisms underlying this compromised performance are unclear but are hypothesised to be linked to increased oxygen demands and a reduced capacity for metabolic depression at elevated temperatures. Here, we investigated how water temperature (both acute and chronic exposures) affected the diving physiology of juvenile estuarine crocodiles (*Crocodylus porosus*, N = 7 per thermal acclimation treatment; total length, mean ± s.d. 56.6 ± 9.6 cm; body mass, mean ± s.d. 488.0 ± 251.6 g). Diving oxygen uptake, diving heart rate, post-dive plasma-lactate concentrations and blood oxygen-carrying capacity were assessed at two test temperatures, reflective of different climate change scenarios (i.e. current summer water temperatures, 28∘C and 'high' climate warming, 34∘C). Diving oxygen uptake rate increased threefold between 28∘C and 34∘C (Q_{10} = 7.4). The capacity to depress oxygen demands was reduced at elevated temperatures; with animals lowering oxygen demands from surface levels by 52.0 ± 27.8% and 27.8 ± 16.4% at 28∘C and 34∘C, respectively. Post-dive plasma-lactate concentrations were independent of water temperature (p = 0.54; lme) and animals terminated dives once lactate accumulated to a threshold of 11.2 ± 0.6 mmol l^{-1}. Together these results suggest the aerobic dive limit of *C. porosus* is significantly reduced at elevated temperatures and animals terminated dives earlier at elevated temperatures rather than increasing reliance on anaerobic metabolism.

Introduction

The performance and survival of many ectothermic species is predicted to be compromised under global climate change (Pörtner and Farrell, 2008). Altered thermal regimes are particularly threatening to ectotherms (almost all fish, amphibians and reptiles) as body temperature is closely tied to the thermal environment. Ectotherm performance is optimised within a limited range of body temperatures (i.e. thermal performance breadth) and ongoing climate warming will likely drive temperatures beyond sustainable limits (Rummer et al., 2014). Body temperatures surpassing thermal performance optima are generally accompanied by a marked decline in fitness-related traits such as locomotor capacity (Johansen and Jones, 2011), developmental rates/growth (McLeod et al., 2013), immune competence (Yu et al., 2009) and survival (Rohr and Palmer, 2013).

The consequences of elevated temperatures on ectotherm performance are well-documented (Bellard et al., 2012; Kingsolver et al., 2013) but the physiological basis for loss of performance remains unclear. Performance decrements at stressfully high temperatures are hypothesised to stem from oxygen demands exceeding oxygen supply capacities (i.e. cardio-respiratory system failure; Oxygen- and capacity-limited thermal tolerance hypothesis; OCLTT) (Pörtner, 2001; Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner, 2010; Eliason et al., 2011). Compromised aerobic performance may occur at high temperatures when maximal rates of oxygen uptake ($\dot{V}O_{2MAX}$) plateau or decrease but resting/standard oxygen uptake rates ($\dot{V}O_{2STANDARD}$) increase exponentially, reducing absolute aerobic scope (AAS, $AAS = \dot{V}O_{2MAX} - \dot{V}O_{2STANDARD}$). A narrowed aerobic scope is thought to translate into a reduced capacity for activities including growth, movement, digestion and reproduction (Pörtner, 2002), and the thermal effects on individuals may scale up to affect population and community dynamics (Pörtner and Peck, 2010). A narrowed aerobic scope at high temperatures has been demonstrated in a number of tropical ectotherms (Munday et al., 2009; Nilsson et al., 2009; Johansen and Jones, 2011; Rummer et al., 2014); however the OCLTT hypothesis is not universal (Clark et al., 2013; Ern et al., 2014; Norin et al., 2014; Ern et al., 2015; Ern et al., 2016). Many ectotherms experience compromised performance at temperatures below those affecting aerobic scope (Norin et al., 2014), suggesting the

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mechanistic explanation may be multi-faceted and thus remains unresolved in many taxa.

Air-breathing, diving ectotherms (e.g. sea snakes, marine iguanas, turtles and crocodylians) appear vulnerable to increases in water temperature as dive capacity (i.e. time spent submerged/dive duration) is inversely related to water temperature (Fuster et al., 1997, Prassack et al., 2001; Priest and Franklin, 2002). Shortened dive times translate into less time available for obligate underwater activities such as foraging/hunting, predator avoidance, sleep/rest and social interactions. Submergence times of free-ranging crocodylians and turtles are reduced in summer months compared to winter months (Carr et al.,1980; Gordos et al., 2003; Hochscheid et al., 2005; Bradshaw et al., 2007; Hochscheid et al., 2007; Campbell et al., 2010a), suggesting these animals are not fully compensating for present-day seasonal thermal fluctuations. Dive durations are predicted to be further reduced by ongoing increases in water temperature in marine and freshwater habitats; with submergence times of some species forecasted to halve under a moderate rate of climate warming (SRES A1B storyline; 50th percentile of IPCC global warming range; Rodgers et al., 2015; Chapter 2). A limited or non-existent capacity to thermally acclimate/acclimatise to elevated temperatures following long-term (i.e. chronic) exposure elevates the susceptibility of this group to climate change (Clark et al., 2008; Rodgers et al., 2015; Chapter 2), but physiological mechanisms constraining performance at high temperatures remain uncertain.

The aerobic dive limit (ADL) conceptually represents the maximum duration an animal can remain submerged before oxygen debt is incurred (Butler, 2006). An individual's ADL is dependent on total body oxygen (TBO) stores and the rate at which these stores are consumed; with smaller stores and/or a faster rate of oxygen uptake reflective of a shorter ADL (Butler, 2006). Oxygen can be stored in the lungs, blood and tissue, and the percent contribution of stores varies between species (Kooyman et al., 1989). In estuarine crocodiles (*Crocodylus porosus*), for instance, pulmonary (i.e. lungs) oxygen represents the majority of stores (~67.0%), followed by blood oxygen (~28.9 %), and tissue oxygen contributes the least (~4.1%) (Wright, 1985). Ectotherm oxygen stores decline with increasing temperature (Pough, 1976; Fuster et al., 1997); however dive durations are reduced at elevated temperatures beyond the extent expected from

reduced TBO alone (Hayward et al., 2016). The thermal sensitivity of ectotherm diving performance is hypothesised to be linked to a reduction in the ADL as a result of diving metabolic rate (i.e. oxygen consumption) increasing exponentially with rising water temperature (Hayward et al., 2016). The validity of this hypothesis remains untested in crocodylians, but shorter dive durations have been associated with reductions in ADLs in an endothermic diver, the steller sea lion (Gerlinsky et al., 2013). Submergences can be extended beyond the ADL with the use of anaerobic pathways but this incurs the cost of longer post-dive surface intervals (PDSIs) to clear accumulated lactate (Kooyman et al., 1980; Costa et al., 2004). Post-dive surface intervals did not increase with water temperature in Chapter 2, suggesting anaerobic pathways were not employed. The hypothesis that an increase in water temperature increases the reliance on anaerobic metabolism has not been directly tested in ectotherms, and represents a potential compensatory option.

Vertebrates enter a unique physiological state when diving, with a suite of cardiovascular alterations occurring (Andersen, 1966). This state is termed the 'dive response' and includes a marked decrease in heart rate (i.e. diving bradycardia), a redistribution of blood stores to essential organs (i.e. peripheral vasoconstriction) and a cardiac-shunt (in some species; Blix and Folkow, 1983; Butler and Jones, 1997). These alterations facilitate prolonged submergences by lowering oxygen demands and animals enter a hypometabolic state where oxygen demands are lower than surface metabolic rates (Davis et al., 2004; Hastie et al., 2007). The initiation of the 'dive response' appears to be context specific in some species (Gaunt and Gans, 1969; Noren et al., 2012); with crocodylians, for example, markedly reducing heart rate (65 ± 6% reduction) during predator avoidance dives (i.e. 'fright-dives') and only small cardiovascular changes (14 ± 6% reduction) are observed during voluntary, undisturbed dives (Wright et al., 1992). The mechanism underlying the thermal sensitivity of diving performance likely differs from the OCLTT, which does not consider hypometabolic states. High temperatures may not only elevate diving oxygen uptake rates ($\dot{V}O_{2DIVE}$) but also compromise a diver's capacity to depress metabolic rates from resting/surface levels.

The aim of this study was to investigate how the diving physiology of juvenile estuarine crocodiles is affected by increases in water temperature. Crocodylians are primarily aquatic, and free-ranging animals have been recorded to dive 50 - 70 times per day (Campbell et al., 2010a). The ability to remain submerged for extended periods of time is thought to be adaptive because predator avoidance, foraging, sleep/recovery and social interactions occur underwater (Seebacher et al., 2005b; Campbell et al., 2010b). The dive capacity of juvenile *C. porosus* is thermally sensitive, with marked reductions in dive durations at temperatures above 28∘C (Rodgers et al., 2015; Chapter 2) but the physiological mechanism underlying this pattern is unresolved. We assessed the effect of water temperature on $\dot{V}O_{2DIVE}$, diving heart rate and reliance on anaerobic metabolism (i.e. post-dive lactate accumulation). We hypothesised $\dot{V}O_{2DIVE}$ would increase markedly with temperature and align with compromised performance (i.e. shorter dive durations) (H_1). Similarly, we predicted that *C. porosus*' relative capacity for metabolic depression (i.e. reductions in heart rate and oxygen consumption compared to surface rates) would be impaired at elevated temperatures (H_2). Thirdly, we hypothesised reliance on anaerobic metabolism would increase at high temperatures, exemplified by high post-dive lactate accumulation, to compensate for a reduced ADL (H_3). The assessment of diving physiology was subsequently used to uncover the mechanisms underlying impaired performance at elevated temperatures.

Materials and Methods

Animal maintenance

Estuarine crocodiles (*Crocodylus porosus*; Schneider, 1801) were obtained from two sources; eggs were collected from a single clutch at David Fleay Wildlife Park (Burleigh Heads, Queensland, Australia) and juveniles were obtained from three clutches at Cairns Crocodile Farm (Gordonvale, Queensland, Australia; *N* = 14 from 4 clutches; 6 reared from eggs; 8 obtained as juveniles). Eggs were transported to The University of Queensland (St Lucia, Queensland, Australia) where they were incubated in an *R*-com 50 egg incubator (Auto Elex Co. Ltd, GimHae, Korea) for 85 days at 31.5 ± 1°C and 70 - 90% humidity. Upon hatching animals were maintained in an environment aimed at optimising healthy growth for 12 months prior to testing. Crocodiles were fed regularly (twice weekly, totalling 15% of their body mass) a mixture of minced beef, chicken and

pilchards supplemented with powdered calcium and vitamin D (Vetafarm, Wagga Wagga, NSW, Australia). Enclosures were cleaned, with complete water changes after feeding. All animals were acclimated to a common water temperature of 31.5°C for 3 months prior to testing, to counteract differences in thermal histories. Animals were between 15 - 22 months old at the time of testing (total length, mean ± s.d. 56.6 ± 9.6 cm; snout-vent length, mean ± s.d. 28.9 ± 5.2 cm; body mass, mean ± s.d. 488.0 ± 251.6 g). All experiments complied with The University of Queensland animal ethics requirements (Approval No. SBS/018/14/ARC/AUST ZOO).

Experimental design and thermal acclimation treatments
Crocodiles were randomly assigned to one of two thermal acclimation treatments; one representing summer water temperatures presently experienced by *C. porosus* (28°C) or one representing a high degree of climate warming (34°C; N = 7 per treatment). Thermal acclimation treatments were identical apart from water temperature and enclosures were large wooden tanks (3.35 m × 0.85 m × 0.75 m; length × width × height) designed to emulate thermally heterogeneous environments conducive to thermoregulatory behaviour. Water temperatures were maintained using 300 W submersible heaters attached to thermostats (Aquasonic, Wauchope, AUS). Enclosures contained freshwater filled to a depth of 0.15 m. Dry platforms were situated at each end of the tanks, one being a relatively 'warm' platform situated underneath a ceramic heat lamp (250 W; OzWhite, Enfield, South Australia; suspended 26 cm above the platform) and a UV-B light (25 W; Exo Terra®, Montreal, Ca) and the other a relatively 'cool' platform with no lamps. Basking opportunity (i.e. time heat lamp was switched on) was 8 h day^{-1} (08.00 – 16.00 h) for both treatments, with substrate temperature underneath the heat lamp averaging 40 ± 3°C (mean ± s.d.). A summer photoperiod was used, with a constant 14 h:10 h light:dark regimen (05.00–19.00 h light) for both treatments. Animals were left to acclimate to thermal treatments for 60 days prior to performance testing and were fasted for 48 h prior to dive trials.

Dive trials

Dive trials were held in a large experimental tank (1.8 m × 2.0 m × 1.9 m, length × width × height) custom-built from foam fibreglass. The dive tank was evenly partitioned into three sections with opaque plastic partitions allowing three dive trials to run concurrently. The dive tank contained filtered freshwater to a depth of 1.3 m and water temperature was finely controlled using a spa heater (900 EVO; Elecro Engineering, Stevenage, UK). Thermal profiling of the dive tank using Thermocron temperature loggers (iButtonLink Technology, Whitewater, WI, USA) confirmed uniformity of temperature throughout the water column. Each partitioned section of the dive tank contained a floating rest platform (0.6 m × 0.15 m × 0.05 m; length × width × height), where crocodiles could rest and breathe on the water surface whilst their body remained submerged.

Diving performance was assessed in animals from both thermal acclimation treatments (i.e. 28°C-acclimated and 34°C-acclimated) at two test temperatures (28°C and 34°C). Position in the dive tank (i.e. partition assignment) and the order of test temperature were randomised. Animals were given an extended habituation period (minimum of 8 h) to ensure blood lactate levels returned to resting levels after handling stress (Franklin et al., 2003). Following the habituation period, either a single 'fright-dive' trial or a sustained 'fright-dive' trial began. The single 'fright-dive' condition involved just one dive, whereas the sustained 'fright-dive' condition involved a bout of four consecutive dives (i.e. sustained diving) with fixed surface intervals of 3.6 ± 0.6 s. 'Fright-dives' were initiated by an experimenter lightly tapping a crocodile with a blunt wooden pole. Dive durations were directly observed and timed.

Blood sampling and analyses

Crocodiles were immediately captured upon surfacing at the end of a trial for blood sampling. Blood samples (0.5 - 2 ml) were drawn from a branch of the jugular vein (venous blood) using ½ inch 23 G needles attached to heparinised (lithium salt; Sigma) syringes. An aliquot (~5 µl) of whole blood was used to determine haemoglobin concentrations and two microcapillary tubes were filled to measure haematocrit. Microcapillary tubes were spun at 5000 g for two minutes (micro-haematocrit centrifuge; Hawksley, Sussex, UK) and percent packed red cell volume (i.e. haematocrit;

% hct) was measured. A colorimetric assay kit (Sigma-Aldrich; MAK115) was used to determine duplicate haemoglobin concentrations [H_b]. Remaining blood was centrifuged (microfuge®18, Beckman Coulter™, Lane Cove, AUS) in Eppendorf tubes at 2 - 5 RCF for 3 min and plasma was subsequently withdrawn and stored in a -80°C freezer until lactate analyses. Plasma-lactate concentrations (mmol l^{-1}) were measured using a lactate meter (Lactate Pro 2, Phill Bates sports promotions, Carlton, AUS); where 5 µl of thawed plasma was placed on test strips. Blood samples were also taken from resting and exhausted animals for reference points. Resting blood samples were obtained by capturing animals in their holding tank and immediately sampling. Exhausted samples were taken from animals following 10 min of running stimulated by lightly touching the animal on the tail and hind legs (for hct and [Hb] analyses). Post-dive blood samples were obtained within 3 min of the animal surfacing to avoid lactate generated by handling stress contaminating the sample.

Diving heart rate

Surface and diving heart rates (f_H; beats min^{-1}) were measured in crocodiles from both acclimation treatments (28◦C-acclimated and 34◦C-acclimated) at two test temperatures (28◦C and 34◦C). Animals were placed in a plastic dive tank (37 × 39 × 56 cm; length × width × height) containing freshwater (depth = 26 cm) within a controlled-temperature room. Two electrocardiogram (ECG) wires (MLA1203 Needle electrode, AD Instruments, Sydney, Australia) were inserted underneath the intersection of two ventral scales, anterior and posterior to the heart and held in place using strapping tape (Elastoplast rigid sports strapping, Beiersdorf, Hamburg, Germany). Instrumentation was complete in under two minutes. The ECG wires were run into an adjacent room, attached to a BioAmp (ML132, AD Instruments) and the bioamp was connected to a PowerLab (4/30 series ML866, AD Instruments, Sydney, Australia). Heart rate recordings were visualised on a laptop using LabChart software (ADInstruments Pty Ltd, Bella Vista, New South Wales, Australia) with a sampling rate of 100 Hz. A video camera (Microsoft LifeCam Studio, Microsoft, USA) was placed above the dive tank and recordings were synchronised with heart rate readings so that dive events could be easily isolated. Three 'fright-dives' were initiated by the experimenter entering the room and lightly touching the animal. Animals were allowed to rest for 30 min between each 'fright-dive'. Surface and diving heart rates were determined by extracting and

averaging multiple stable recordings (3 per animal) between 60 - 300 s in duration. Surface and diving heart rates were averaged for each animal at each test temperature. Relative (%) bradycardia was calculated as:

$$(1) \ \% \ brady = \left[\left(f_{H,surface} - f_{H,diving} \right) / f_{H,surface} \right] \times 100$$

where $f_{H,surface}$ is mean heart rate when the animal is at the surface of the water and $f_{H,diving}$ is mean heart rate when the animal is performing a 'fright-dive'. Animals were left to recover after instrumentation and equilibrate body temperature with water temperature for a minimum of one hour before trials began.

Diving oxygen uptake rate

Diving metabolic oxygen uptake rate ($\dot{V}O_{2DIVE}$) was determined for crocodiles from each thermal acclimation treatment at two test temperatures (28°C and 34°C) using flow-through respirometry. Animals were fasted for a minimum of five days prior to testing to eliminate metabolic responses to feeding (i.e. specific dynamic action; Gienger et al., 2011), and left to adjust to dive tank conditions for an hour prior to testing to ensure body temperature had equilibrated with water temperature. $\dot{V}O_{2DIVE}$ was measured inside a custom built diving column (height = 1.33 m, base diameter = 0.42 m, top diameter = 0.25 m) placed inside the dive tank at a water depth of 1.3 m. The water surface was sealed using a custom-fitted piece of Styrofoam with a dome-shaped respiratory hood (volume = 3.6 L) fitted with inflow and outflow air outlets. The diving column and respiratory hood were designed to ensure the only available air space was inside the respiratory hood (Appendix 3.1, Fig. A3.1). A pull-system was utilised with flow-rate (FR) ranging between 590 – 1240 ml min^{-1}, depending on animal body mass, using a mass-flow controller (*SS3*, Sable Systems International, North Las Vegas, USA; calibrated with a Bubble-O -Meter; Ohio, USA). Outflowing air was scrubbed of water vapour by passing it through a drying column (Drierite; Sigma, Sydney, AUS). Fractional concentrations of carbon dioxide (CO_2) and oxygen (O_2) were measured by passing dry air into a CO_2 analyser (LI-820, LI-COR, Nebraska, USA) and subsequently into an O_2 analyser (Oxzilla, Sable Systems International, North Las Vegas, USA). The CO_2 meter malfunctioned beyond correction for many trials, so rates of O_2 consumption ($\dot{V}O_2$, ml min^{-1}) were calculated using equation 11.2 from Lighton (2008) and respiratory

quotients (i.e. ratio of carbon dioxide produced compared to oxygen consumed at a given time point) were estimated for each test temperature using Grigg's (1978) equation (2) derived from juvenile *C. porosus* ($N = 11$; $P < 0.001$):

$$(2)\ RQ = 1.098 - 0.0203T$$

where RQ is the respiratory quotient and T is test temperature (◦C). Surface oxygen consumption rates ($\dot{V}O_{2SURFACE}$) were measured for one hour, followed by a 'fright-dive'. Post-dive oxygen consumption ($\dot{V}O_{2POSTDIVE}$) was measured to estimate oxygen debt accumulated during submergence, and oxygen debt was assumed to be cleared once $\dot{V}O_{2POSTDIVE}$ equalled $\dot{V}O_{2SURFACE}$. $\dot{V}O_{2DIVE}$ was calculated using equation (3) (Hurley and Costa, 2001);

$$(3)\ \dot{V}O_{2DIVE} = {O_{2DEBT}}/{DD}$$

where O_{2DEBT} represents oxygen debt accumulated during submergence (ml min^{-1}) and DD is dive duration (i.e. total time submerged; min) (sample calculation Appendix 3.2). Animals were motionless during surface periods and dives (excluding ascending and descending movements). Baseline measurements (in the absence of animals) were taken before and after trials for a minimum of 2 h to detect drifts in fractional concentrations of O_2 and CO_2.

Statistical analyses

Data analyses were performed in R Studio (version 3.1.3; R Core Team, 2012) using the nlme (linear and nonlinear mixed effects models; Pinheiro et al., 2012) package. A series of linear mixed effects models were used to determine the effects of test temperature and thermal acclimation treatments on diving performance (i.e. minutes submerged), post-dive plasma-lactate accumulation (mmol l^{-1}), diving oxygen uptake rate ($\dot{V}O_{2DIVE}$) and diving heart rate (% bradycardia). Test temperature (2-level factor), acclimation treatment (2-level factor), body mass and dive tank partition number were included as fixed effects and animal source (i.e. David Fleay Wildlife Park or Cairns Crocodile Farm) and identification number were included as random effects (ID nested within source) in all models. A repeated measures analysis of covariance (ANCOVA) was used to assess

the effect of blood sampling time (min) on plasma-lactate accumulation and compare lactate accumulation between different conditions (i.e. resting, single 'fright-dive' and sustained 'fright-diving'). Statistical significance was accepted at $p \leq 0.05$.

Results

Diving performance

Single and sustained diving performance (i.e. total time spent submerged during four consecutive dives) were thermally sensitive, with performance decrements experienced at test temperatures of 34°C compared to 28°C ([single] $p < 0.05$; $F_{1,9} = 19.2$, lme; [sustained] $p < 0.0001$; $F_{1,10} = 59.9$, lme) (Fig. 3.1 A - B). Single submergences performed at 28°C lasted 18.5 ± 2.4 min (mean ± s.e.) and reduced to 9.0 ± 1.0 min (mean ± s.e.) at 34°C ($Q_{10} = 0.30$; Fig. 3.1 A). Similarly, crocodiles diving at 28°C spent an average of 66.7 ± 5.9 min (mean ± s.e.) underwater, during sustained dive trials, whereas animals diving at 34°C spent an average of 28.2 ± 1.9 min (mean ± s.e.) submerged ($Q_{10} = 0.24$; Fig. 3.1 B). Both sustained and single diving performances were independent of thermal acclimation treatment, with no observed differences in *C. porosus* acclimated to 28°C compared to 34°C ([single] $p = 0.45$, $F_{1,11} = 0.6$, lme; [sustained] $p = 0.87$, $F_{1,10} = 0.03$) (Fig. 3.1 A - B). Covariate interactions among body mass ($p \geq 0.91$) and partition number ($p \geq 0.70$) were not significant.

Diving heart rate

Surface heart rates ($f_{H,surface}$) were significantly higher at test temperatures of 34°C (65 ± 4 beats min^{-1}) compared to 28°C (46 ± 3 beats min^{-1}) (Fig. 3.1 C; $p < 0.001$, $F_{1,6} = 43.7$, lme) but independent of thermal acclimation treatment ($p = 0.54$, $F_{1,5} = 0.4$, lme). Diving heart rates ($f_{H,diving}$) were independent of thermal acclimation treatment ($p = 0.30$, $F_{5,1} = 1.3$) but test temperature had a significant effect ($p = 0.05$, $F_{1,6} = 5.8$, lme); with elevated $f_{H,diving}$ at 34°C (24 ± 2 beats min^{-1}) compared to 28°C (19 ± 1 beats min^{-1}) (Fig. 3.1 C). All animals exhibited diving bradycardia, with heart rates reducing by 12 - 55 beats min^{-1} from surface levels. Relative bradycardia (i.e. % reduction from $f_{H,surface}$) was thermally insensitive (test temperature $p = 0.44$, $F_{1,6} = 0.3$; acclimation treatment $p = 0.65$, $F_{1,5} = 0.2$, lme; Fig. 3.2 A). Body mass had no significant effect on $f_{H,surface}$, $f_{H,diving}$ and relative bradycardia ($p > 0.29$; lme).

Diving oxygen uptake rate

Surface ($\dot{V}O_{2SURFACE}$) and diving ($\dot{V}O_{2DIVE}$) metabolic rates were significantly higher at test temperatures of 34∘C compared to 28∘C (Fig. 3.1 D; [$\dot{V}O_{2SURFACE}$] $p < 0.01$, $F_{1,5} = 19.1$, $Q_{10} = 2.32$, lme; [$\dot{V}O_{2DIVE}$] $p < 0.05$, $F_{1,4} = 9.1$, $Q_{10} = 7.4$, lme). Thermal acclimation treatments and body mass had no effect on $\dot{V}O_{2SURFACE}$ ($F_{1,5} = 0.1$; body mass $p = 0.16$, $F_{1,5} = 0.2$, lme) and $\dot{V}O_{2DIVE}$ (thermal acclimation treatment $p = 0.37$, $F_{1,5} = 0.98$, body mass $p = 0.13$, $F_{1,4} = 3.5$ lme). Diving metabolic rates were depressed from surface levels by 52.9 ± 27.8% at 28°C and 27.8 ± 16.5% at 34°C. Test temperature had a significant effect on relative metabolic depression (Fig. 3.2 B; $p = 0.05$, $F_{1,4} = 6.8$, lme) but was independent of thermal acclimation treatment ($p = 0.32$, $F_{1,5} = 0.7$, lme) and body mass ($p = 0.40$, $F_{1,4} = 4.2$, lme). Post-dive oxygen debt and recovery durations were thermally insensitive, averaging 13.5 ± 2.6 ml kg $^{-1}$ and 18.0 ± 2.0 min for both test temperatures (Fig. 3.3 A - B; [O_2 debt] $p = 0.07$, $F_{1,5} = 5.7$, lme; [recovery duration] $p = 0.21$, $F_{1,4} = 2.2$, lme), and were independent of thermal acclimation treatment (([O_2 debt] $p = 0.14$, $F_{1,5} = 3,1$, lme; [recovery duration] $p = 0.29$, $F_{1,5} = 1.4$, lme) and body mass ([O_2 debt] $p = 0.13$, $F_{1,4} = 3.8$, lme; [recovery duration] $p = 0.1$, $F_{1,4} = 2.4$, lme).

Post-dive plasma-lactate accumulation

Post-dive plasma-lactate levels were independent of thermal acclimation treatment ($p = 0.82$, $F_{1,9} = 0.06$ [single]; $p = 0.26$, $F_{1,9} = 5.3$ [sustained]; lme), test temperature (Fig. 3.3 C; $p = 0.54$, $F_{1,8} = 0.42$ [single]; $p = 0.26$, $F_{1,1} = 1.6$ [sustained], lme) and body mass ($p = 0.59$, $F_{1,8} = 0.31$ [single]; $p = 0.39$, $F_{1,1} = 2.0$ [sustained], lme) in both single and sustained dive trials. Lactate accumulation was significantly greater following a sustained dive trial (i.e. four consecutive dives) compared to a single dive; with plasma-lactate averaging 11.2 ± 0.6 mmol l^{-1} for single dives but reaching 14.0 ± 0.7 mmol l^{-1} for sustained diving ($p < 0.01$, repeated measures ANCOVA). Resting plasma lactate samples were independent of thermal acclimation treatment ($p = 0.52$, $F_{1,6} = 0.47$, lme) test temperature ($p = 0.56$, $F_{1,6} = 0.32$, lme) and body mass ($p = 0.50$, $F_{1,4} = 0.57$, lme). Post-dive plasma-lactate concentrations were higher than resting levels (28°C = 1.9 ± 0.2 mmol l^{-1}; 34°C = 2.2 ± 0.5 mmol l^{-1}; mean ± s.e.m.); with ~sixfold and ~sevenfold increase for single and sustained dive trials, respectively ($p < 0.01$, repeated measures ANCOVA; Fig. 4). Lactate concentrations were independent of blood sampling times ($p = 0.95$, repeated measures ANCOVA; Fig. 5 A - B).

Haematocrit and haemoglobin

Resting haematocrit (hct, %) and haemoglobin ([Hb], g l^{-1}) were independent of thermal acclimation treatment (hct p = 0.49, $F_{1,8}$ = 0.5; [Hb] p = 0.58, $F_{1,5}$ = 0.4, lme), with haematocrit averaging 18 ± 1% and [haemoglobin] averaging 64 ± 6 g l^{-1} in all animals. Post-dive hct and [Hb] were also independent of both thermal acclimation treatment (hct p = 0.43, $F_{1,5}$ = 0.7; [Hb] p = 0.85, $F_{1,5}$ = 0.04, lme) and test temperature (hct p = 0.40, $F_{1,4}$ = 0.9; [Hb]; p = 0.74, $F_{1,3}$ = 0.1, lme). There was no change in hct or [Hb] between resting and post-dive states (hct p = 0.53; [Hb] p = 0.97, lme). Blood sampling time had no effect on hct or [Hb] (hct p = 0.11, $F_{1,4}$ = 4.1; [Hb] p = 0.19, $F_{1,3}$ = 2.8, lme)

Discussion

Ectotherm functioning and performance is often compromised at elevated temperatures akin to forecasted climate change (Pörtner and Farrell, 2008), but the underlying mechanisms are frequently unresolved. Here we partially illuminated the physiological mechanisms constraining dive capacity at elevated temperatures in juvenile estuarine crocodiles. In line with previous findings (Rodgers et al., 2015; Chapter 2), diving performance of *C. porosus* was markedly compromised at elevated temperatures, with submergence times halving between 28°C and 34°C. Performance decrements appear to be linked to reductions in ADLs at elevated temperatures, stemming from increased oxygen demands and a reduced capacity for metabolic depression (supporting H$_1$ and H$_2$). Reliance on anaerobic metabolism did not differ between test temperatures, with crocodiles terminating dives at a plasma-lactate threshold of 11mmol l^{-1} (rejecting H$_3$). Cumulatively these results suggest *C. porosus* terminated dives earlier at the elevated temperature due to a faster depletion of body oxygen stores and animals do not compensate for this decreased aerobic capacity by increasing use of anaerobic pathways.

Thermal sensitivity of diving oxygen uptake and metabolic depression

Body temperature has long been identified as the most influential abiotic factor governing aerobic metabolism in ectotherms (Brett, 1971). Surface metabolic rates ($\dot{V}O_{2SURFACE}$) increased markedly between 28°C and 34°C (Q_{10} = 2.32), reflecting the typical metabolic response of ectotherms to acute thermal increases (Schulte, 2015). Similar Q_{10} values have been reported for resting *C. porosus* over a comparable

temperature range ($\dot{V}O_{2STANDARD,}Q_{10}$ = 2.68, range = 20 - 33∘C; Grigg 1978); suggesting metabolic thermal sensitivity does not differ between these contexts. In line with H_1, diving metabolic rates increased threefold between 28∘C and 34∘C. This increase in oxygen demands translates into a faster depletion of TBO during submergence. Rapid depletion of TBO likely decreases dive duration and increases the frequency at which aerially-respiring divers must surface and replenish oxygen stores.

Diving metabolic rates were more thermally sensitive than surface metabolic rates (i.e. $\dot{V}O_{2DIVE}$ Q_{10} = 7.4; $\dot{V}O_{2SURFACE}$ Q_{10} = 2.32) and this is likely due to a compromised capacity for metabolic depression at the 34∘C. Diving oxygen requirements lowered from surface levels by 52% and 28% at 28∘C and 34∘C, respectively. A reduction in relative metabolic depression suggests the capacity for hypometabolism is compromised beyond the extent to be expected from Q_{10} effects only. The molecular and biochemical mechanisms responsible for initiating the 'dive response' (i.e. metabolic depression) are not well understood, particularly in ectotherms (Withers and Cooper, 2010). Diving bradycardia is controlled by interactions between the cholinergic and adrenergic nervous systems governing pacemaker heart cells (Alboni et al., 2011). Stimulation of cold receptors (particularly those located in facial regions), coupled with inputs from nasal, lung and carotid chemoreceptors appear to be key in the initiation of the 'dive response' (Drummond and Jones, 1979; Alboni et al., 2011). Cold receptors are unlikely to receive stimulation at elevated temperatures and may underlie the elevated heart rate observed in *C. porosus* at 34∘C. Further to this, chemoreceptor functioning (e.g. intrapulmonary and brainstem) can be dependent on body temperature in lizards (Douse and Mitchell, 1988; Zena et al., 2016), and it is possible that chemoreceptor functioning was compromised in *C. porosus* diving at 34∘C. Crocodiles were able to maintain significant declines in heart rate during submergence (i.e. % bradycardia) at both test temperatures, suggesting other components of the 'dive response' were compromised. For example, limited capacity for either initiation of the 'dive response', peripheral vasoconstriction (Altimiras et al., 1998) or cardiac shunting may have contributed to reduced metabolic depression at 34∘C, but further experimentation is required for confirmation.

Aerobic dive limits and reliance on anaerobic metabolism

Post-dive lactate levels (11.2 ± 0.6 mmol l^{-1}) surpassed resting lactate levels (1.9 ± 0.2 mmol l^{-1}) indicating animals extended submergences beyond aerobic dive limits (ADL). Prolonged submergences (> 40 min) exceeding estimated ADLs have been recorded in wild, adult crocodylians (Campbell et al., 2010a) and these dives are thought to be associated with predator avoidance behaviour (Campbell et al., 2010b), similar to the 'fright-dives' performed here. These prolonged dives were followed by extended recovery durations on the water surface, potentially indicative of oxygen debt clearance times (Campbell et al., 2010a). In contrast, surpassing ADLs is thought to be rare in routine dives as many free-ranging ectotherms dive well within their estimated ADL (Burggren et al., 1989; Hochscheid et al., 2005; Pratt et al., 2010). Short (4.5 min), repetitive dives in response to no stimuli are entirely supported by aerobic metabolism in juvenile *C. porosus* (Wright, 1987). Taken together, we can postulate that predator avoidance dives in wild crocodiylians are fuelled by both aerobic and anaerobic metabolism, whereas repetitive short dives are likely entirely aerobic.

Crocodiles terminated dives at a lactate threshold of 11.2 ± 0.6 mmol l^{-1} regardless of test/body temperature, demonstrating a behavioural strategy to surface sooner (i.e. reduced dive duration) rather than further rely on anaerobic metabolism. This behaviour may be beneficial as it avoids further accrual of lactate and concomitant extended recovery times. My hypothesis that reliance on anaerobic metabolism would increase at 'high' temperatures is rejected. Instead, post-dive oxygen debt and recovery durations were 'defended' at the high temperature, so that animals spent no additional recovery time at the water surface following a dive at 34◦C compared to 28◦C. This behaviour contrasts to observations of juvenile *C. porosus* swimming at elevated temperatures (Campbell et al., 2013b). Swimming performance (i.e. distance swum) of juvenile *C. porosus* can be maintained between the temperatures of 28 - 33◦C, but maintaining performance at 33◦C comes at the cost of increased oxygen debt (~tenfold) and extended recovery durations (~threefold) (Campbell et al., 2013b). This discrepancy between metabolic partitioning may be due to behavioural strategies which optimise different outputs (i.e. total time underwater versus total distance swum), or the endpoint of dive trials may be more natural/volitional compared to swimming trials

in recirculating flumes. Nonetheless, responses to elevated temperatures may be trait specific and are important to consider.

Haematocrit and haemoglobin

Chronic increases in temperature have elicited compensatory responses in ectotherms, whereby the carrying capacity of blood is augmented by increased [Hb] and hct (Houston and Cyr, 1974; Gallaugher and Farrell, 1992; Jayasundara and Somero, 2013; Lilly et al., 2015). In contrast to these findings, [Hb] and hct remained unchanged in juvenile *C. porosus* following long-term exposure to an elevated temperature (i.e. 34°C), suggesting a lack of plasticity. An inability to adjust [Hb] and hct may underlie the absence of thermal acclimation in 'fright-dive' performance. However, blood oxygen stores only account for approximately one third (28.9%; Wright, 1985) of TBO stores while pulmonary oxygen accounts for ~67%; therefore a lack compensation in $\dot{V}O_{2DIVE}$ likely played a greater role in compromised 'fright-dive' performance (Wright, 1985). Haemoglobin concentration and haematocrit levels rise in some vertebrates during hypoxia and/or diving (Thornton and Hochachka, 2004), likely stemming from recruitment of red blood cells from the spleen (Hurford et al., 1985). This response was not observed here and [Hb] and hct remained unchanged following a 'fright-dive' suggesting splenic contraction does not occur during predator avoidance dives in juvenile *C. porosus.*

Error associated with estimating respiratory quotients

Respiratory quotients (*RQ*) were estimated using an equation derived by Grigg (1978) for the majority of $\dot{V}O_2$ calculations and a small margin of error may have been introduced into the absolute values. The *RQs* were derived from $\dot{V}O_{2STANDARD}$ measurements on the same species (i.e. *Crocodylus porosus*) with a similar body mass (180 – 6200 g) and age (juveniles) ranges, making estimates comparable (Grigg, 1978). Using Grigg's (1978) *RQ* assumes equal thermal sensitivity of $\dot{V}O_{2STANDARD,}$ $\dot{V}O_{2PRE-DIVE}$ and $\dot{V}O_{2POST-DIVE.}$ This assumption may be valid as animals are stationary when resting at the water surface, similar to resting conditions when measuring $\dot{V}O_{2STANDARD}$. Estimating *RQs* for conditions where anaerobic metabolism is partially fuelling activity, as is the case here with diving, is often avoided as lactate accumulation can shift the bicarbonate-CO_2 equilibrium towards CO_2 (Lighton and Halsey, 2011). Reliance on anaerobic metabolism

was equal at both test temperatures here, so any error introduced from anaerobiosis would be the same and have no overall impact on the relative effect of test temperature on $\dot{V}O_{2DIVE}$. Further comforting, is that the *RQs* calculated when the CO_2 meter was functioning ($\dot{V}O_{2PRE\text{-}DIVE}$ *RQ* = 0.65 ± 0.02, mean ± s.e.; $\dot{V}O_{2POST\text{-}DIVE}$ *RQ* = 0.55 ± 0.0, mean ± s.e.) fall within the range measured by Grigg (1978; i.e. range: 0.32 – 0.74; mean ± s.d.: 0.49 ± 0.13).

Ecological consequences of reduced ADLs

The ADL of juvenile *C. porosus* was reduced by ~ 50% at a water temperature reflective of predicted climate change (i.e. 34∘C), compared to present-day summer temperatures (28∘C). This finding suggests predator avoidance dive times may be shortened if water temperatures continue to increase in marine and freshwater habitats. Additional concern stems from the finding that diving performance was equally compromised at 34°C in crocodiles from both thermal acclimation treatments (i.e. 28∘C-acclimated and 34∘C-acclimated). This finding demonstrates a lack of thermal phenotypic plasticity, which is thought to be a defining safeguard in long-lived species which may experience substantial thermal increases within a single lifetime. A non-existent thermal acclimation capacity at elevated temperatures has been demonstrated previously in *C. porosus* (Rodgers et al*.,* 2015; Chapter 2). Similarly, many other diving ectotherms have limited thermal acclimation capacity (Graham et al., 1971; Clarke et al., 2008; Heatwole et al., 2012) and some species are unable to compensate for seasonal thermal increases (Carr et al., 1980;; Gordos et al., 2003; Hochscheid et al., 2005; Campbell et al., 2010a). Underlying this lack of acclimation capacity in diving performance of *C. porosus* is likely the inability to compensate rates of oxygen consumption ($\dot{V}O_{2DIVE}$ and $\dot{V}O_{2SURFACE}$), heart rate and blood oxygen-carrying capacity (haemoglobin and haematocrit) to elevated temperatures- as seen here.

A lack of thermal acclimation capacity brings into question how this species will fare as water temperatures in natural habitats continually increase with climate change. 'Fright-dive' durations will likely decrease, with concomitant increases in surfacing frequency. Surfacing frequency has been shown to increase with rising water temperature in several diving ectotherms, including in sea snakes (Udyawer et al., 2016), marine and freshwater turtles (Southwood et al., 2003; Storey et al., 2008),

newts (Samajova and Gvozdik, 2009) and freshwater crocodiles (Campbell et al., 2010a). Unless behavioural compensation is employed (e.g. diving in deep, cool water pockets or migrating polewards into cooler climates), juvenile *C. porosus* may need to frequently replenish oxygen stores at the potential cost of becoming increasingly conspicuous to predators.

Figures
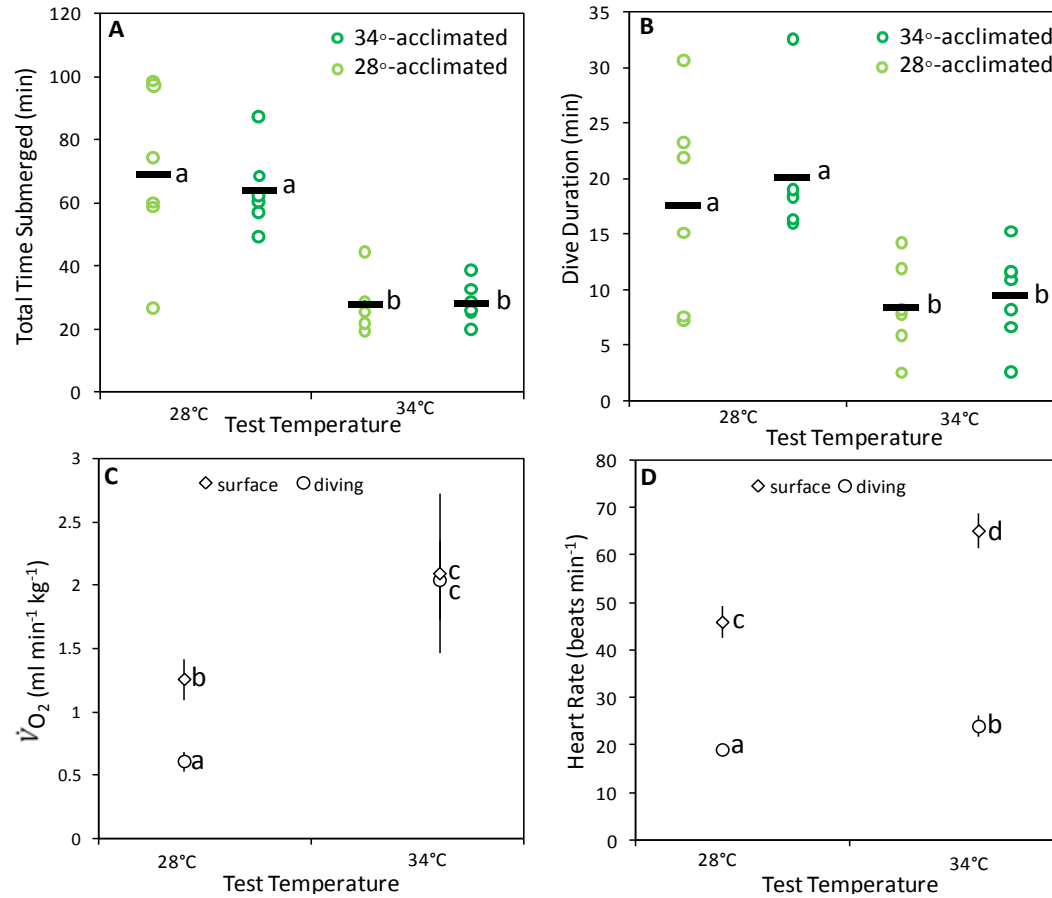
Figure 3.1: Thermal sensitivity of diving performance (min submerged; **A** - **B**), rates of oxygen consumption ($\dot{V}O_2$; ml min^{-1} kg^{-1}; **C**), and heart rate (beats min^{-1}; **D**) and in juvenile estuarine crocodiles (*Crocodylus porosus*). Both sustained (**A**) and single (**B**) 'fright-dive' performance was reduced at test temperatures of 34°C compared to 28°C ([single] $p < 0.05$; *d.f.* = 6; [sustained] $p < 0.0001$; *d.f.* = 6; lme; N = 7 per acclimation treatment). Surface and diving heart rates and diving oxygen consumption rates increased with rising temperature (**C** - **D**: [$f_{H,surface}$] $p < 0.001$, *d.f.* = 6, lme; [$f_{H,diving}$] $p = 0.052$ *d.f.* = 6, lme; [$\dot{V}O_{2SURFACE}$] $p < 0.05$, Q_{10} = 2.32, *d.f.* = 7, lme; [$\dot{V}O_{2DIVE}$] $p < 0.01$, Q_{10} = 7.4, *d.f.* = 7, lme). Values are shown as raw data points in **A** - **B**, and values are represented as mean ± s.e. from pooled thermal acclimation treatments in **C** - **D**. Different lowercase letters indicate statistically significant differences between treatment groups.
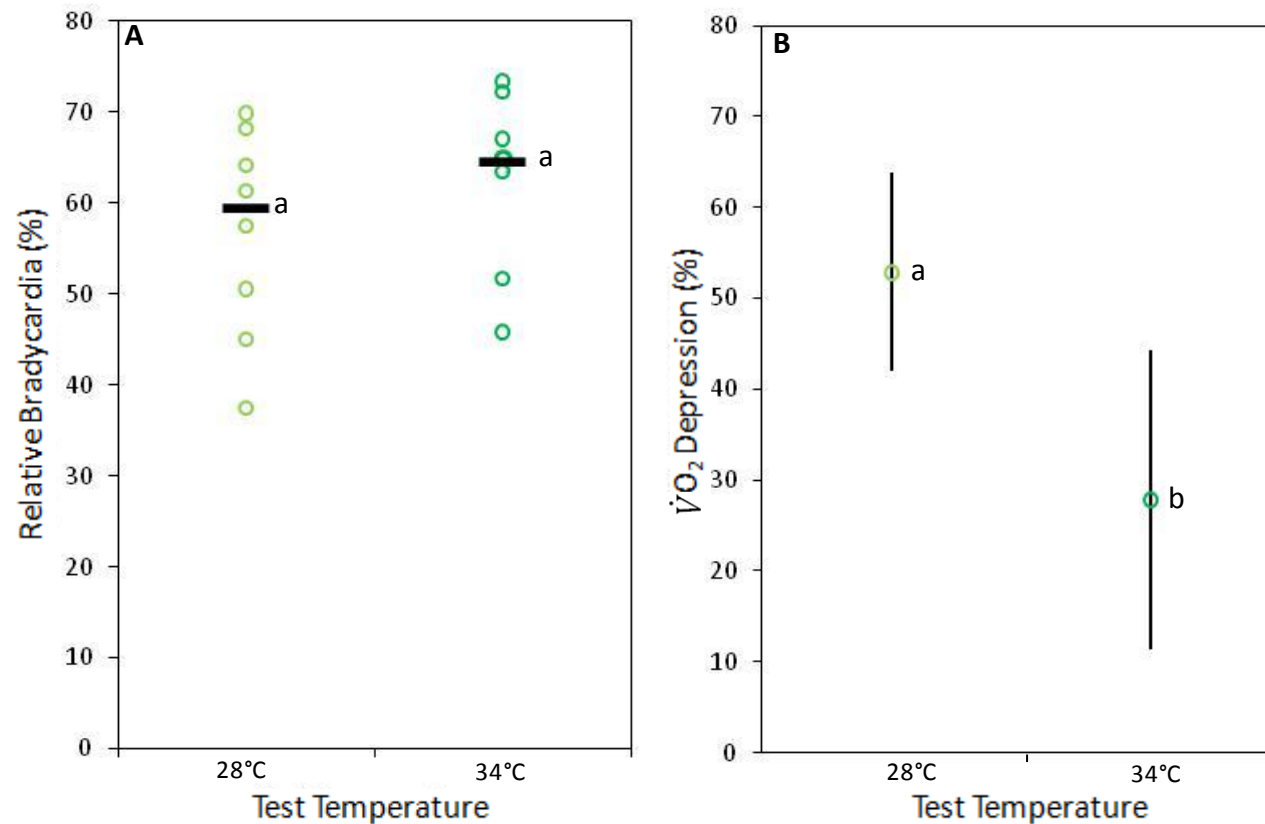
Figure 3.2: (**A**) Thermal sensitivity of relative diving bradycardia (i.e. % reduction from $f_{H,surface}$) of juvenile estuarine crocodiles (*Crocodylus porosus*; N = 8 per test temperature). Relative bradycardia was independent of test temperature (p = 0.18, *d.f.* =7, lme). (**B**) Effect of test temperature on diving metabolic depression (i.e. % reduction from $\dot{V}O_{2SURFACE}$) in juvenile estuarine crocodiles (*Crocodylus porosus*; N = 8 per test temperature). Diving metabolic depression was reduced at test temperatures of 34°C compared to 28° (p = 0.05, *d.f.* =7, lme). Values are shown as raw data points in **A** and values are represented as mean ± s.e. in **B**. Different lowercase letters indicate statistically significant differences between groups.
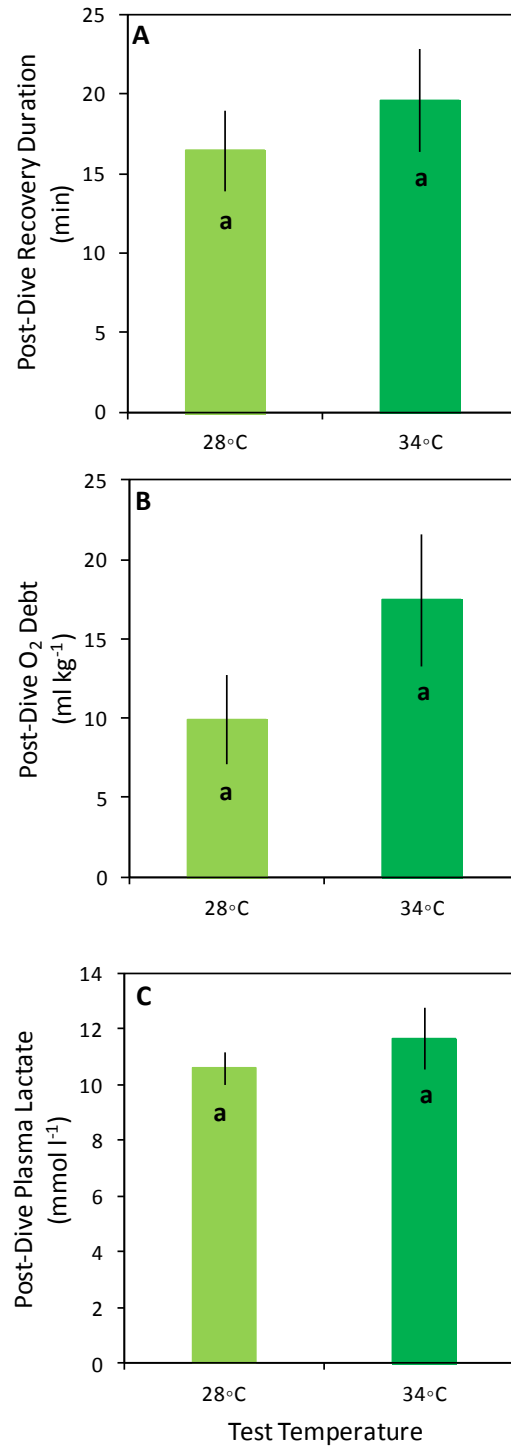
Figure 3.3: Thermal sensitivity of post-dive (**A**) recovery duration (i.e. time required for $\dot{V}O_{2POSTDIVE} = \dot{V}O_{2PREDIVE}$), (**B**) oxygen debt (ml kg⁻¹) and (**C**) plasma-lactate accumulation (mmol l⁻¹) in juvenile estuarine crocodiles (*Crocodylus porosus*) ($N = 8$ per test temperature, pooled from thermal acclimation treatments). Test temperature had no significant effect on post-dive recovery duration ($p = 0.21$, *d.f.* = 7; linear mixed-effects model, lme), oxygen debt ($p = 0.07$, *d.f.* = 7, lme) or lactate accumulation ($p = 0.54$, *d.f.* = 13; lme). Values are represented as mean ± s.e. Different lowercase letters indicate statistically significant differences between groups.

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CHAPTER 4

THERMAL SENSITIVITY AND PLASTICITY OF AEROBIC CAPACITY: COMPARISONS OF RESTING, DIVING AND MAXIMUM METABOLISM

Abstract

Reductions in ectotherm aerobic capacity are predicted to occur with climate change but thermal acclimatisation is set to play a pivotal protective role. Resting cardiorespiratory functions (physiological floors) are typically thermally phenotypically plastic whilst maximum capacities (physiological ceilings) are generally fixed in fishes, lending to the principle of 'plastic floors and concrete ceilings'. The applicability of this principle to semi-aquatic ectotherms remains untested. Moreover, it is unknown how performance constrained by 'physiological floors' (e.g. diving performance) fits within theoretical frameworks of aerobic capacity. Here we assessed the thermal sensitivity and plasticity in resting oxygen consumption ($\dot{V}O_{2STANDARD}$), maximum oxygen consumption ($\dot{V}O_{2MAX}$) and absolute aerobic scope ($AAS = \dot{V}O_{2MAX} - \dot{V}O_{2STANDARD}$) in juvenile estuarine crocodiles (*Crocodylus porosus*, N = 4 - 6 per thermal acclimation treatment; total length, mean ± s.d. 57.5 ± 9.6 cm; body mass, mean ± s.d. 499.2 ± 252.3 g) at temperatures emulating climate warming scenarios (i.e. current summer water temperatures, 28∘C and 'high' climate warming, 34∘C). The thermal sensitivity of $\dot{V}O_{2STANDARD}$, $\dot{V}O_{2MAX}$ and diving metabolism ($\dot{V}O_{2DIVE}$) were compared to assess if diving performance is constrained by aerobic capacity. Resting rates of oxygen consumption increased markedly between 28 - 36∘C (Q_{10} = 3.15, p < 0.001, lme) and did not differ between thermal acclimation treatments. In contrast, $\dot{V}O_{2MAX}$ was thermally insensitive but plastic; warm-acclimated animals (i.e. 34∘C-acclimated) exhibited a twofold increase in $\dot{V}O_{2MAX}$ compared to animals acclimated to 28∘C (p < 0.01, lme). Absolute aerobic scope maintained thermal independence between 28 - 36∘C and increased in warm-acclimated animals due to elevated $\dot{V}O_{2MAX}$ (p < 0.05, lme). The thermal sensitivity of $\dot{V}O_{2DIVE}$ (Q_{10} = 7.4) was threefold greater than $\dot{V}O_{2STANDARD}$ (Q_{10} = 2.28), and thermal thresholds marking decrements in diving performance did not align with reductions in aerobic capacity. Together, these findings oppose the 'plastic floors and concrete ceilings' hypothesis and suggest assessing the vulnerability of diving ectotherms based solely on aerobic scope measurements underestimates susceptibility to elevated temperatures.

Introduction

Ectotherms comprise the majority of biodiversity and are projected to experience population declines, local extinctions and shifts in geographic ranges as climate change drives temperatures beyond sustainable limits (Perry et al., 2005; Doney et al., 2012). For ectotherms, escaping thermal extremes within a lifetime is limited to behavioural thermoregulation and physiological compensation due to the tight relationship between body temperature and environmental temperature (Huang et al., 2014; Seebacher et al., 2015). The functional capacity of ectotherms is optimised within a limited range of body temperatures as a result of molecular, cellular and metabolic processes being thermally sensitive (Hochachka and Somero, 1968; Hochachka,1967). The majority of obligate daily activities (e.g. feeding and predator avoidance) are underpinned by aerobic capacity (i.e. metabolic consumption of oxygen) and concomitant thermal constraints (Clarke and Johnston, 1999; Gillooly et al., 2001). For this reason, it is essential to assess the thermal sensitivity and plasticity of aerobic capacity to predict how climate change may affect these organisms.

Ectotherm metabolic rate is generally thermally sensitive and resting rates of oxygen consumption ($\dot{V}O_{2STANDARD}$) represent the basal energetic 'cost of living' in resting (i.e. not paying off oxygen debt), inactive, post-absorptive, non-gravid animals (Fry and Hart, 1948). $\dot{V}O_{2STANDARD}$ typically increases exponentially with rising temperature and the energetic 'cost of living' is projected to increase under climate warming, leaving less energy available for growth and daily activities (Pörtner, 2001). Maximum oxygen consumption ($\dot{V}O_{2MAX}$) lies at the opposite end of the scale and coincides with maximal energetic outputs (e.g. maximum swimming or running capacity) preceding use of anaerobic pathways that can lead to acidosis, an oxygen debt and fatigue. Ectotherm $\dot{V}O_{2MAX}$ generally increases with rising temperature but declines or reaches a plateau at thermal extremes (Pörtner, 2001). Aerobic capacity can be represented as absolute aerobic scope (i.e. $AAS = \dot{V}O_{2MAX} - \dot{V}O_{2STANDARD}$) or factorial aerobic scope (i.e. $FAS = \dot{V}O_{2MAX} / \dot{V}O_{2STANDARD}$). Due to the typical disparity in the thermal sensitivity of $\dot{V}O_{2STANDARD}$ (exponential increase) and $\dot{V}O_{2MAX}$ (declines or reaches a plateau) at thermal extremes, AAS narrows (Fig. 4.1 A - B). Limitations on aerobic capacity at elevated temperatures are hypothesised to underlie decrements in growth, movement, digestion and reproduction (Pörtner, 2002), and thermal effects on

individuals are theorised to scale up to affect population and community dynamics (Pörtner, 2002; Pörtner and Peck, 2010).

The thermal sensitivity of aerobic capacity and degree of underlying plasticity can vary greatly between species (Clark et al., 2011). For example, *AAS* can narrow at thermal extremes (Munday et al., 2009; Nilsson et al., 2009; Johansen and Jones., 2011; Rummer et al., 2014), continually increase with rising temperature up to lethal limits (Norin et al., 2014), or be thermally independent (Ern et al., 2014; Verhille et al., 2016; Poletto et al., 2017). Likewise, the thermal plasticity of metabolism can differ within a species depending on level of assessment (i.e. $\dot{V}O_{2STANDARD}$ or $\dot{V}O_{2MAX}$; Sandblom et al., 2016). For example, European perch (*Perca fluviatilis, L.*), Atlantic halibut (*Hippoglossus hippoglossus*) and shorthorn sculpin (*Myoxocephalus scorpius*) can offset increased basal energy expenditure ($\dot{V}O_{2STANDARD}$) following long term warming exposure, but acclimation capacity of $\dot{V}O_{2MAX}$ is absent (Gräns et al., 2014; Sandblom et al., 2014; Sandblom et al., 2016). These findings led to the 'plastic floors and concrete ceilings' theoretical framework, whereby resting cardiorespiratory functions are postulated to be plastic but maximum cardiorespiratory functions are fixed/less plastic (Sandblom et al., 2016). This framework has been applied to fishes but remains untested in semi-aquatic, air-breathing ectotherms.

This study assessed the thermal sensitivity of $\dot{V}O_{2STANDARD}$, $\dot{V}O_{2MAX}$, *AAS* and *FAS* in juvenile estuarine crocodiles (*Crocodylus porosus*). The effects of thermal acclimation on $\dot{V}O_{2STANDARD}$ and $\dot{V}O_{2MAX}$ were examined to test if *C. porosus* fits the 'plastic floors and concrete ceilings' framework. The estuarine crocodile is a tropical species with a large geographic range, spanning northern Australia, East Timor, Papua New Guinea, Solomon Islands, Indonesia, Brunei, Malaysia, Singapore, Sri Lanka and Bangladesh (Webb et al., 2010). Concerns regarding the persistence of *C. porosus* in a warming climate stem from the thermal sensitivity of dive capacity and an inability to acclimate both diving performance and diving metabolism($\dot{V}O_{2DIVE}$) to elevated temperatures (Rodgers et al., 2015; Chapter 2 - 3). Diving represents an activity constrained by 'physiological floors' as predator avoidance dives are typified by reductions in metabolism from surface levels ($\dot{V}O_{2SURFACE}$; Andersen, 1966; Chapter 3). An inability to thermally acclimate diving metabolism suggests other resting cardiorespiratory functions (e.g. $\dot{V}O_{2STANDARD}$ and resting heart rate) may show limited phenotypic

plasticity. Moreover, swimming performance of *C. porosus* has shown complete thermal compensation at cold temperatures (Glanville and Seebacher, 2006), suggesting greater plasticity may apply to maximum cardiorespiratory functions. Diving metabolism is highly thermally sensitive (Q_{10} = 7.4, Chapter 3) but it is unknown how $\dot{V}O_{2DIVE}$ fits within existing aerobic scope frameworks. Aerobic capacity underlying diving performance may reflect changes in *AAS* if $\dot{V}O_{2DIVE}$ is equally thermally sensitive and plastic as $\dot{V}O_{2STANDARD}$ (Fig. 4.1 C). Alternatively, aerobic capacity underlying diving performance may be more accurately depicted by the thermal sensitivity of resting cardiorespiratory functions in isolation. The aim of this study was threefold: i) to assess the thermal sensitivity of aerobic capacity at temperatures mimicking climate change, ii) to compare the thermal plasticity in $\dot{V}O_{2STANDARD}$ and $\dot{V}O_{2MAX}$ and iii) to assess how diving metabolism fits within existing aerobic scope frameworks. It was hypothesised that H$_1$: aerobic capacity (i.e. *AAS*) would decrease at elevated temperatures due to an exponential increase in $\dot{V}O_{2STANDARD}$ and a decline/plateau of $\dot{V}O_{2MAX}$, H$_2$: thermal plasticity would be observed in $\dot{V}O_{2MAX}$ but absent in $\dot{V}O_{2STANDARD}$, in direct opposition to the 'plastic floors and concrete ceilings' principle, and H$_3$: $\dot{V}O_{2DIVE}$ and $\dot{V}O_{2STANDARD}$ would be equally thermal sensitive and plastic, and decrements in diving performance would align with a narrowing of aerobic scope.
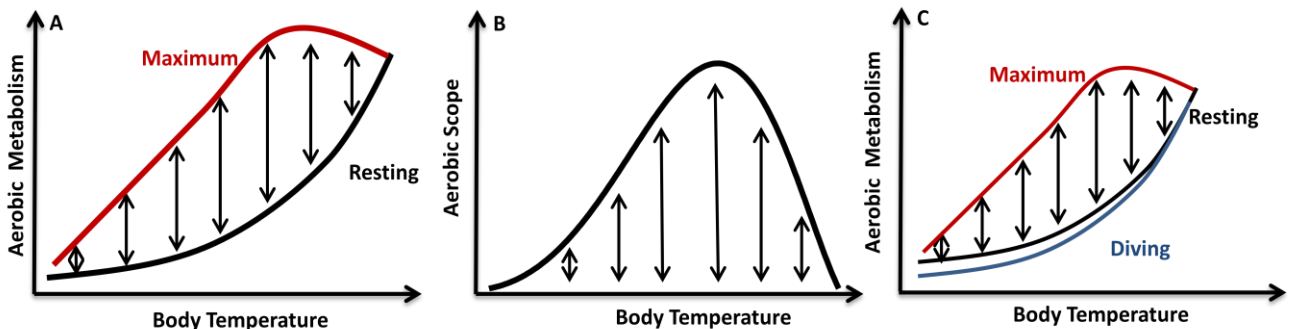
Figure 4.1: (**A**) Hypothetical resting (black line) and maximum (red line) rates of oxygen consumption as a function of ectotherm body temperature. Resting rates of oxygen consumption typically increase exponentially with rising body temperature. In contrast, maximum rates of oxygen consumption generally decline or reach a plateau at very high temperatures. Arrows represent the magnitude of absolute aerobic scope (i.e. maximum oxygen consumption – resting oxygen consumption). (**B**) Hypothetical thermal sensitivity of ectotherm aerobic scope arising from disparities in the thermal sensitivity of resting and maximum rates of oxygen consumption. Aerobic scope is typically reduced at very low and high body temperatures. Adapted from Pörtner (2010). (**C**) Diving metabolism (blue line) is hypothesised to be equally thermally sensitive as resting oxygen consumption.

Materials and Methods

Animal maintenance

Estuarine crocodiles (*Crocodylus porosus*; Schneider, 1801) were obtained from two sources; eggs were collected from a single clutch at David Fleay Wildlife Park (Burleigh Heads, Queensland, Australia) and juveniles were obtained from three clutches at Cairns Crocodile Farm (Gordonvale, Queensland, Australia) ($N = 10$ from 4 clutches; 4 reared from eggs; 6 obtained as juveniles). Eggs were transported to The University of Queensland (St Lucia, Queensland, Australia) where they were incubated in an *R*-com 50 egg incubator (Auto Elex Co. Ltd, GimHae, Korea) for 85 days at 31.5 ± 1°C and 70 - 90% humidity. Upon hatching animals were maintained in an environment aimed at optimising healthy growth for 12 months prior to testing. Crocodiles were fed regularly (twice weekly, totalling 15% of their body mass) a mixture of minced beef, chicken and pilchards supplemented with powdered calcium and vitamin D (Vetafarm, Wagga Wagga, New South Wales, Australia). Enclosures were cleaned, with complete water changes after feeding. All animals were acclimated to a common water temperature of 31.5°C for 3 months prior to testing, to counteract differences in thermal histories. Animals were between 20 - 27 months old at the time of testing (total length, mean ± s.d. 57.5 ± 9.6 cm; snout-vent length, mean ± s.d. 31.3 ± 4.8 cm; body mass, mean ± s.d. 499.2 ± 252.3 g). All experiments complied with The University of Queensland animal ethics requirements (Approval No. SBS/018/14/ARC/AUST ZOO).

Experimental design and thermal acclimation treatments

Crocodiles were randomly assigned to one of two thermal acclimation treatments; one representing summer water temperatures presently experienced by *C. porosus* (28°C, $N = 4$) or one representing a high degree of climate warming (34°C) ($N = 6$). Thermal acclimation treatments were identical apart from water temperature and enclosures were large wooden tanks (3.35 m × 0.85 m × 0.75 m; length × width × height) designed to emulate thermally heterogeneous environments conducive to thermoregulatory behaviour. Water temperatures were maintained using 300 W submersible heaters attached to thermostats (Aquasonic, Wauchope, AUS). Enclosures contained freshwater filled to a depth of 0.15 m. Dry platforms were situated at each end of the tanks, one being a relatively 'warm' platform situated underneath a ceramic heat lamp (250 W; OzWhite, Enfield, South Australia; suspended 26 cm above the platform) and a UV-B

light (25 W; Exo Terra®, Montreal, Ca) and the other a relatively 'cool' platform with no lamps. Basking opportunity (i.e. time heat lamp was switched on) was 8 h day^{-1} (08.00–16.00 h) for both treatments, with substrate temperature underneath the heat lamp averaging 40 ± 3°C (mean ± s.d.). A summer photoperiod was used, with a constant 14 h : 10 h light:dark regimen (05.00–19.00 h light) for both treatments. Animals were left to acclimate to thermal treatments for six months prior to testing.

Respirometry: resting and maximum rates of oxygen consumption

Resting and maximum rates of oxygen consumption ($\dot{V}O_2$) were measured in crocodiles from each acclimation treatment at three test temperatures (28°C, 34°C and 36°C) using flow-through respirometry. Test temperature order and acclimation treatment were randomised. Measurements occurred within a temperature-controlled room using sealed, cylindrical respirometers constructed from PVC-piping (small respirometer: length × diameter, 54.0 × 8.6 cm; large respirometer: 63.5 (l) × 15.7 cm (d)) with inflow and outflow air outlets at either end. A push-system was utilised with flow-rates ranging between 590 – 1240 ml min^{-1}, depending on animal body mass, using a mass-flow controller (*SS3*, Sable Systems International, North Las Vegas, USA, calibrated with a Bubble-O -Meter; Ohio, USA). Both inflowing and outflowing air was scrubbed of water vapour by passing it through drying columns (Drierite; Sigma, Sydney, Australia). Fractional concentrations of carbon dioxide (CO_2) and oxygen (O_2) were measured by passing dry air into a CO_2 analyser (LI-820, LI-COR, Nebraska, USA) and subsequently into an O_2 analyser (Oxzilla, Sable Systems International, North Las Vegas, USA). Respirometers and crocodiles were rubbed with a cloth rinsed with diluted ethanol (70%) prior to experiments to minimise microbial background respiration. Baseline measurements (in the absence of animals) were taken before and after trials for a minimum of 2 h to detect drifts in fractional concentrations of O_2 and CO_2.

Animals were fasted for a minimum of five days prior to testing to eliminate metabolic responses to feeding (i.e. specific dynamic action; Gienger et al., 2011). Prior to testing, animals were weighed and left inside the temperature controlled room for 1 h to recover from handling stress and ensure equilibration of body and room temperatures. Resting metabolic rate ($\dot{V}O_{2STANDARD}$) was measured in a dark room over a minimum of 12 h, typically between 1800 – 0600 because 24 h pilot testing showed

lower oxygen consumption during this time of day, suggesting quiescence. Maximum metabolic rate ($\dot{V}O_{2MAX}$) was measured after exercising animals until exhaustion (typically 5 - 7 min). Exercise was induced by stimulating crocodiles to run by lightly touching their tails, and gently flipping animals on their back which elicited a 'righting' response. Animals were considered exhausted when they were unable to right themselves and were immediately transferred into the respirometer. Animals were rested between trials for a minimum of eight days to ensure complete recovery.

Rates of O_2 consumption and CO_2 production were visualised using LabChart software (ADInstruments Pty Ltd, Bella Vista, New South Wales, Australia) and data were exported to Microsoft Excel for extraction. Resting metabolic rates were calculated by averaging the lowest 10% of measurements over the 12 h period and excluding outliers (i.e. values outside the mean ± 2 s.d.) as recommended by Clark et al. (2013). Maximum metabolic rates were calculated by averaging the highest recordings maintained for any two minute period following exhaustion. Following data extraction, oxygen consumption ($\dot{V}O_2$; ml O_2 min^{-1}) and carbon dioxide production ($\dot{V}CO_2$; ml CO_2 min^{-1}) were calculated using equations 10.6 and 10.7, respectively from Lighton (2008).

Statistical analyses and temperature quotient comparisons
The ratio of rates (i.e. metabolic rate) over a 10∘C temperature range (i.e. temperature quotients, Q_{10}) were calculated for resting ($\dot{V}O_{2STANDARD}$), surface ($\dot{V}O_{2SURFACE}$), diving ($\dot{V}O_{2DIVE}$) and maximum ($\dot{V}O_{2MAX}$) rates of oxygen consumption in crocodiles acclimate to 28°C, using the following equation (1):

$$(1)\ Q_{10} = (R_2/R_1)^{10/(T2-T1)}$$

where R_2 represents the mean metabolic rate at temperature 2 (T2) and R_1 is the mean metabolic rate at temperature 1 (T1). Mean $\dot{V}O_{2DIVE}$ and $\dot{V}O_{2SURFACE}$ were derived from Chapter 3. Temperature quotients were calculated between 28∘C (i.e. T1) and 34∘C (i.e. T2) in animals acclimated to 28°C. Statistical analyses were performed in R Studio (version 3.1.3; R Core Team, 2012) using the nlme (linear and nonlinear mixed effects models; Pinheiro et al., 2012) package. A series of linear mixed effects models were used to determine the effects of test temperature and thermal acclimation treatments on

$\dot{V}O_{2STANDARD}$, $\dot{V}O_{2MAX}$, *AAS* and *FAS*. Test temperature (3-level factor), acclimation treatment (2-level factor) and body mass were included as fixed effects and animal identification number as a random effect in all models. Time to exhaustion was included as a fixed effect in the $\dot{V}O_{2MAX}$ model. *P*-values ≤ 0.05 were considered statistically significant.

Results

Thermal sensitivity and plasticity of aerobic metabolism

Resting oxygen consumption ($\dot{V}O_{2STANDARD}$) increased 2.5-fold between 28∘C and 36∘C (Fig. 4.2 A, $F_{2,12}$ = 18.71, *p* < 0.001, lme; Q_{10} = 3.16) and was independent of thermal acclimation treatment (Fig. 4.2 A , $F_{1,8}$ = 0.67, *p* = 0.44, lme). Maximum oxygen consumption ($\dot{V}O_{2MAX}$) was thermally insensitive (Fig. 4.2 B, $F_{2,13}$ = 1.749, *p* = 0.21, but plastic; $\dot{V}O_{2MAX}$ was ~twofold higher in 34∘C-acclimated animals compared to 28∘C-acclimated animals at 34°C and 36°C (Fig. 4.2 B, $F_{1,8}$ = 12.29, *p* < 0.01, lme), but not 28°C. Time to exhaustion had no effect on $\dot{V}O_{2MAX}$ ($F_{1,13}$ = 0.89, *p* = 0.36, lme). Both $\dot{V}O_{2STANDARD}$ and $\dot{V}O_{2MAX}$ increased with animal body mass ($\dot{V}O_{2STANDARD}$ -$F_{1,12}$ = 13.78, *p* < 0.001, lme; $\dot{V}O_{2MAX}$ - $F_{1,13}$ = 11.26, *p* < 0.01, lme).

Absolute aerobic scope (*AAS*) was thermally insensitive ($F_{1,12}$ = 1.26, *p* = 0.32, lme) but wider in 34∘C-acclimated animals compared to 28∘C-acclimated at 34°C and 36°C (Fig. 4.2 C, $F_{1,8}$ = 8.29, *p* < 0.05, lme). For example, *AAS* averaged 3.9 ± 0.2 ml min^{-1} kg^{-1} body mass (mean ± s.e.) and 10.9 ± 2.0 ml min^{-1} kg^{-1} bm (mean ± s.e.) at 36∘C, in 28∘C-acclimated and 34∘C-acclimated animals, respectively. In contrast, factorial aerobic scope (*FAS*) was thermally insensitive (Fig. 4.2 D, *p* = 0.36, $F_{2,12}$ = 1.13, lme) and did not differ between thermal acclimation treatments (Fig. 4.2 D, *p* = 0.74, $F_{1,8}$ = 0.11, lme).

Comparisons of temperature quotients (Q_{10})

Comparisons of temperature quotients between resting ($\dot{V}O_{2STANDARD}$), surface ($\dot{V}O_{2SURFACE}$), diving ($\dot{V}O_{2DIVE}$) and maximum ($\dot{V}O_{2MAX}$) rates of oxygen consumption show $\dot{V}O_{2DIVE}$ to be the most thermally sensitive (Q_{10} = 7.4), followed by surface (Q_{10} = 2.33)

and resting (Q_{10} = 2.28), and maximum oxygen consumption was thermally insensitive (Q_{10} = 1.05) (Fig. 4.3).

Discussion

Ectotherm aerobic capacity and associated performance are predicted to be severely reduced under climate warming, but aerobic capacity in *C. porosus* maintained thermal independence between 28 - 36°C. Moreover, *AAS* was thermally plastic and increased in warm-acclimated animals due to elevated $\dot{V}O_{2MAX.}$ In contrast, $\dot{V}O_{2STANDARD}$ was not phenotypically plastic, and increased markedly with rising temperature in both acclimation groups. Collectively, these findings oppose the 'plastic floors and concrete ceilings' hypothesis and also suggest aerobic capacity may be maintained under climate warming.

Thermal independence of aerobic scope

A narrowing of aerobic scope at elevated temperatures typically stems from $\dot{V}O_{2STANDARD}$ rising beyond the rate of increase in $\dot{V}O_{2MAX}$ (Pörtner and Knust, 2007). Resting oxygen consumption increased markedly between 28°C and 36°C ($Q_{10 =}$ 3.16), reflecting the typical metabolic response of ectotherms to acute thermal increases (Schulte, 2015). Similar Q_{10} values have been reported for resting *C. porosus* over a comparable temperature range ($\dot{V}O_{2STANDARD,} Q_{10}$ = 2.68, range = 20 - 33°C; Grigg 1978). Temperature quotients at upper test temperatures (i.e. 34°C - 36°C; Q_{10} = 3.6) were higher compared to lower temperatures (i.e. 28°C - 34°C; Q_{10} = 3.01). This pattern has been observed in juvenile *C. porosus* at lower temperatures and reflects an exponential curve (Wright, 1986). Exponential rises in $\dot{V}O_{2STANDARD}$ suggest basal energy requirements will be elevated under climate warming.

In contrast, maximum oxygen consumption did not change with test temperature. Maximum oxygen consumption may be optimised at temperatures below 28°C. However, previous measurements of $\dot{V}O_{2MAX}$ in juvenile *C. porosus* derived lower values at temperatures below 28°C (i.e. 20°C $\dot{V}O_{2MAX}$ = 2.33 ml O$_2$ min^{-1} kg^{-1}; 25°C $\dot{V}O_{2MAX}$ = 2.67 ml O$_2$ min^{-1} kg^{-1}; Wright, 1986) suggesting $\dot{V}O_{2MAX}$ reached a plateau at maximal levels in this study. Values derived for $\dot{V}O_{2MAX}$ align with values recorded in *C. porosus* following exhaustive exercise on a treadmill, suggesting the chase method used here is

equally effective in eliciting maximal cardiorespiratory responses (Owerkowicz and Baudinette, 2008).

Aerobic scope (both *AAS* and *FAS*) declined slightly with increasing temperature in 28∘C-acclimated animals, but this pattern was not significant. Despite disparate sensitivities of $\dot{V}O_{2STANDARD}$ and $\dot{V}O_{2MAX}$, aerobic scope remained thermally insensitive. Thermal independence of aerobic scope in *C. porosus* was unexpected (rejecting H$_1$), but similar findings have been documented in Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) (Verhille et al., 2016; Poletto et al., 2017). For example, *AAS* of *O. tshawytscha* was maintained across a wide thermal range approaching upper tolerance limits (i.e. 12 - 23∘C; Poletto et al., 2017). Constraints on aerobic scope at elevated temperatures appear species specific. Aerobic capacity does not universally narrow at elevated temperature, and even continually increases in some species at temperatures approaching lethal limits (Norin et al., 2014).

Thermal plasticity of aerobic capacity
The 'plastic floors and concrete ceilings' framework postulates $\dot{V}O_{2STANDARD}$ to be thermally plastic and $\dot{V}O_{2MAX}$ to be thermally fixed in fishes (Sandblom et al., 2016). *Crocodylus porosus* showed the opposite pattern (supporting H$_2$); maximum rates of oxygen consumption doubled in 34∘C-acclimated animals and consequently widened *AAS*. Resting rates of oxygen consumption were not thermally (phenotypically) plastic and $\dot{V}O_{2STANDARD}$ increased at similar rates between 28 - 36∘C in both acclimation treatments. Although these findings differ to the 'plastic floors and concrete ceilings' principle, they align with previous investigations of physiological plasticity in *C. porosus.* Thermal acclimation capacity at elevated temperatures is absent in predator avoidance diving performance/metabolism in *C. porosus* (Chapters 2 - 3). Predator avoidance dives are constrained by 'physiological floors' as animals are inactive and enter a hypometabolic state where oxygen requirements are reduced from surface levels (Andersen, 1966). Absent thermal plasticity in both resting and diving metabolism suggest physiological floors are constrained in this species.

Crocodylians have low basal energy requirements compared to other ectotherms (Grigg and Kirshner, 2015) and a lack of plasticity in 'physiological floors' may reflect cardiorespiratory functions which are already evolved to minimal limits. Similarly,

$\dot{V}O_{2MAX}$ capacities in athletic ectotherms (e.g. salmonids) may already be maximised, leading to fixed maximal cardiorespiratory functions but plastic physiological floors. It appears that ectotherms can thermally compensate either $\dot{V}O_{2MAX}$ or $\dot{V}O_{2STANDARD}$ to elevated temperatures but not both; however a greater number of assessments are required to gauge broad scale patterns.

Relationship between diving metabolism and aerobic capacity

Performance decrements (e.g. diving, swimming, growth and reproduction) are hypothesised to occur at temperatures beyond optima for aerobic scope (Pörtner, 2001; Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner, 2010; Eliason et al., 2011). In contrast to this, diving performance in *C. porosus* declines at temperatures above 28◦C (Chapters 2 - 3) but aerobic scope is maintained between 28 – 36◦C. Taken together, this suggests aerobic scope cannot be used as a proxy to predict the thermal sensitivity of diving performance. Diving performance is likely entirely independent of $\dot{V}O_{2MAX}$. Likewise, the thermal sensitivity of diving metabolism was threefold higher than $\dot{V}O_{2STANDARD}$ and estimations of aerobic limits on diving based on $\dot{V}O_{2STANDARD}$ measurements likely underestimate thermal sensitivity.

Diving is not the only performance trait which declines at elevated temperatures independently of changes in aerobic scope. Decrements in cardiac output, growth, swimming performance and reproductive output have been observed in ectotherms despite maintained or increased aerobic scope across the same thermal range (Clark et al., 2011; Johansen and Jones, 2011; Healy and Schulte, 2012; Ern et al., 2014; Gräns et al., 2014). These findings suggest mechanisms other than oxygen demands exceeding supply/delivery capacities limit performance (Clarke et al., 2013). Indeed, aerobic scope may be a performance trait in itself with a thermal optima differing to optimal temperatures of other traits (i.e. multiple performances, multiple optima, Clark et al., 2013). My findings suggest assessments solely measuring aerobic scope to predict the vulnerability of diving ectotherms to climate change underestimate susceptibility. Moreover, assessing the sensitivity and flexibility of both resting and maximum cardiorespiratory functions is essential as disparities and trade-offs appear to be present and species specific (Sandblom et al., 2016).

Figures
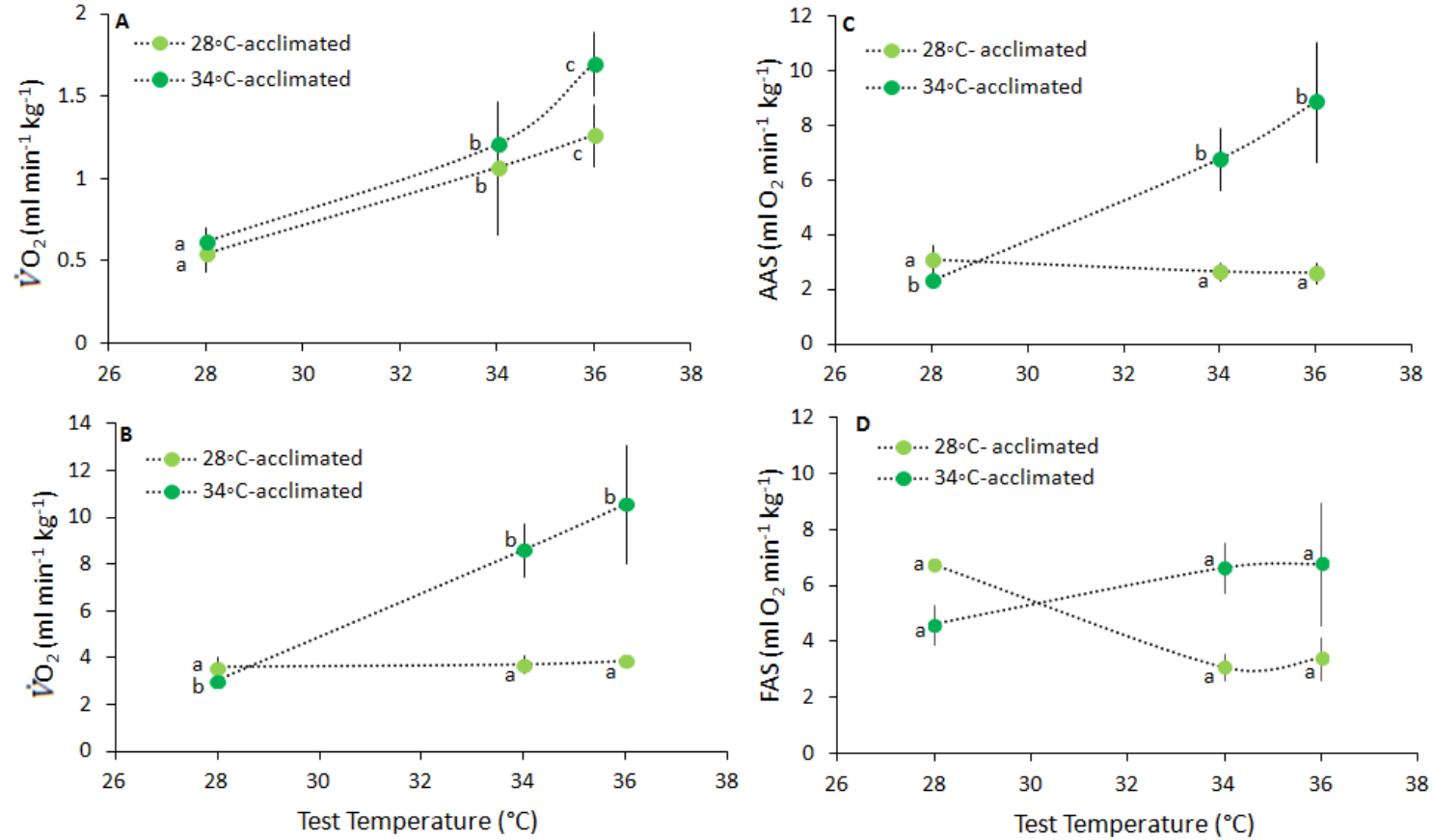
Figure 4.2: Thermal sensitivity and plasticity of (**A**) resting oxygen consumption ($\dot{V}O_{2STANDARD}$), (**B**) maximum oxygen consumption ($\dot{V}O_{2MAX}$) (**C**) absolute aerobic scope (*AAS*) and (**D**) factorial aerobic scope (*FAS*) in juvenile estuarine crocodiles (*Crocodylus porosus*) acclimated to either 28◦C (light green circles, *N* = 4) or 34◦C (dark green circles, *N* = 6). $\dot{V}O_{2STANDARD}$ increased 2.5-fold between 28◦C and 36◦C ($p < 0.001$, lme) and was independent of thermal acclimation treatment ($p = 0.44$, lme). $\dot{V}O_{2MAX}$ was thermally insensitive ($p = 0.058$, lme) but plastic ($p < 0.01$, lme). *AAS* was thermally insensitive ($p = 0.32$, lme) but wider in 34◦C-acclimated animals compared to 28◦C-acclimated ($p < 0.05$, lme). *FAS* was thermally insensitive ($p = 0.36$, lme) and did not differ between thermal acclimation treatments ($p = 0.74$, lme). Different lowercase letters indicate statistically significant differences between groups.
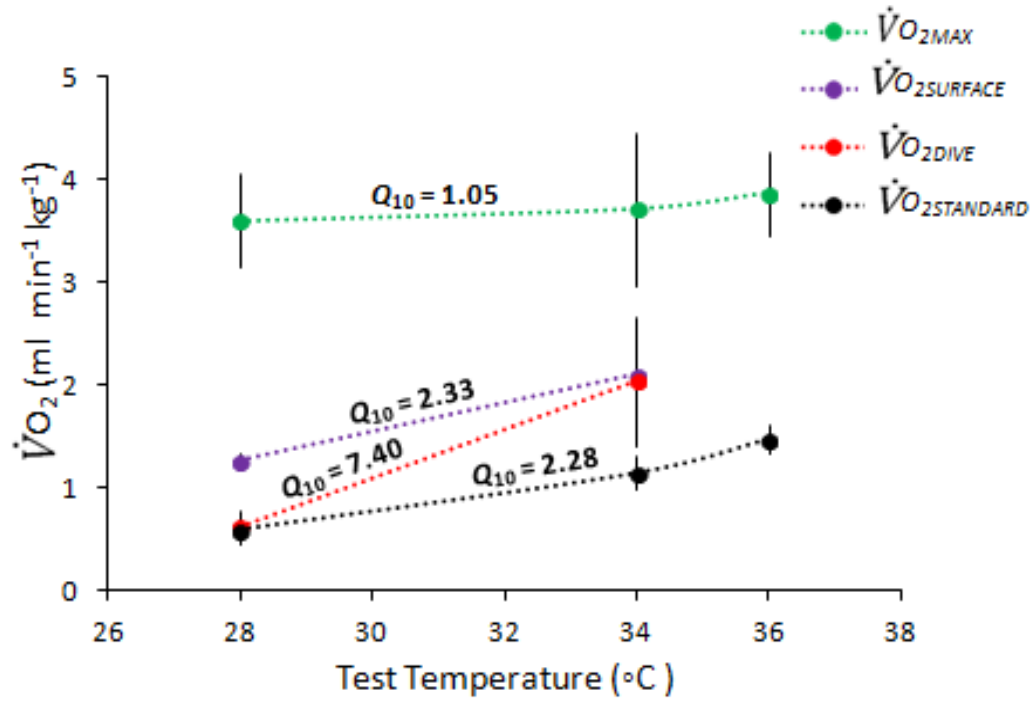
Figure 4.3: Comparisons of temperature quotients (Q_{10}) between resting ($\dot{V}O_{2STANDARD}$), surface ($\dot{V}O_{2SURFACE}$), diving ($\dot{V}O_{2DIVE}$) and maximum ($\dot{V}O_{2MAX}$) rates of oxygen consumption in juvenile estuarine crocodiles (*Crocodylus porosus*) acclimated to 28°C. $\dot{V}O_{2DIVE}$ was the most thermally sensitive ($Q_{10} = 7.4$), followed by $\dot{V}O_{2SURFACE}$ ($Q_{10} = 2.33$) and $\dot{V}O_{2STANDARD}$ ($Q_{10} = 2.28$), and $\dot{V}O_{2MAX}$ was thermally insensitive ($Q_{10} = 1.05$).

CHAPTER 5

THERMAL SWITCH IN DIVING BEHAVIOUR: SEASONAL TEMPERATURE INCREASES ASSOCIATED WITH REDUCTIONS IN SUBMERGENCE TIMES

Abstract

Rises in water temperature accompanying climate change are predicted to compromise the dive capacity of air-breathing ectotherms, and assessments of how animals are faring with present-day thermal fluctuations are an essential starting point. Submergence behaviour of 24 adult *C. porosus* (total length range: 2.02 – 4.60 m) free-ranging throughout the Wenlock River (Cape York Peninsula, QLD, AUS; S 12.350°, E 142.107°) was assessed during the second hottest year on record, globally (2015). Satellite transmitters with wet-dry sensors were fixed to the nuchal shield of animals and used to continuously record dive events between August and December. Benthic river water temperature (Tw) was recorded every 30 min using conductivity-temperature-depth loggers. Dive duration (i.e. minutes submerged) and frequency (i.e. dives h^{-1}) were modelled as a function of Tw. Aerobic dive limits (cADL) were estimated for animals in the coldest (i.e. August; mean Tw = 26∘C) and warmest month (i.e. December; mean Tw = 30∘C), and compared to observed submergence times. Submergence times were inversely related to Tw, with mean and maximum dive durations reducing by 40% (Q_{10} = 0.35; $p < 0.001$; GAMM) and 18% (Q_{10} = 0.68; $p < 0.001$; lme), respectively, over the 5∘C differential (Tw range = 26 - 31∘C). The majority (95%) of routine dives fell within cADLs regardless of month or Tw, but a greater percentage of maximum dives exceeded cADLs in December (27.4%) compared to August (18.7%) ($p < 0.01$; GLMM). These results suggest the aerobic dive capacity of *C. porosus* is influenced by present-day seasonal thermal increases.

Introduction

Prolonged submergences of air-breathing divers are thought to facilitate obligate underwater activities such as foraging/hunting, predator avoidance, sleep/rest and social interactions (Kramer, 1988). Optimising time underwater is constrained by the need to surface and replenish oxygen stores and many divers routinely restrict dive times within aerobic limits (Burggren et al., 1989; Hochscheid et al., 2005; Pratt et al., 2010). The aerobic dive limit (ADL) is a conceptual representation of the maximum submergence time an animal can sustain prior to the use of anaerobic metabolism and associated lactate accumulation and acidosis (Butler, 2006). A diver's ADL can be calculated by dividing its total body oxygen (TBO) stores by the rate at which oxygen is consumed (i.e. metabolic rate). Ectothermic ADLs are sensitive to changes in environmental temperature due to the tight relationship between ectotherm body temperature and ambient temperature. As the body temperature of an ectotherm equilibrates with water temperature (Tw), its metabolic rate and hence oxygen depletion rate become directly correlated to Tw, with diving oxygen stores consumed more rapidly as temperature rises (Udyawer et al., 2016; Chapter 3).

Increases in water temperature, be it seasonal fluctuations or forced climate change, present a unique threat to ectothermic obligate divers (e.g. sea snakes, marine iguanas, turtles and crocodylians). Time available for dive-dependent activities may be severely reduced, with animals required to surface and replenish oxygen stores more frequently. An inverse relationship between dive duration and Tw, coupled with increased surfacing frequency has been documented in numerous species, including sea snakes (Udyawer et al., 2016), marine and freshwater turtles (Southwood et al., 2003; Storey et al., 2008), newts (Samajova and Gvozdik, 2009) and freshwater crocodiles (Campbell et al., 2010a). Submergence times are predicted to decline precipitously with climate change (Campbell et al., 2010a; Rodgers et al., 2015; Chapter 2), but it is unclear how some species are faring with present-day thermal fluctuations (e.g. seasonal changes).

Seasonal changes in Tw vary with latitude, with some environs fluctuating < 1∘C year^{-1} (typically tropical and polar systems) and others varying > 30∘C year^{-1} (typically temperate) (Mundahl, 1989). Increases in seasonal Tw may alter diving behaviour in several forms dive durations may be shortened due oxygen being consumed at a faster rate, or dive durations may remain unchanged but animals increasingly rely on anaerobic metabolism, incurring the cost of longer post-dive surface intervals (PDSI) to clear anaerobic debt (Kooyman et al., 1980; Costa et al., 2004). Alternatively, divers may compensate for gradual thermal changes by modifying their physiology to express seasonal phenotypes (Fry, 1958; Wilson and Franklin, 2002).

The capacity to responsively alter underlying physiology (e.g. cardiac output, diving metabolic rates) to environmental perturbations is termed acclimation (in the laboratory) or acclimatisation (in the field) (Wilson and Franklin, 2002). Ectothermic divers generally have a limited capacity to thermally acclimate/acclimatise diving performance to elevated temperature (Gordos et al., 2003; Bradshaw et al., 2007). Diving performance in the Mary River turtle (*Elusor macrurus*), for example, only partly acclimates to temperatures of 28°C (Clark et al., 2008). Likewise, submergence times of free-ranging marine and freshwater turtles are reduced in summer months compared with winter months (Carr et al.,1980; Gordos et al., 2003; Bradshaw et al., 2007), suggesting these ectotherms are not fully compensating for present-day seasonal changes.

The estuarine crocodile (*Crocodylus porosus*) is an aerially-respiring (Wright, 1986), ectothermic diver and experiences seasonal shifts in Tw of approximately 6∘C (Grigg and Kirshner, 2015). The survival and reproductive success of crocodylians are thought to be linked to dive capacity because predator avoidance, foraging, sleep/recovery and social interactions occur underwater (Seebacher et al., 2005b; Campbell et al., 2010b). Our understanding of the physiological mechanisms supporting dives in *C. porosus* predominantly stems from experimental studies on juveniles. Voluntarily dives are short in duration (~5 min) but can be prolonged up to 30 min in animals diving responsively to a perceived threat (e.g. loud noise produced by experimenter; Wright, 1987; Rodgers et al., 2015). Physiological alterations, termed the 'dive response', often accompany submergences and include a suite of cardiovascular modifications (Andersen, 1966). These alterations facilitate prolonged submergences by

lowering oxygen demands from surface levels (Davis et al., 2004; Hastie et al., 2007). The dive response is more pronounced in animals performing predator avoidance dives compared to undisturbed dives (Gaunt and Gans, 1969; Noren et al., 2012). Aerobic limits on predator avoidance dives are therefore lessened (i.e. longer ADLs) compared to routine dives due to a slower depletion of TBO stores.

Dive durations of juvenile *C. porosus* show precipitous declines at temperatures exceeding 28◦C under experimental conditions (Rodgers et al., 2015; Chapters 2 – 3). This marked drop in performance stems from the thermal sensitivity of both diving metabolic rate and metabolic depressive capacity (Chapter 3). For example, between the temperatures of 28◦C - 34◦C, diving oxygen requirements increased threefold, and metabolic depressive capacity reduced by ~46% (Chapter 3). Juvenile *C. porosus* appear unable to acclimate diving performance at temperatures exceeding 28◦C (Rodgers et al., 2015), but summer Tw regularly exceeds this threshold (Bureau of Meteorology Australia, 2017). Taken together, these observations suggest *C. porosus* may be inhabiting microclimates nearing its upper thermal limits to maintain diving performance and slight increases in Tw could pose a threat (Sunday et al., 2014).

Very little is known about the free-ranging diving behaviour of *C. porosus*, despite it being the largest, extant, semi-aquatic predator. A prior attempt to remotely monitor diving behaviour of *C. porosus* yielded data for only a single animal (9.75 kg) over an 8 d recording period due to tag dysfunctions (Grigg et al., 1985). This individual performed very short dives (3 - 5 min) and two prolonged dives (22 min and 30 min); whether these data are representative of typical diving behaviour is unclear. Estuarine crocodiles dive in response to unfavourable surface water currents, to depths of 2 - 5 m (resting on the river substratum) potentially as an energy-saving strategy (Campbell et al., 2010c), but the diving repertoire of *C. porosus* remains largely uncharacterised.

The aim of this study was threefold: i) to describe the diving repertoire of free-ranging *C. porosus* (average dive frequency, depth and duration), ii) to examine the relationship between seasonal thermal increases in Tw and diving behaviour (i.e. dive duration, frequency and cumulated time spent underwater) and iii) to estimate the effect of Tw on cADLS and compare estimates to observed dive durations. Crocodiles were fitted with depth and wet-dry sensors and dive behaviour was monitored

throughout 2015; the second hottest year on record globally (NOAA, 2016). We predicted: H_1) dive durations would be inversely related to Tw reflecting a faster depletion of oxygen stores, H_2) cumulative time spent underwater water would decrease with increasing Tw, H_3) dive frequency would increase with rising Tw to compensate for shorter submergence times, and H_4) aerobic dive limits would be exceeded more frequently during summer months compared to winter months due to *C. porosus* already living close to its upper thermal limits for diving performance.

Materials and Methods

Study Site and Animal Tagging

Adult estuarine crocodiles (*Crocodylus porosus*; $N = 24$) were trapped along the Wenlock River, Cape York Peninsula, Australia during annual trips each August between 2008 – 2015 (S 12.350◦, E 142.107◦; Fig. 5.1). Traps were baited with wild pig (*Sus scrofa*) and were positioned along the river bank or floated on the water surface. Once captured, animals were manually restrained and body size (total length, TL; and snout-vent length, SVL) and sex were recorded (Table 5.1).

Coded acoustic transmitters (VEMCO V16T, 98 [L] x 16 [D] mm, 36 g, Nova Scotia, Ca) with inbuilt pressure sensors for depth measurements (maximum depth of 34 m, resolution = 0.1 m) were surgically implanted in 19 animals behind the left forelimb. Implantation of tags behind the forelimb ensured transmitters were always submerged and detected when animals were in the water (Franklin et al., 2009). Local anaesthetic (Lignocaine, Troy Laboratories, Smithfield, Aus) was administered prior to surgery and the procedures outlined in Franklin et al., (2009) were followed. Transmitter data, along with a unique identification code were acoustically transmitted on 69 kHz at a power output of 158 dB, at a pseudo-random time intervals between 90 and 120 s.

Satellite transmitters (TGM-4310, Telonics, Arizona, U.S.A) were attached to the nuchal shield of animals and held in place with plastic-coated, stainless steel wire threaded through holes drilled in the osteoderms. Six animals were fitted with satellite transmitters in August 2014/2015. Satellite tags had wet-dry sensors which were used to identify dive events. Wet-dry sensors had a conductivity switch, whereby the switch was activated during submergence in seawater and became inactive when dry or in

freshwater. Satellite tags were positioned on the animal's nuchal shield to ensure the wet-dry sensor became dry (i.e. no conductivity) when the crocodile's head was out of the water (Fig. 5.2). All tags weighed < 3% of the smallest animal's estimated body mass and the tagging procedures took ≤ 60 min (Franklin et al., 2009). Satellite transmitters were designed to fall off animals within 18 months of tagging and acoustic transmitters were designed to be retained for 5 - 8 years, without any subcutaneous movement or biofouling (Franklin et al., 2009). Data from the first 2 d following release were discarded to eliminate atypical behaviour associated with capture (Seebacher et al., 2005b). The geographic fixes and dive data (i.e. average dive duration, maximum dive duration and percent time spent submerged) were recorded by the ARGOS satellite system every 3 h.

Acoustic Array and Environmental Data

An acoustic tracking system was established, consisting of 31 omni-directional hydrophones deployed (VR2-W, VEMCO, Nova Scotia, Ca) throughout a 120 km stretch of the Wenlock River system (Fig. 5.1), with distance between hydrophones ranging between 3 – 17 km. Conductivity-temperature-depth (CTD; DST milli-CRD, Star Oddim Gardabear, Iceland) loggers were attached to two hydrophones to monitor benthic/deep water temperature and salinity fluctuations. CTD loggers recorded measurements every 30 min. Data were downloaded from hydrophones and CTDs annually.
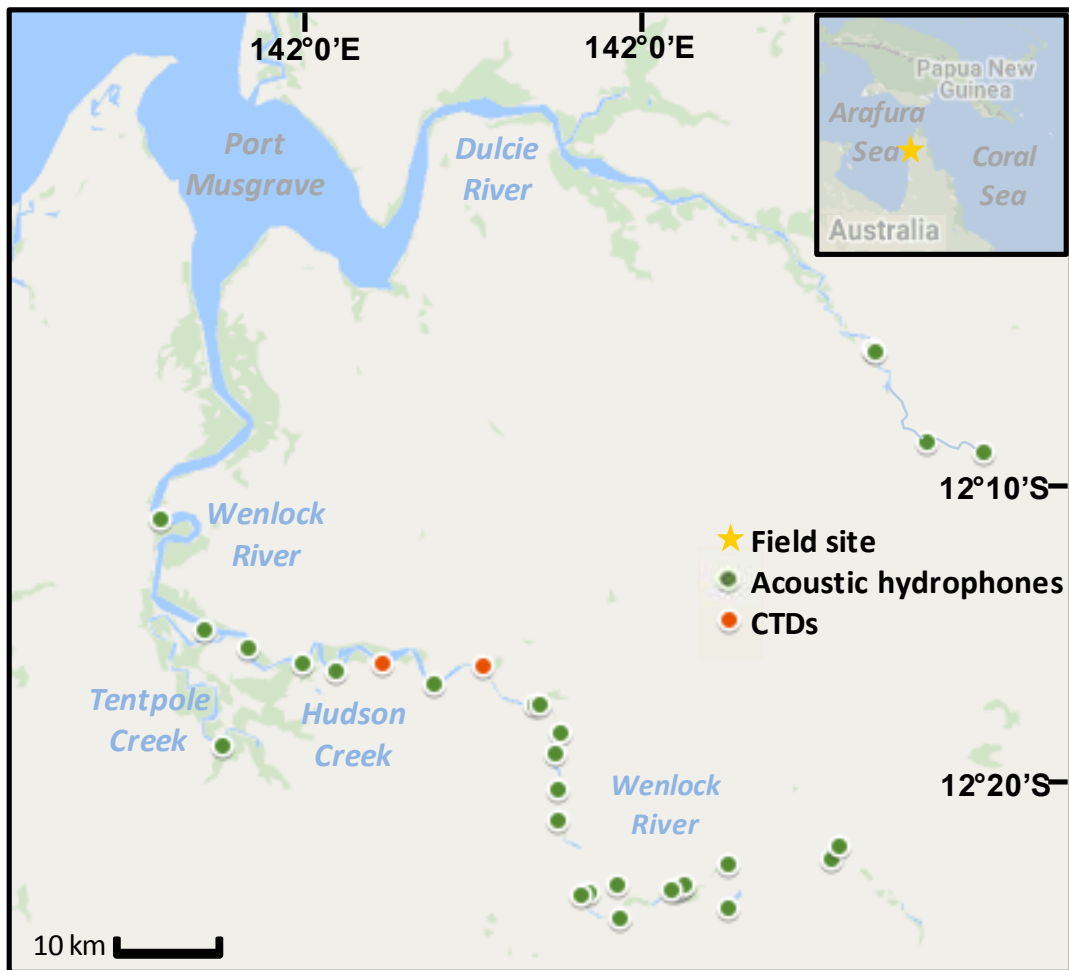
Figure 5.1: Field site, the Wenlock River system, Cape York Peninsula (S 12.20°, E 142.05°). Yellow star on inset marks river position within Queensland, Australia. Green circles mark locations of acoustic hydrophones and red circles show locations of water temperature loggers (i.e. CTDs).

Data Management

Dive events recorded by the satellite wet-dry sensors were synchronised with the closest water temperature recording in time and geographic space. Dives which occurred > 5 km from the CTD loggers were excluded from analyses (only ARGOS location classes 1-3 were included). Wet-dry sensors did not operate in freshwater and satellite fixes of animals were used to confirm when animals entered freshwater river reaches, allowing real zeros in dive frequency counts to be distinguished from zeros arising from freshwater inactivation of tags. Dive depth profiles were extracted from the acoustic fixes in R-Studio (version 3.1.3; R Core Team, 2012) using the behavioural event qualifier (BEQ) function in V-Track (Campbell et al., 2012); where a dive was defined using a minimum depth threshold (≥ 0.5 m) and a maximum time between detections threshold (60 min), to ensure animals did not leave the field of detection before resurfacing. The termination depth threshold was set to ≤ 0.5 m to qualify the end of a dive event. Sensitivity analyses were performed on a subset of acoustic data in order to parameterise the time threshold, with the aim of ensuring all dive events were extracted and maximum dive duration was not overestimated (Appendix 5.1, Fig. A5.1 A - B). One animal migrated 200 km south of the study site and resided in the Kendall River (QLD, AUS) during 2015 and was therefore excluded from analyses.

Figure 5.2: Adult estuarine crocodile (*Crocodylus porosus*) with a wet-dry satellite transmitter attached to its nuchal shield. The wet-dry sensor is entirely exposed to air when the animal is at the water surface. Photo credit: Craig Franklin.

Estimating total body oxygen stores and aerobic dive limits

Pulmonary, blood and tissue oxygen stores were estimated based on animal body size, and summed to determine total body oxygen (TBO) stores. Crocodile body mass (M_b) was estimated using a known mass-length relationship derived by Grigg and Kirshner (2015):

$$(1)\ M_b = 2.658\ TL^{3.242}$$

Where M_b is body mass (kg) and TL is total length (m). Lung volume (V_L , ml) of *C. porosus* scales allometrically with M_b, with an exponent of 0.906 (N = 24; r^2 = 0.96; p < 0.01; Wright and Kirshner, 1987). Blood volume was assumed to be the same as the American alligator (*Alligator mississippiensis*; 72.7 ml kg^{-1}, Huggins, 1961) and tissue volume was assumed to be 500 ml kg^{-1} (Wright, 1985). Equations derived by Wright (1985) were used to estimate blood and tissue oxygen stores at body temperatures of 25∘C and 30∘C; such that:

$$(2)\ pulmonary\ O_2 = 13.8 \times 10^{-3} M_b{}^{.906}\ (25∘C); O_2 = 12.8 \times 10^{-3} M_b{}^{.906}\ (30∘C)$$

$$(3)\ tissue\ O_2 = 0.44 \times 10^{-3} M_b\ (25∘C); O_2 = 0.42 \times 10^{-3} M_b\ (30∘C)$$

$$(4)\ blood\ O_2 = 6.2 \times 10^{-3} M_b\ (25∘C); O_2 = 4.7 \times 10^{-3} M_b\ (30∘C)$$

Calculated aerobic dive limits (cADL; min) were estimated using Butler's (2006) equation:

$$(5)\ cADL = TBO/SMR$$

Where TBO is the sum of pulmonary, tissue, blood O_2 stores (ml) and SMR is standard metabolic rate (ml min^{-1}). cADLs were estimated for both summer and winter water temperatures (i.e. 30∘C summer; 25∘C winter), accounting for the thermal sensitivity of both TBO (Wright, 1985) and SMR (Seymour et al., 2013). Aerobic limits were estimated for dives with no metabolic alterations (i.e. at $\dot{V}O_{2STANDARD}$ and dives with a 52% depression of $\dot{V}O_{2STANDARD}$ based on empirical values determined in Chapter 3.

Statistical analyses

Data analyses were performed in R Studio (version 3.1.3; R Core Team, 2012) using the mgcv (mixed GAM computation vehicle; Wood, 2000), nlme (linear and nonlinear mixed effects models; Pinheiro et al., 2012), lattice (Sarkar, 2008), cosinor (Sachs, 2014) and V-Track (Campbell et al., 2012) packages. Time stamps for dive events were adjusted from coordinated universal time (UTC) to Australian Eastern standard time (AEST) and converted into radians. Diel rhythms in diving behaviour (i.e. time spent submerged, dive frequency, average dive durations and maximum dive durations) were assessed by fitting cosinor curves to data (appropriate for circular data). A series of general additive mixed models (GAMM) and linear mixed effects models (lme) were used to determine the effects of water temperature on dive durations (minutes submerged; Gaussian distribution), dive frequency (dives h^{-1}; Gaussian distribution) and proportion of time spent underwater (%; binomial distribution). Water temperature was included as a fixed effect, total length as a fixed covariate and time of day and animal identification number (ID) as nested random effects. Response variables were log-transformed and smoothers were applied where necessary. The use of GAMMs allowed for a non-linear relationship between the response variable and multiple explanatory variables, in conjunction with the non-independence of errors arising from multiple measurements on the same crocodiles. GAMM random structure was optimised using residual maximum likelihood (REML) and the fixed structure was subsequently optimised using maximum likelihood (ML) (Zuur et al., 2009). GAMM simplification was achieved by comparing Akaike information criterion (AIC) and Bayesian information criterion (BIC) values. The proportion of dives exceeding cADLS (%; binomial distribution) was compared between August and December using a general linear mixed model (GLMM), with month as a binary indicator variable and ID as a random effect. *P*-values < 0.01 were considered statistically significant for GAMM analyses as suggested by Zuur et al., (2009), and a $p \leq 0.05$ significance threshold was used for all other analyses.

Results

Diving repertoire

Dive durations were 4.4 ± 4.8 min (median ± interquartile range, IQR) and maximum submergence times were 24.2 ± 19.7 min (median ± IQR; Fig. 5.3 A). The longest recorded dive (with a matching Tw recording) lasted 4 h and was performed by a 2.9 m (TL) animal in November at a water temperature of 30.8∘C. Crocodiles spent an average of 42.6 ± 30.0% (mean ± s.d.) of the day underwater and dived to depths of 0.7 ± 1.0m (median ± IQR), with maximum dive depth recorded as 14.3 m (Fig. 5.3 B). Cosinor curves showed significant diurnal acrophases (i.e. peaks) between 10.00-13.00 in percent time underwater (amplidutde, amp. = 94.7; acrophase, acro. = 0.8, $p < 0.001$), maximum dive duration (amp. = 79.3; acro. = 0.8, $p < 0.001$) and average dive duration (amp. = 17.5; acro. = 0.8, $p < 0.001$) (Fig. 5.3 C - D). Dive frequency displayed no diel rhythm (amp. = 3.28, acro. = -1.2, $p = 0.6$), with animals performing 7.1± 4.1 dives h^{-1} (median ± IQR) regardless of time of day

Thermal sensitivity of submergence behaviour

Mean and maximum dive durations were inversely correlated with water temperature (mean, GAMM; $F_1 = 22.88$, $p < 0.0001$; max., lme, $F_{1,1279} = 13.8$, $p < 0.001$; Fig. 5.4 A, Appendix 5.2, Fig. A5.2 A). Dives performed at 26∘C were 6.9 ± 5.9 min (median ± IQR), compared to 4.1 ± 3.6 min (median ± IQR) at 31∘C (~40% reduction; Fig. 5.4 A). Similarly, maximum submergence times were reduced by ~18% between 26 - 31∘C (Fig. 5.4 A). Mean submergence times were independent of body size (i.e. TL) (GAMM, $F_1 = 0.68$, $p = 0.79$), but maximum dive durations decreased with body size (lme, $F_{1,31} = 9.9$, $p < 0.01$). Dive frequency increased with Tw between 27 - 29.5∘C and reached a plateau between 29.5 - 31∘C (GAMM; $F_1 = 8.86$, $p < 0.0001$, Fig. 5.4 B, Appendix 5.2, Fig. A5.2 B). Percentage of time spent submerged decreased with water temperature (GAMM; $F_1 = 8.9$, $p < 0.001$, Fig. 5.4 B, Fig. 5.5, Appendix 5.2, Fig. A5.2 C).

Total body oxygen stores and aerobic dive limits

Total body oxygen stores at submergence were estimated to average 11.1 ± 0.2 ml kg^{-1} body mass (bm) and 9.3 ± 0.2 ml kg^{-1} bm (mean ± s.d.) for animals with body temperatures of 25∘C (~August water temperature) and 30∘C (~December water temperature), respectively. Aerobic dive limits for routine dives (i.e. no metabolic

depression) were estimated to average 27.0 ± 0.6 min and 16.7 ± 0.4 min (mean ± s.e.) in August and December, respectively. Aerobic limits on dives with a 52% reduction of standard metabolic rates were lengthened compared to routine dives, with cADLs of 54.0 ± 13.0 min (August) and 33.4 ± 0.8 (December). Mean dive durations exceeded cADLS more frequently in December (4.3 ± 1.4%) compared to August (1.6 ± 0.0%) (p <0.01, GLMM), but this effect was eliminated once metabolic depression (i.e. 48% of $\dot{V}O_{2STANDARD}$) was factored in (Fig. 5.6 A - B, p = 0.09, GLMM). Approximately 41.7 ± 3.8% of maximum dives with no metabolic depression exceeded cADLS regardless of month (p = 0.07, GLMM), but once metabolic depression was accounted for more dives exceeded ADLs in December (27.4 ± 3.7%) compared to August (18.7 ± 1.1%) (Fig. 5.6 A - B, p < 0.01, GLMM).

Discussion

Biotelemetry advancements (e.g. miniaturisation and extended battery lives) have opened the door to accurate, long term monitoring of species behavioural responses to environmental changes (Campbell et al., 2015). Submergences of free-ranging, adult *C. porosus* were remotely monitored to further our understanding of this species' diving behaviour and concurrently gauge the influence of thermal fluctuations. Routine dives of *C. porosus* were short in duration (~ 4.5 min) and occurred at shallow depths (~ 1 m) but prolonged (up to 4 h) and deep (up to 14 m) dives were also observed. Water temperature had an overbearing influence on diving behaviour, with both submergence times and time spent underwater significantly reduced with temperature increases (supporting H_1 and H_2). These findings suggest temperature increases presently experienced by *C. porosus* limit aerobic dive capacity.

Diving repertoire

Diving repertoires can vary greatly between species with respect to dive duration and depth (Schreer et al., 2001). Submergence behaviour of *C. porosus* was similar to that of freshwater crocodiles (*C. johnstoni*) with both species routinely performing short (< 12 min) and shallow (< 2 m) dives, well within estimated ADLs (Campbell et al., 2010b). Submergence times recorded here fall within the range recorded in a single *C. porosus* by Grigg et al. (1985), suggesting that individual shared a similar diving repertoire. Short and shallow dives have also been recorded in other ectotherms, including:

migrating green sea turtles, *Chelonia mydas* (Hays et al., 2001) and Arafura file snake
Acrochordus arafurae (Pratt et al., 2010). In contrast, some turtles inhabiting pelagic
zones can dive to depths beyond 100 m (e.g. leatherback turtle, *Dermochelys coriacea*;
Eckert et al., 1986; Eckert et al., 1989), and many freshwater turtles (e.g. painted turtle,
Chrysemys picta) can remain submerged for months during hibernation (Jackson, 2002).

Inter-species disparities in diving patterns led to the distinction between air-
breathers predominantly living at the water surface (i.e. < 50% at the surface), termed
'divers', and air-breathers predominantly living underwater (i.e. > 50% underwater),
termed 'surfacers' (Kooyman, 1989; Renaud and Carpenter, 1994). Crocodiles spent
42.6% of the day underwater, classifying this species as a 'diver', but partitioning of
time above and below the water surface was sensitive to both the time of day and water
temperature.

Distinctive dive patterns can be identified within a species' diving repertoire,
reflecting different functions (Hochscheid et al., 1999b; Hays et al., 2000). Multi-channel
loggers recording both dive events and animal activity (e.g. accelerometers and tail-beat
frequency monitors) can distinguish between active and inactive dives. A range of
functions have been attributed to varying dive profiles, including: short and shallow
foraging dives, subsurface travelling dives, and deep inactive resting dives (Hochscheid
et al., 1999a; Hochscheid et al., 1999b; Hays et al., 2004; Fossette et al., 2012).
Freshwater crocodiles, for instance, show two distinctive dive types: inactive resting
dives (~12 min) and active, short dives assumed to be associated with foraging
behaviour (< 1 min) (Campbell et al., 2010b). Dive functions of *C. porosus* were
indistinguishable as swimming activity was not monitored but diel patterns in
behaviour can provide insight. Time spent underwater peaked diurnally between 10.00
-13.00 and overlapped with the daily peak in maximum dive durations; potentially
reflecting long, resting dives conducive to a nocturnal nature.

Diving within aerobic limits
The large majority of routine dives (~95%) fell within estimated ADLs. Diving within
aerobic limits is considered an adaptive strategy, as the alternative, extending dives
anaerobically, requires prolonged post-dive surface intervals (PDSI) to clear
accumulated lactate and repay oxygen debt (Costa et al., 2004). Anaerobically

supported dives are likely reserved for life-threatening situations, such as predator avoidance or aggressive interactions with conspecifics. A greater number of maximum submergences exceeded ADLs in December (34%) compared to August (28%), with underwater activity concomitantly reduced, likely reflecting extended PDSIs. The maximum dive duration recorded here was 4 h, which to be supported aerobically would have required a 93% depression of $\dot{V}O_{2STANDARD}$ (i.e. diving metabolic rate = TBO stores/dive duration). This level of metabolic depression is unlikely as juvenile *C. porosus* and free-ranging *C. johnstoni* can only reduce heart rate by 65% and 12.5%, respectively (Wright et al., 1992; Seebacher et al., 2005b). This 4 h dive was likely partially fuelled by anaerobic metabolism and may have been followed by an extended surface period to clear anaerobic by-products.

Thermal sensitivity of diving behaviour
Seasonal fluctuations in temperature often elicit thermal acclimation/acclimatisation responses in ectotherms where underlying physiology is reorganised, to the end that thermal effects on performance are blunted (Johnston and Dunn, 1987). In distinction, dive durations of *C. porosus* were inversely related to water temperature. Mean dive durations reduced by 40% between the coolest (i.e. August) and warmest (i.e. December) months, despite a ~12 week time lag. Thermal acclimation responses typically occur within 2 - 6 weeks, giving the animals, here, a large time frame (Angilletta, 2009). A lack of thermal acclimatisation response may reflect many scenarios. For instance, several tropical ectotherms possess the capacity to acclimate to decreases in environmental temperature but not increases (relative to current habitat temperature), suggesting these animals are nearing their upper thermal limits (Somero, 2010). Alternatively, the cost of acclimatisation may be greater than the net benefits (Angilletta, 2009). Very little is known regarding the energetic (or other) costs associated with acclimatisation, but if functions other than diving are optimised at different temperatures (i.e. multiple performances- multiple optima hypothesis; Clark et al., 2013), acclimatisation responses may be complex.

Crocodiles transitioned from being 'surfacers' to 'divers' at water temperatures above 28∘C. For example, *C. porosus* spent an average of 13.1 h day^{-1} submerged in August and only 8.5 h day^{-1} in December. This dramatic decline in cumulated time spent

underwater likely stems from shortened dive durations in the absence of sufficient up-regulation of dive frequency. Increased dive frequency at elevated temperatures is considered a form of behavioural compensation and has been recorded in many ectotherms including: newts (Samajova and Gvozdik, 2009), sea snakes (Udyawer et al., 2016) and marine and freshwater turtles (Southwood et al., 2003; Storey, et al., 2008). Dive frequency of *C. porosus* increased with Tw between 27 - 29.5°C but reached a plateau at Tw exceeding this threshold (partially supporting H$_3$). Costs associated with travelling between the water surface and substratum above this thermal threshold may outweigh the benefit derived from up-regulating dive frequency. Alternatively, further increases in dive frequency may be unfeasible if animals are forced to spend time at the water surface clearing oxygen debt. Similar patterns are apparent in free-ranging *C. johnstoni*; dive durations declined with thermal increases and extended PDSIs followed prolonged dives (> 40 min) in summer, suggestive of lactate clearance and reestablishment of homeostasis (Seebacher et al., 2005b; Campbell et al., 2010a).

Although only associative in nature, the relationship between water temperature and submergence times derived similar Q_{10} values to experimental assessments; where juvenile *C. porosus* were acutely exposed to thermal increases across a similar range (Rodgers et al., 2015; Chapters 2 - 3). The mechanism underlying shortened dive durations at elevated Tws, likely stems from ectotherm body temperature equilibrating with Tw; thereby indirectly driving a faster depletion of TBO stores. Further work, synchronising diving behaviour with heart rate recordings, as a proxy for oxygen consumption, could test this hypothesis.

Water temperature appears to strongly influence diving behaviour of *C. porosus* but it is important to recognise the limitations inherent to all field studies. The animals are likely responding to a suite of environmental cues and may be concurrently exposed to stressors other than elevated Tw. Establishing causation is not viable but we are presented with an ecologically relevant representation of how water temperature influences diving behaviour. Nonetheless, time required for obligate underwater activities such as foraging for aquatic prey, social interactions, resting on the river substratum and predator avoidance was likely cut short during warmer months. It is unknown how much underwater time can be lost before fitness is compromised. However, time lost towards feeding opportunities likely reduces growth and increased

conspicuousness to predators may reduce survival rates. Tagging animals with a range of technologies, to gain higher resolution behavioural recordings (e.g. accelerometry) in combination with physiological recordings (e.g. body temperature and heart rate) could greatly enhance the transparency of these data (e.g. Clark et al., 2010).

Figures and Tables

Table 5.1: Overview of diving data from individual estuarine crocodiles (*Crocodylus porosus*) with satellite and acoustic tags. * indicates the individual excluded from analyses.

ID	Sex	TL (m)	SVL (m)	Total no. detections	Total no. dives	Median dive duration (min)	Max. dive duration (min)	Tag Type
171*	M	3.46	1.78	1211	33 037	2.37	310.72	Satellite (wet-dry)
134	M	3.56	1.83	163	3450	3.55	108.70	Satellite (wet-dry)
197	M	3.23	1.66	90	2089	4.63	124.08	Satellite (wet-dry)
196	M	4.60	2.31	746	19 148	1.75	228.47	Satellite (wet-dry)
189	M	3.89	2.04	1259	31 400	4.28	244.57	Satellite (wet-dry)
186	F	2.93	1.51	355	4628	1.80	237.85	Satellite (wet-dry)
1	M	3.57	1.75	5165	101	N.A.	N.A.	Acoustic (depth)
4	F	2.75	1.41	108	223	N.A.	N.A.	Acoustic (depth)
5	M	4.12	2.14	4424	158	N.A.	N.A.	Acoustic (depth)
6	F	2.80	1.44	3246	325	N.A.	N.A.	Acoustic (depth)
7	M	2.02	1.01	134	32	N.A.	N.A.	Acoustic (depth)
8	M	2.90	1.49	4977	88	N.A.	N.A.	Acoustic (depth)
10	F	3.27	1.65	107	13	N.A.	N.A.	Acoustic (depth)
11	M	2.13	1.08	9039	180	N.A.	N.A.	Acoustic (depth)
13	F	2.62	1.35	1173	325	N.A.	N.A.	Acoustic (depth)
14	M	3.58	1.86	4977	76	N.A.	N.A.	Acoustic (depth)
15	M	1.94	0.99	11866	18	N.A.	N.A.	Acoustic (depth)
25	M	3.15	1.74	2676	14	N.A.	N.A.	Acoustic (depth)
29	M	3.69	1.87	4495	162	N.A.	N.A.	Acoustic (depth)
40	M	3.61	1.82	5541	170	N.A.	N.A.	Acoustic (depth)
41	F	2.91	1.49	1491	236	N.A.	N.A.	Acoustic (depth)
43	M	4.17	2.12	526	128	N.A.	N.A.	Acoustic (depth)
47	M	3.11	1.54	74423	23	N.A.	N.A.	Acoustic (depth)
69	M	4.51	2.39	1148	64	N.A.	N.A.	Acoustic (depth)
71	M	4.34	2.21	17906	9	N.A.	N.A.	Acoustic (depth)
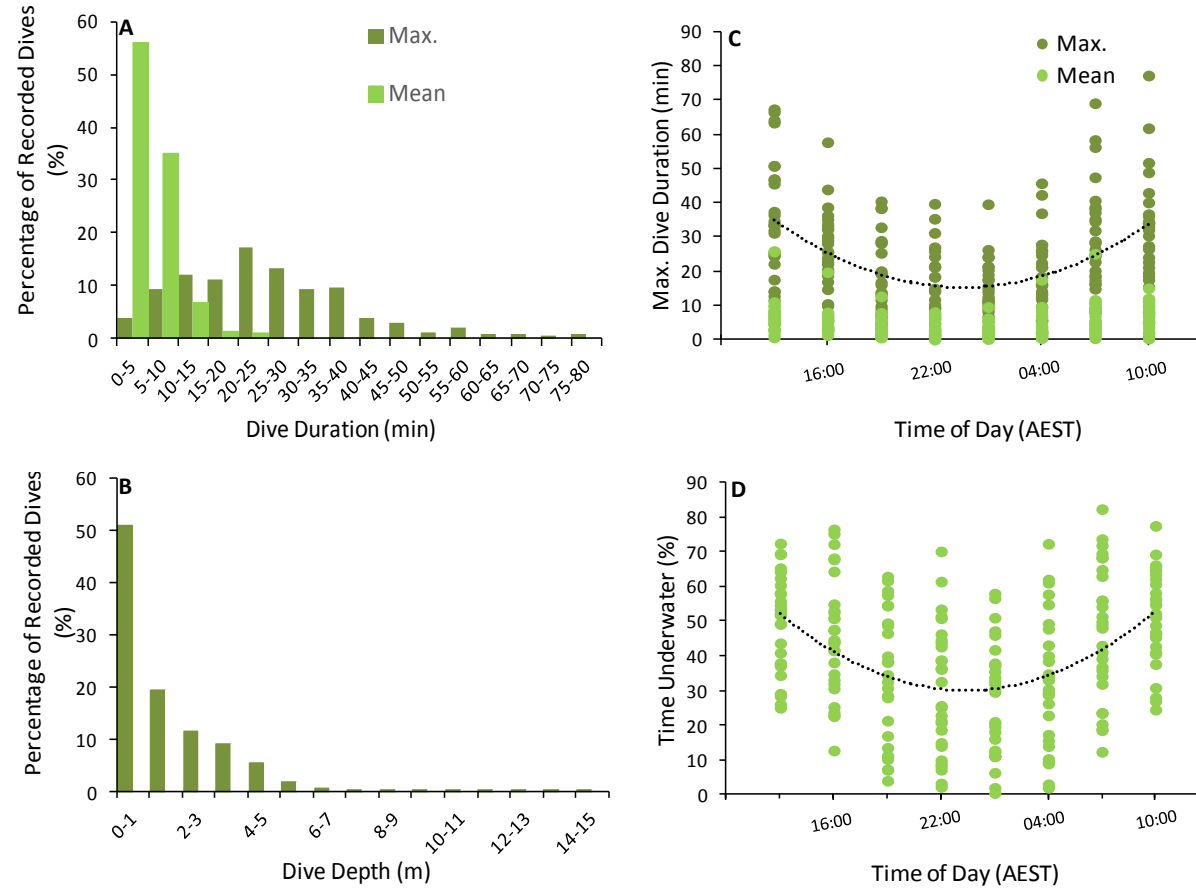
Figure 5.3: Diving repertoire of free-ranging adult estuarine crocodiles (*Crocodylus porosus*; *N* = 24) recorded in the Wenlock River, Cape York Peninsular (QLD, AUS). (**A**) Frequency histogram of maximum (dark green bars) and mean (light green bars) dive durations (i.e. min submerged), (**B**) Frequency histogram of diving depths (m), (**C**) Plot of diel cycle of maximum dive durations, and (**D**) Plot of diel pattern in time spent submerged (i.e. % of 3 h spent underwater).
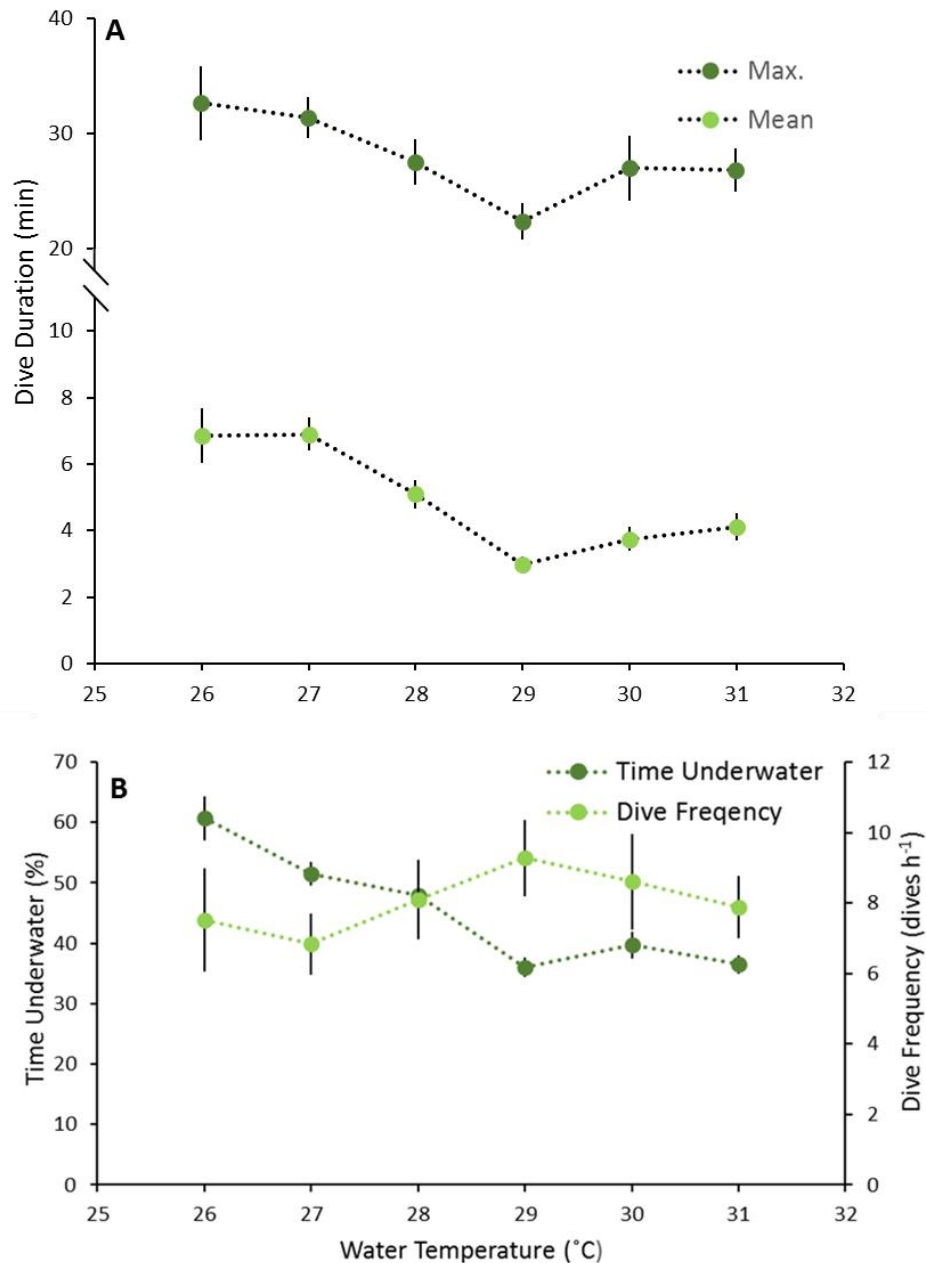
Figure 5.4: (A) Dive durations (i.e. min submerged) and (B) proportion of time spent underwater (%) and dive frequency (i.e. dives h^{-1}) of free-ranging, adult estuarine crocodiles (*Crocodylus porosus*; N = 5) as functions of water temperature (Tw). Dives were recorded in the Wenlock River, Cape York Peninsular (QLD, AUS) between August – December 2015. Maximum (dark green circles) and mean (light green circles) dive durations were inversely correlated with Tw (Max. $p < 0.001$, GAMM; Mean $p < 0.001$ GAMM). Dive frequency increased with Tw between 27 - 29.5∘C and reached a plateau between 29.5 - 31∘C (GAMM; $F_1 = 8.86$, $p < 0.0001$). Proportion of time spent submerged decreased with rising Tw (GAMM; $F_1 = 8.9$, $p < 0.001$). Values are represented as median ± s.e.
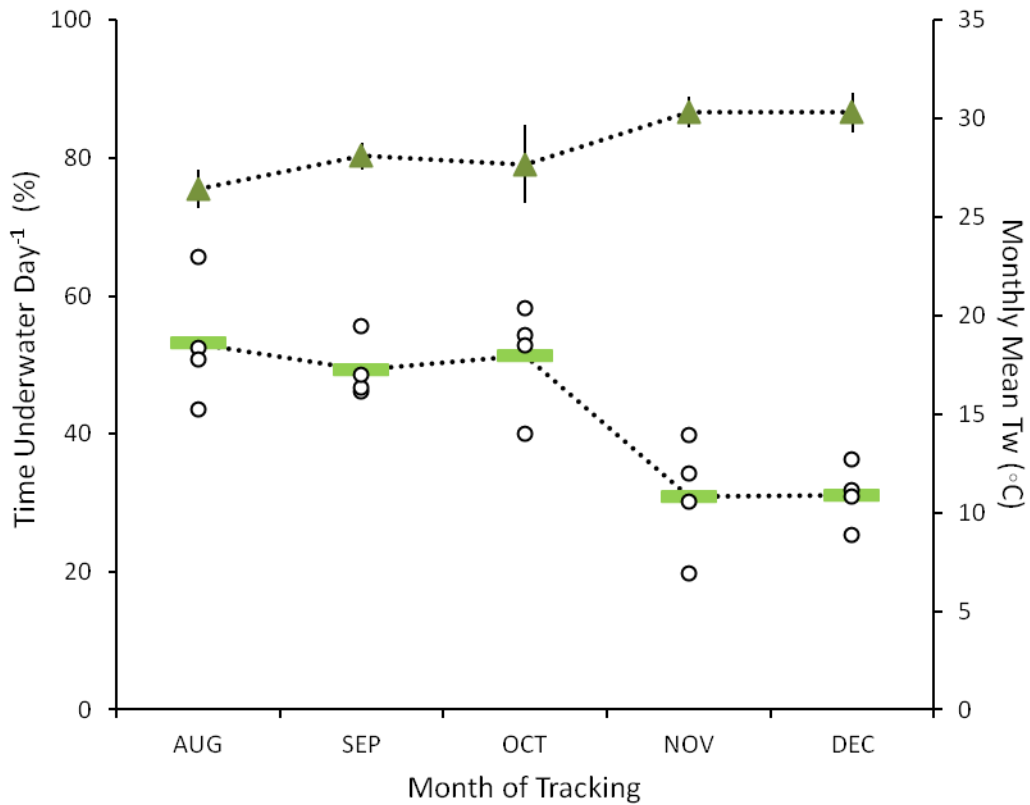
Figure 5.5: Percent time spent underwater (open circles) of free-ranging, adult estuarine crocodiles (*Crocodylus porsus*; *N* = 5) during different months over the recording period (i.e. August – December 2015) in the Wenlock River, Cape York Peninsular (QLD, AUS). Solid light green lines represent mean time spent underwater day^{-1} (%). Dark green triangles represent monthly deep water temperature (mean ± s.d.) recorded with CTD (i.e. conductivity-temperature-depth) loggers.
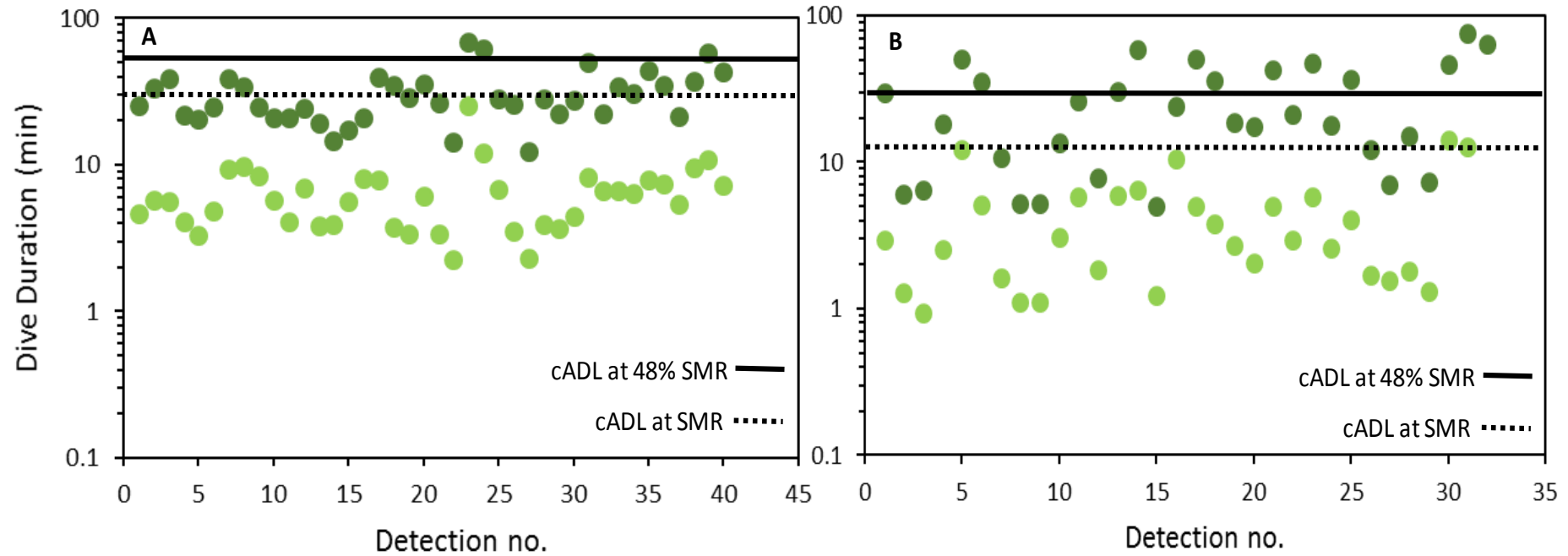
Figure 5.6: Mean (light green circles) and maximum (dark green circles) dive durations (i.e. minutes submerged) of free-ranging, adult estuarine crocodiles (*Crocodylus porosus*, $N = 5$) with calculated aerobic dive limits (cADL; min) overlaid. Dives were recorded in the Wenlock River, Cape York Peninsular (QLD, AUS) during (A) August 2015 (i.e. coldest month; mean water temperature (T_w) = 26.4∘C) and (B) December 2015 (i.e. warmest month; mean T_w = 30.3∘C). Dotted horizontal lines mark the mean cADL for dives with no metabolic alterations (i.e. at standard metabolic rate; $\dot{V}O_{2STANDARD}$ / SMR) and solid horizontal lines mark the cADL for dives with a 52% depression of $\dot{V}O_{2STANDARD}$. A greater proportion of mean dive durations exceeded ADLs at $\dot{V}O_{2STANDARD}$ in December (4.3 ± 1.4%) compared to August (1.6 ± 0.0%, $p < 0.01$, GLMM) but no dives exceeded cADLs once metabolic depression was accounted for. Approximately 41.7 ± 3.8% of maximum dives with no metabolic depression exceeded cADLS regardless of month ($p = 0.07$, GLMM), but once metabolic depression was accounted for more dives exceeded ADLs in December (27.4 ± 3.7%) compared to August (18.7 ± 1.1%) ($p < 0.01$, GLMM).

General Discussion

Air-breathing, diving ectotherms represent a crucial component of aquatic ecosystems but to this group, the threat of climate change is particularly salient. The inverse relationship between water temperature and ectotherm dive durations has been identified in over 12 species but only at ecologically-relevant temperatures (Table 6.1). The potential impacts of projected thermal increases have been overlooked, but simple extrapolation of these data to higher environmental temperatures begs the question of: *will ectotherm dive durations and time available for obligate underwater activities (e.g. foraging and predator avoidance) be cut short under climate change?* This thesis offers insight to this question by providing the first comprehensive investigation of the thermal sensitivity and plasticity of diving performance, diving behaviour and aerobic capacity in an ectothermic diver- the estuarine crocodile (*Crocodylus porosus*). Key findings from Chapters 2 - 5 are discussed within a climate change vulnerability framework (i.e. exposure-sensitivity-resilience; Huey et al., 2012) and related to existing literature.

Influence of seasonal thermal fluctuations on diving behaviour

Chapter 5 characterised the natural diving repertoire of *C. porosus* and evaluated the associations between seasonal thermal fluctuations and diving behaviour. Submergence behaviour is highly utilised in estuarine crocodiles; animals performed more than 100 dives day^{-1} and spent over half the day underwater in cooler months. Substantial decreases in dive durations and cumulated underwater activity were observed between the coldest (i.e. August) and warmest (i.e. December) months, indicating diving behaviour is strongly tied to the thermal environment.

It is unknown whether these reductions in dive durations are of a natural magnitude or linked to presently experienced climate warming, because long term data are unavailable. The existing field set-up, with animals tagged with dive loggers (outlined in Chapter 5), offers the opportunity to track year-to-year and even decadal alterations in thermal regimes and diving behaviour. This set-up could allow assessments of very slow rates of warming on diving behaviour, compared to experimental assessments where temperatures are generally increased over the course of hours, days or weeks. Such systems are rare, but a recent investigation showed European perch (*Perca fluviatilis*) exposed to a slow rate of warming

over three decades exhibited compensation in growth rates and resting cardiorespiratory function (Sandblom et al., 2016).

Monitoring diving behaviour of across multiple life stages (e.g. hatchlings, juveniles, sub-adults and adults) allows identification of the most vulnerable stage to climate warming. Comparisons of temperature quotients (Q_{10}) for dive durations suggest juvenile *C. porosus* (Q_{10} = 0.17; Chapter 2) are more sensitive to thermal increases than adults (Q_{10} = 0.35; Chapter 5). However, Q_{10} values derived in Chapters 2 and 5 have limited comparability because a myriad of factors likely influenced dive durations in the field compared to controlled experimental conditions. Nonetheless, this pattern likely stems from lengthened ADLs in adults compared to juveniles resulting from increased TBO stores and lower mass-specific metabolic rates (Gillooly et al., 2001). Estuarine crocodiles can undergo a 20, 000-fold increase in body size throughout a lifetime (e.g. 0.05 kg hatchling - 1000 kg adult; Grigg and Kirshner, 2015) and a similar increase in lung oxygen stores (scaling exponent = 0.906; Wright and Kirshner, 1987). Moreover, oxygen storage capacity may increase throughout ontogeny beyond enlarged pulmonary stores. For example, blood oxygen-carrying capacity (i.e. haematocrit and haemoglobin) and muscle oxygen stores (i.e. myoglobin) increase throughout development in sea lions (Richmond et al., 2006; Fowler et al., 2007). Longitudinal field studies, where individuals are tracked throughout development, could provide a robust representation of ontogenetic related changes in the thermal sensitivity of diving behaviour. Nevertheless, the results here show diving behaviour of both juveniles and adults to be strongly tied to the thermal environment.

Exposure, sensitivity and resilience to climate change
Climate change is projected to differentially affect global ecosystems and the exposure of different species largely depends on geographic range (Deutsch et al., 2008). The rate and magnitude of forced warming is predicted to be slower and reduced in the tropics compared to temperate regions (IPCC, 2007; IPCC, 2014). Despite reduced warming exposure at low latitudes, tropical ectotherms are expected to be more vulnerable to climate change compared to temperate counterparts (Tewksbury et al., 2008; Wright et al., 2009). Even slight increases in temperature may pose a threat to tropical ectotherms as many inhabit environments nearing upper thermal limits (Sunday et al., 2014). Moreover, tropical ectotherms have evolved in relatively constant thermal environments (i.e. little seasonal thermal variation) under selective pressures for thermal specialisation (i.e. performance optimised across a

narrow range of temperatures) and absent/limited thermal acclimation capacity (Janzen, 1967; Ghlamabor et al., 2006; Tewksbury et al., 2008; c.f. Seebacher et al., 2005a).

   As a tropical species, *C. porosus* is projected to be deleteriously affected by thermal increases. Record high temperatures have recently (2016) been recorded in tropical regions overlapping with the geographic distribution of *C. porosus* (NOAA, 2017; Fig. 6.1). Diving performance of *C. porosus* is highly thermally sensitive, with submergence times forecasted to halve under a moderate rate of climate warming (SRES A1B storyline; 50th percentile of IPCC 2007 global warming range; Rodgers et al., 2015; Chapter 2). The thermal sensitivity of diving behaviour was also apparent in free-ranging *C. porosus* and average dive durations declined by 40% between temperatures of 26 and 31∘C (Chapter 5). The thermal optima for diving performance was not identified, but marked declines in performance were observed at temperatures above 28∘C (Rodgers et al., 2015; Chapter 2). Water temperatures throughout the geographic range of *C. porosus* regularly exceed this threshold (e.g. Fig. 6.2.; Bureau of Meteorology, Australia, 2017) suggesting further warming will push temperatures towards critical limits for diving performance.
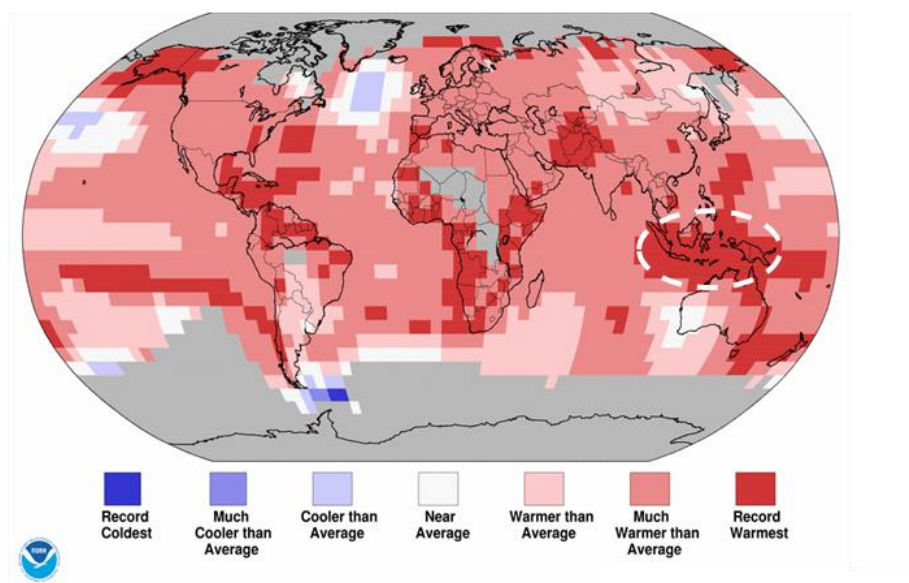
Figure 6.1: Land and ocean temperature percentiles from 2016 (source: NOAA's National Centers for Environmental Information, 2017). Colours represent the magnitude of warming compared to previously recorded years (i.e. 1880 - 2015). The overlaid white circle marks the geographic distribution of *C. porosus*, showing record high temperatures.
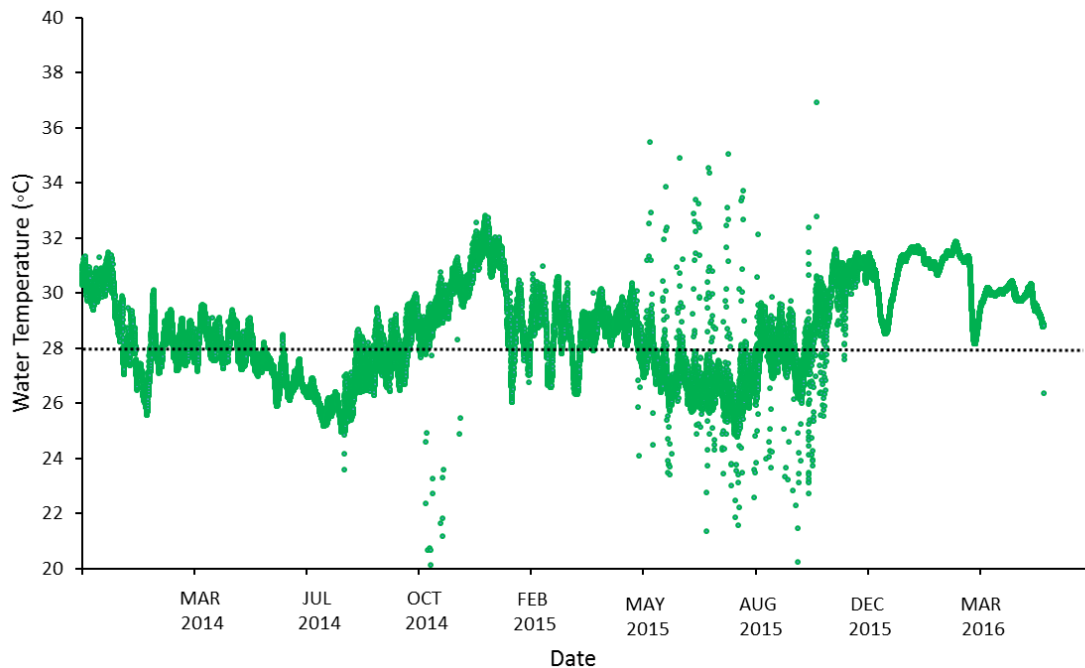
Figure 6.2: Water temperature (◦C) between December 2013 and May 2016 in part of *C. porosus'* habitat, the Wenlock River (Cape York Peninsula, QLD, AUS). Temperatures regularly exceeded 28◦C (marked by the horizontal black dotted line) - temperatures above 28°C are associated with decreases in diving performance (Chapters 2 – 3). Water temperatures were recorded using conductivity-temperature-depth loggers (DST milli-CRD, Star Oddim Gardabear, Iceland).

Compensatory responses may mediate the effects of climate change on ectotherm functioning (Stillman, 2003; Seebacher et al., 2015). Species can change their thermal tolerance limits by genetic adaptation (requiring multiple generations in a short space of time), acclimatise to altered thermal regimes, behaviourally compensate, or shift their geographical range to thermally preferable habitat (typically towards the poles or higher altitudes; Peck, 2011; Huey et al., 2012). My findings reveal thermal acclimation is unlikely to buffer estuarine crocodiles from the thermal effects on dive capacity (Rodgers et al., 2015; Chapter 2). This lack of phenotypic thermal plasticity was linked to diving metabolic rates and blood oxygen-carrying capacity remaining unadjusted following long-term exposure to elevated temperatures (Chapter 3). Transgenerational effects may facilitate thermal acclimation responses between generations (Mousseau and Fox, 1998; Rossiter, 1996). However, transgenerational adaptation is likely limited in *C. porosus* as sexual maturity is not reached until ~15 years of age (Grigg and Kirshner, 2015) and substantial thermal increases could occur within this timeframe.

A lack of acclimation capacity at thermal highs combined with late maturity, suggests *C. porosus* will be reliant on behavioural strategies to buffer thermal extremes (Basson and Clusella-Trullas, 2015). Crocodiles may seek refuge in deep, thermally stratified, cool water pockets, as seen in yellow-bellied sea snakes (*Hydrophis platurus*; Cook and Brischoux, 2014). Additionally, migrations to higher latitudes may be undertaken, but distances to cool refuges are greatest in tropical species (Wright et al., 2009) and are considered energetically expensive. However, estuarine crocodiles are highly mobile and use ocean currents to facilitate long-distance travel, suggesting southerly migrations are viable (e.g. travelling > 400 km in 20 days; Read et al., 2007; Campbell et al., 2010c; Campbell et al., 2013a). In summary, *C. porosus* appears to be living at temperatures beyond optima for diving performance in the absence of thermal acclimation capacity. Consequently, diving behaviour and concomitant activities will likely be disrupted under climate change, unless behavioural compensation is employed.

Applicability of findings to other ectothermic vertebrate divers

A large diversity of diving ectothermic vertebrates exist and the findings here may cautiously be hypothesised to apply to this group as a whole. Inter-species differences may exist in the thermal sensitivity of dive capacity, thermal acclimation capacity and geographic proximity to upper performance limits. Thermally sensitive dive capacity is a shared trait among ectothermic divers (Table 6.1). Experimental assessments show rises in water temperature to causally reduce dive durations in over ten species (Table 6.1). Likewise, seasonal increases in water temperature have been associated with reductions in submergence times in at least four ectothermic divers (Table 6.1). Temperature quotients for diving performance centre on 0.31, representing a 70% reduction in dive duration for every 10∘C rise in temperature (Q_{10} range = 0.07 – 0.43; Table 6.1). These values span a similar range to Q_{10} values derived for juvenile and adult *C. porosus* (i.e. juveniles Q_{10} = 0.17; adults Q_{10} = 0.35; Chapters 2 and 5). As a group, dive capacity of ectothermic vertebrates appears to be highly thermally sensitive.

Thermal acclimation capacity remains unassessed in most ectothermic divers and only two comparisons are available (Table 6.1). Complete acclimation was reported in maximal diving performance of Arafura filesnakes (*Acrochordus arafurae*) at 32∘C but average dive durations were unadjusted (Bruton et al., 2012). Partial cold-acclimation was shown in Mary River turtles (*Elusor macrurus*) but acclimation at warm temperatures (i.e. 28∘C) was absent (Clark et al., 2008). It can be hypothesised that acclimatisation capacities may be present in

temperate divers such as the alpine newt (*Triturus alpestris*) and Murray River turtle (*Emydura macquarii*) but absent in tropical species such as elegant sea snakes (*Hydrophis elegans*) (Table 6.1). Building on this, acclimation capacity may be more apparent in fully aquatic species (e.g. spine-bellied sea snake, *Hydrophis curtus*) compared to semi-aquatic species (e.g. freshwater crocodiles, *Crocodylus johnstoni*) as shown in global-scale assessments (Seebacher et al., 2015). Broad scale trends in thermal acclimation capacity lend hypotheses but more data are required before the capacity of this group can be assessed.

Ectothermic divers supported by bimodal respiration (i.e. aquatic and aerial) have been hypothesised to respond in a different manner to elevated temperatures compared to air-breathers (e.g. Pratt and Franklin, 2010). Bimodal breathers can extract aquatic oxygen cutaneously and/or by specialised structures (e.g. cloacal bursae) and include species such as the white-throated snapping turtle (*Elseya albagula*), Fitzroy River turtle (*Rheodytes leukops*) and spine-bellied sea snake (*Hydrophis curtus*). These species are expected to up-regulate aquatic respiration at elevated temperatures thereby reducing the need to surface and replenish oxygen stores aerially (Pratt and Franklin, 2010). This hypothesis has been rejected in the majority of assessments (Table 6.1; c.f. Clark et al., 2008; Dabruzzi et al., 2012). Instead, bimodal breathers generally increased reliance on aerial respiration at elevated temperatures, with increased surfacing frequency and decreased submergence times (Prassack et al., 2001; Pratt and Franklin, 2010; Bruton et al., 2012; Udyawer et al., 2016). For this reason, bimodal breathers are likely just as vulnerable as air-breathers to the effects of climate change on dive capacity. In summary, diving performance appears to be highly thermally sensitive in ectothermic, vertebrate divers as a group. The dive capacity of these species will likely be severely compromised under climate warming, unless safeguards (e.g. acclimatisation, behavioural compensation and transgenerational adaptation) are effective.

Extensions of diving physiology knowledge

Increased metabolic demands at elevated temperatures are hypothesised to underlie the thermal sensitivity of diving performance (Hayward et al., 2016). This hypothesis has been tested in three species of diving sea snake (Table 6.1). As predicted, diving metabolism increased with rising temperature, with temperature quotients (Q_{10}) centred on 2.5 (test temperature range 20 - 32∘C) (Pratt and Franklin, 2010; Udyawer et al., 2016). Chapter 3 provides the first assessment of thermal effects on diving metabolism at temperatures mimicking climate change scenarios. Diving metabolic rate increased threefold in *C. porosus* between 28∘C and 34∘C and derived a very high temperature quotient (Q_{10} = 7.4). Variations in

Q_{10} values may reflect greater exponential increases in diving metabolism at temperatures approaching upper thermal limits, interspecies variation, or methodological differences (i.e. 'fright-dives' compared to undisturbed dives).

A major advancement in understanding the thermal constraints on ectotherm dive capacity was realised in Chapter 3. Decrements in diving performance at elevated temperatures were linked not only to increased metabolic demands but also a compromised capacity to depress oxygen demands from surface levels. For example, relative diving oxygen requirements lowered from surface levels by ~52% and ~28% at water temperatures of 28∘C and 34∘C, respectively. Metabolically adjusted dives (e.g. predator avoidance dives) are likely to be more thermally sensitive compared to dives with no cardiovascular changes (e.g. short foraging dives) as a product of thermal constraints acting on both diving metabolism and metabolic depression (i.e. 'dive response'). Despite thermal constraints acting on metabolic depression capacity, significant bradycardia was maintained, suggesting other components of the 'dive response' were compromised. The next progression could examine the effects of elevated temperatures on the initiation of the dive response, peripheral vasoconstriction and cardiac shunting.

Theoretical frameworks proposed to underlie ectotherm loss of performance at thermal extremes have overlooked hypometabolic states, such as diving. The oxygen- and capacity-limited thermal tolerance hypothesis (OCLTT; Pörtner, 2001; Pörtner and Knust, 2007), for instance, only examines the interplay between resting and maximal rates of oxygen consumption with rising temperatures. Prior to the analyses in Chapter 4, it was unknown how diving metabolism compares to resting and maximal rates with respect to sensitivity and plasticity. The thermal sensitivity of diving metabolism was threefold higher than resting metabolism (Chapter 4). Aerobic scope was maintained across a broad thermal range (i.e. 28 - 36∘C) despite diving performance declining at temperatures above 28∘C (Chapter 4). Decrements in dive capacity in *C. porosus* are therefore not linked to a narrowing of aerobic scope and it is recommended that the OCLTT framework is applied with caution when estimating the vulnerability of diving ectotherms to climate change. Building on this, the 'plastic floors and concrete ceilings' hypothesis was tested in *C. porosus*; which postulates resting cardiorespiratory functions to be thermally sensitive and maximal cardiorespiratory functions to be relatively fixed (Sandblom et al., 2016). This hypothesis holds true in several fishes (Sandblom et al., 2016), but the opposite was observed in *C. porosus* (Chapter 4). Maximal rates of oxygen consumption doubled in warm-acclimated animals (i.e. 34∘C-

acclimated), whereas resting metabolic rates remained unadjusted following chronic exposure (Chapter 4). Future efforts could work to establish if thermal inflexibility in resting and diving metabolism is a shared trait among ectothermic divers or specific to *C. porosus.*

Reproducibility and statistical power

Reproducibility of scientific findings is a cornerstone of the scientific method but concerns are mounting due to replications of published findings failing to yield similar results (Parker et al., 2016; Clark, 2017). One issue inherent to working with crocodylians is small sample sizes due to space requirement, ethical limits and extensive safety requirements. Experiments with small sample sizes inherently have low statistical power, and if a significant difference is detected, the magnitude of that effect (e.g. difference between treatment group means) is necessarily large. This phenomenon is known as the 'winner's curse' because the experimenter fortunate enough to detect an effect with a small sample size is also 'cursed' because they likely overestimated the strength of that effect (Ioannidis, 2008). The effect of water temperature on diving performance was assessed in Chapters 2 and 3 in separate experiments using different animals (from 5 clutches), but the same results were obtained. This consistency suggests the thermal sensitivity of diving performance in juvenile *C. porosus* is a reproducible finding. The magnitude of this effect may still be overestimated but plots displaying the spread/variation within data sets have been incorporated to increase transparency (Weissgerber et al., 2015). Moreover, temperature quotients obtained for the thermal sensitivity of diving performance fall within the range (Q_{10} range = 0.07 – 0.43) recorded in other ectothermic divers from studies with larger sample sizes (Table 6.1). Collectively, these findings and comparisons show the results presented in this thesis are reproducible, and suggest the effect sizes were unlikely to be over-estimated. Nonetheless, strengthening collaborations between crocodile farms and laboratory groups could enable experiments on a larger number of animals and circumvent sample size issues.

Future directions

This thesis provides a comprehensive understanding of the thermal constraints acting on the diving physiology, behaviour and aerobic capacity of *C. porosus*, but concurrently gives rise to more research questions. Outlined below are research questions aimed at furthering our understanding of how climate warming may affect the functional capacity of diving ectotherms.

Qu. 1: *Which dive-dependent activities (e.g. underwater foraging or predator avoidance) are likely to be compromised in <u>C. porosus</u> by rising water temperatures?*

Chapters 2 - 3 focused specifically on predator avoidance dives (i.e. 'fright dives') as these submergences are linked to the survival of hatchlings and juveniles and may indirectly affect recruitment rates and population growth. However, crocodylians are thought to dive for a range of reasons, including foraging for aquatic prey and resting on the substratum and avoiding fast flowing surface currents (Seebacher et al., 2005b; Campbell et al., 2010b; Campbell et al., 2010c). The thermal sensitivity of diving performance may differ between dive functions. Foraging dives in crocodylians are reported to be short (< 1 min) and active (Campbell et al., 2010b), and are unlikely accompanied by cardiorespiratory alterations (i.e. 'dive response'; Blix and Folkow, 1983; Butler and Jones, 1997). These short dives fall within regular ventilatory periods of *C. porosus* (i.e. no breath holding; Wright, 1987), and it can therefore be hypothesised that foraging dive durations will be independent of water temperature. The thermal sensitivity of foraging and 'fright' dive durations could be compared in juvenile *C. porosus* under experimental conditions. Heart rate loggers could be surgically implanted into animals, to ascertain the extent of bradycardia accompanying differing dive functions. Building on this, underwater foraging success (e.g. prey captured/unit of time) could be assessed across a range of test temperatures, using food items (e.g. dead pilchards) placed at the bottom of a dive tank. Alternatively, live prey (e.g. crayfish) could be used but the thermal sensitivity of prey escape performance (e.g. burst swim speed) would need to be accounted for.

Activities associated with diving behaviour (e.g. foraging, rest and predator avoidance) in free-ranging, adult *C. porosus* were not recorded in Chapter 5, but forthcoming work could incorporate telemetry technology which aids in identifying dive types. Integrating dive recordings with accelerometers, mandibular angle sensing, gastric pH meters and heart rate monitors could identify active and resting dives (e.g. marked by acceleration), hunting/foraging dives (e.g. marked jaw movements or increased gastric pH) and predator avoidance dives (e.g. marked by pronounced bradycardia). From here, the association between water temperature and durations of differing dive functions could be established. Distinguishing dive types and their associated thermal sensitivities would present a clear picture of how the diving ecology of *C. porosus* may be affected under climate warming.

Qu. 2: *Is there a trade-off between diving performance and post-dive recovery rates at elevated temperatures?*

Decrements in diving performance at elevated temperatures were well-established in this thesis, but the thermal sensitivity of post-dive recovery rates was untested. The relationship between water temperature and dive capacity may be further complicated by the thermal sensitivity of metabolic recovery rates in ectotherms (Gleeson, 1991). Ectotherms can recover from anaerobic activity faster at warmer temperatures due to higher metabolic rates conferring faster biochemical reactions, thereby clearing lactate faster (i.e. converting lactate back to pyruvate) and rapidly restoring glycogen (Gleeson, 1991). In Chapter 3 post-dive oxygen debt was marginally higher (but not significant, $p = 0.07$) in crocodiles diving at 34°C compared to 28°C, but recovery durations were very similar, suggesting accelerated recovery rates at the elevated temperature. This hypothesis could be experimentally assessed by allowing animals to recover (following a bout of diving at a set temperature) across a range of constant test temperatures within a CT (controlled-temperature) room whilst activity levels are monitored and controlled for. Animals would ideally be cannulated, and blood samples could be taken at recovery time points (e.g. 0, 0.5, 2, 4, 6 h post-dive) to monitor plasma-lactate concentrations as a proxy for recovery (i.e. decreases with recovery).

Following this experiment, thermal preference tests could be performed; where temperature preference (e.g. thermal gradient from 25 - 35°C) following a bout of diving or a period of rest (control) is examined. This would test the hypothesis that animals seek out elevated temperatures following diving, presumably to accelerate recovery. Together these experiments may identify an important trade-off at elevated temperatures, with accelerated recovery rates potentially reducing post-dive surface intervals.

Qu. 3: *Is the dive capacity of air-breathing, vertebrate, diving ectotherms, as a group, likely to be compromised under climate warming?*

Assessing the impacts of elevated temperatures, emulating climate warming, on the dive capacity of numerous ectothermic divers could increase the predictive power regarding the vulnerability of this group. The thermal sensitivity of diving performance and metabolism could be examined in a range of species (e.g. marine iguanas, newts, and marine turtles). Specific focus should be applied to assessing thermal acclimation/acclimatisation capacities in dive capacity (as seen in Chapter 2) as data are currently scant (Table 6.1). A larger data base pertaining to air-breathing diving ectotherms, will allow patterns underlying vulnerability to

elevated temperatures to be identified. For example, tropical divers may be more vulnerable to climate change than temperate divers due to limited thermal acclimation capacities (Seebacher et al., 2015). Expanding the experiments conducted in this thesis to a range of diving ectotherms, is a crucial progression in assessing if the dive capacity of this group is threatened as a whole.

Qu. 4: *How will the functional capacity and geographic range of <u>C. porosus</u> be affected by climate change?*

Understanding the thermal constraints on the ecological functioning of *C. porosus* more specifically, requires the assessment of an entire suite of performance traits (e.g. swimming performance, digestive capacity, immune function, growth and fecundity; Kearney and Porter, 2009). Performance traits generally differ in thermal sensitivity and optima, so trade-offs may exist at elevated temperatures (i.e. multiple performances, multiple optima; Clark et al., 2013). For example, whilst diving performance is reduced at elevated temperatures, hatchling growth rates may increase so that juvenile crocodiles are under threat from predation for less time. Likewise, swimming performance of *C. porosus* is maintained across a wider thermal breadth (i.e. 23 - 33∘C; Elsworth et al., 2003; Campbell et al., 2013b) than diving, and reliance on swimming for predator evasion and prey perusal may be increased under climate warming. The thermal sensitivity of reproductive success may also bring its own set of challenges with potential effects on fecundity, embryo survival and sex-ratios. For instance, compared to optimal incubation temperatures (29.1 – 33.0∘C), nests exceeding 34∘C induce elevated rates of hatchling deformities and higher mortality rates (~80% mortality; Webb and Cooper-Preston, 1989).

A wealth of data relating to the thermal sensitivity of locomotor performance, growth, heat exchange, metabolism and fecundity already exist for *C. porosus* (Grigg and Kirsher, 2015). To integrate the complexity of multiple thermal performance optima, physiological data could be compiled into a mechanistic niche model, and microclimate conditions where survival and positive energy balance are maintained (i.e. fundamental niche) can be identified under varying magnitudes of climate change (Kearney and Porter, 2009). This approach will likely provide the most comprehensive estimates of how the persistence and geographic range of *C. porosus* may be altered under climate warming.

In summary, the effects of environmental temperature on ectotherm functional capacity are complex but robust representations of species' responses to climate change are urgently required. Assessing the thermal sensitivity of a single trait (e.g. aerobic scope) is no longer considered to accurately depict species vulnerability to rising temperatures (Clark et al., 2013), particularly in diving ectotherms (Chapter 4). Forthcoming work should aim to answer the above listed questions, and in doing so will increase clarity regarding the vulnerability of *C. porosus* and other diving ectotherms to climate warming.

Conclusions

This body of work highlights a previously overlooked threat to ectothermic divers- climate change. In doing so, our understanding of diving physiology has been broadened, particularly with respect to metabolic depression. Dive capacity of both juveniles and adults was markedly influenced by water temperature (Chapters 2, 3 and 5). Submergence times declined precipitously with rising water temperature and long term exposure to elevated temperatures did not elicit acclimation responses (Chapter 2). Decrements in diving performance at elevated temperatures were linked to increased oxygen demands and a reduced capacity for metabolic depression, irrespective of thermal acclimation treatment (Chapter 3). Alongside this, significant contributions were made to diving physiology theory. The thermal sensitivity and plasticity of metabolic depression was previously unexplored. Moreover, diving metabolism was contextualised within existing frameworks (e.g. OCLTT hypothesis and 'plastic floors and concrete ceilings'), showing decrements in diving performance cannot be explained by a narrowing of aerobic scope and maximum metabolic rates are phenotypically plastic but diving and resting rates of metabolism are fixed (Chapter 4).

From a conservation standpoint, this research strongly suggests climate change will cause serious disruption to the diving behaviour of estuarine crocodiles. Shortened submergence times may see cumulated time available for underwater activities cut short, forcing animals to spend a greater amount of time at the water surface. Forced time at the water surface may make hatchlings and juveniles more conspicuous to predators. Time available for rest and underwater foraging may also diminish, potentially increasing the overall cost of living. Behavioural compensation (e.g. migrating polewards, seeking deep, cool water holes or increasing reliance on terrestrial activity) may be the only safeguard if this species is subject to rapid escalations in environmental temperatures.

Additional concern stems from the similarities between *C. porosus* and other ectothermic, diving vertebrates. Dive capacity in this group appears to be highly thermally sensitive and the few tests of thermal acclimation capacity suggest physiological compensation may be limited (Table 6.1). Many of these species are late to mature which suggests transgenerational acclimation will be an ineffective buffer against climate change. Several ectothermic divers are listed as endangered or vulnerable on the International Union for the Conservation of Nature (IUCN) red list due to threats such as habitat loss and fisheries by-catch but climate change is often unlisted (Table 6.1; IUCN, 2017). The red list categories of these species are likely underestimated and findings presented here call for a re-evaluation. In summary, this thesis brings to light the severity of a previously unnoticed threat to ectothermic divers. Provided the findings here are applicable to this group as a whole, the functional capacity of diving ectotherms will likely be compromised under climate warming.

Table 6.1: Overview of studies investigating the thermal sensitivity of dive capacity in ectothermic, air-breathing, diving vertebrates. For each study the following are reported: species name (common and scientific), respiration mode (aerial or bimodal), aquatic reliance (fully aquatic: does not leave water, primarily aquatic: only leaves water for nesting and rare occasions, and semi-aquatic: regularly leaves water to bask or avoid water currents), geographic distribution (tropical or temperate), test temperatures (°C), relationship between water temperature (Tw) and dive durations (DD), diving performance Q_{10}, diving metabolism Q_{10}, acclimation capacity (unassessed, complete, partial or absent), aquatic respiration thermal sensitivity (increases/decreases/independent of water temperature), seasonal thermal effects, International Union for the Conservation of Nature (i.e. IUCN) red list status and sample size.

Citation	Common name	Scientific name	Respiration mode	Aquatic reliance	Topical/ Temperate	Test temp. (°C)	Relationship between Tw~DD	Diving Q_{10}	Diving metabolism Q_{10}	Acclimation capacity	Aquatic respiration thermal sensitivity	Seasonal thermal effects	IUCN status	Sample size
Storey et al., 2008	white-throated snapping turtle	*Elseya albagula*	bimodal	primarily aquatic	temperate	20, 25, 30	inverse	unreported	unassessed	unassessed	unassessed	N.A	endangered	7
Bentivegna et al., 2003	loggerhead sea turtle	*Caretta caretta*	aerial	primarily aquatic	subtropical-temperate	13-27	inverse	unreported	unassessed	unassessed	N.A.	shorter dive durations in summer	vulnerable	10
Bruton et al., 2012	Arafura file snake	*Acrochordus arafurae*	bimodal	fully aquatic	tropical	22, 32	inverse	unreported	$\dot{V}O_{2STANDARD}$ = 2.9	maximum dive: complete; mean dive: absent	independent	N.A	least concern	8-9
Campbell et al., 2010a	freshwater crocodile	*Crocodylus johnstoni*	aerial	semi-aquatic	tropical	unreported	inverse	unreported	unassessed	unassessed	N.A.	shorter dive durations in summer	least concern	6-9
Clark et al., 2008	Mary River turtle	*Elusor macrurus*	bimodal	primarily aquatic	temperate	17, 28	inverse	0.07	$\dot{V}O_{2STANDARD}$ = 2.46	partial cold acclimation; absent warm acclimation	increase with Tw in acclimated animals	N.A	endangered	6-7

Citation	Common name	Scientific name	Respiration mode	Aquatic reliance	Topical/ Temperate	Test temp. (°C)	Relationship between Tw~DD	Diving Q_{10}	Diving metabolism Q_{10}	Acclimation capacity	Aquatic respiration thermal sensitivity	Seasonal thermal effects	IUCN status	Sample size
Gordos et al., 2003	Fitzroy River turtle	*Rheodytes leukops*	bimodal	primarily aquatic	temperate	16.5 - 27.3	inverse	unassessed	unassessed	unassessed	unassessed	shorter dive durations in summer	vulnerable	6-9
Herbert and Jackson, 1985	Western painted turtle	*Chrysemys picta bellii*	bimodal	primarily aquatic	temperate	3, 10, 15, 20	inverse	unassessed	unassessed	unassessed	unassessed	N.A	least concern	14-23
Hochsheid et al., 2005	loggerhead sea turtle	*Caretta caretta*	aerial	primarily aquatic	subtropical-temperate	14.7-26	inverse	unassessed	unassessed	unassessed	N.A.	shorter dive durations in summer	vulnerable	1
Prassack et al., 2001	painted turtle	*Chrysemys picta*	bimodal	primarily aquatic	temperate	15, 25	inverse	unassessed	unassessed	unassessed	independent	N.A.	least concern	11
Pratt and Franklin, 2010	Arafra file snake	*Acrochordus arafurae*	bimodal	fully aquatic	tropical	20, 32	inverse	0.43	2.52	unassessed	independent	N.A.	least concern	17
Preist and Franklin, 2002	Fitzroy River turtle	*Rheodytes leukops*	bimodal	primarily aquatic	temperate	15, 23, 30	inverse	0.27	unassessed	unassessed	unassessed	N.A.	vulnerable	6
Preist and Franklin, 2002	Murray River turtle	*Emydura macquarii*	primarily aerial	primarily aquatic	temperate	15, 23, 30	inverse	0.36	unassessed	unassessed	unassessed	N.A.	least concern	5
Seebacher et al., 2005b	freshwater crocodile	*Crocodylus johnstoni*	aerial	semi-aquatic	tropical	21.5 - 32	inverse	unreported	unassessed	unassessed	N.A.	shorter dive durations in summer	least concern	6

Citation	Common name	Scientific name	Respiration mode	Aquatic reliance	Topical/ Temperate	Test temp. (°C)	Relationship between Tw~DD	Diving Q_{10}	Diving metabolism Q_{10}	Acclimation capacity	Aquatic respiration thermal sensitivity	Seasonal thermal effects	IUCN status	Sample size
Samajova and Gvozdik, 2009	alpine newt	*Triturus alpestris*	bimodal	primarily aquatic	temperate	10, 15, 20, 25	inverse	unreported	unassessed	unassessed	N.A.	N.A.	least concern	12
Udyawer et al., 2016	spine-bellied sea snake	*Hydrophis curtus*	bimodal	fully aquatic	tropical	21, 24, 27, 30	inverse	0.24	2.5	unassessed	independent	N.A.	unassessed	12
Udyawer et al., 2016	elegant sea snake	*Hydrophis elegans*	bimodal	fully aquatic	tropical	21, 24, 27, 30	inverse	0.35	2.3	unassessed	independent	N.A.	least concern	10
Southwood et al., 2003	green sea turtle	*Chelonia mydas*	aerial	primarily aquatic	tropical - subtropical	17.8 – 31.6	inverse	unreported	unassessed	unassessed	N.A.	shorter dive durations in summer	endangered	6

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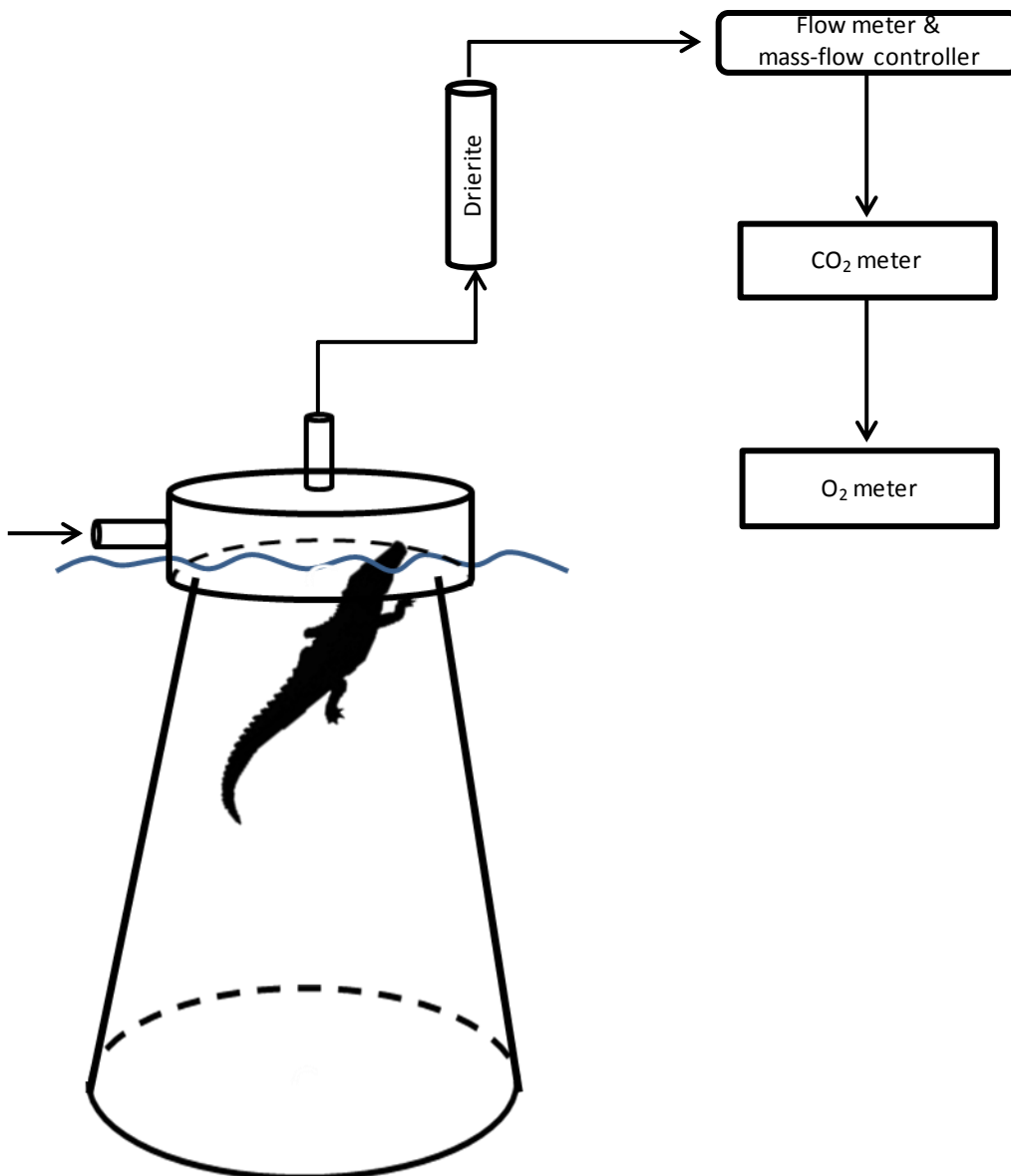
Figure A3.1: Overview of diving respirometry design used in Chapter 3. Diving oxygen consumption was measured inside a custom built diving column (height = 1.33 m, base diameter = 0.42 m, top diameter = 0.25 m) placed inside the dive tank at a water depth of 1.3 m (water line marked by blue line). The water surface was sealed using a custom-fitted piece of Styrofoam with a dome-shaped respiratory hood (volume = 3.6 L) fitted with inflow and outflow air outlets. The diving column and respiratory hood were designed to ensure the only available air space was inside the respiratory hood. A pull flow through system was utilised and arrows show the direction of air flow. Out flowing air was scrubbed of water vapour by passing it through a drying column (Drierite; Sigma, Sydney, AUS) using a flow meter and mass-flow controller (*SS3*; Sable Systems International, North Las Vegas, USA). Fractional concentrations of carbon dioxide (CO_2) and oxygen (O_2) were measured by passing dry air into a CO_2 meter (LI-820, LI-COR, Nebraska, USA) and subsequently into an O_2 meter (Oxzilla, Sable Systems International, North Las Vegas, USA).
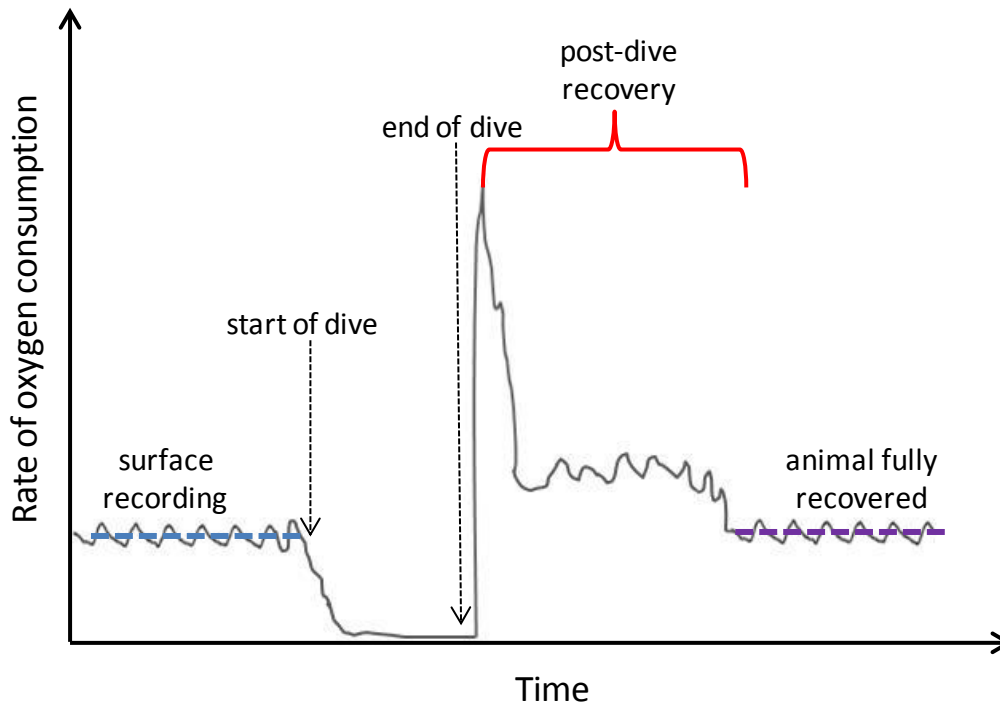
Figure A3.2: Example diving metabolic rate ($\dot{V}O_{2DIVE}$) calculation, adjusted from Hurley and Costa (2001). The sample calculation is based on a 0.39 kg (body mass; #10) crocodile diving at 28°C; dive duration = 23.27 min. The blue horizontal line represents the pre-dive surface metabolic rate (i.e. $\dot{V}O_{2SURFACE}$, recorded for 1 h), calculated from the area under the metabolic curve divided by duration; $\dot{V}O_{2SURFACE}$ = 0.54 ml O_2 min^{-1}. The arrows mark the start and end of a dive. Oxygen consumption drops to zero whilst the animal is submerged and 'spikes' once the animal surfaces. The red bracket marks the post-dive recovery interval (spanning 9.48 min). Post-dive recovery metabolic rate (i.e $\dot{V}O_{2POSTDIVE}$) was also calculated from the area under the metabolic curve divided by duration; $\dot{V}O_{2POSTDIVE}$ = 0.62 ml O_2 min^{-1}. Animals were considered recovered when $\dot{V}O_{2POSTDIVE}$ equalled $\dot{V}O_{2SURFACE}$ (marked by purple horizontal line). Total O_2 consumed during the recovery interval was 0.62 O_2 min^{-1} × 9.48 min = 5.88 ml O_2. The portion of $\dot{V}O_{2POSTDIVE}$ which is accounted for by baseline metabolism is 0.54 ml O_2 min^{-1} × 9.48 min = 5.12 ml O_2. Therefore, the O_2 debt from the dive is 5.88 ml O_2 - 5.12 ml O_2 = 0.76 ml O_2, and diving metabolic rate is equal to 0.76 ml O_2 / 23.27 min = 0.3 ml O_2 min^{-1}. Adjusting for body mass brings this value to 0.08 ml O_2 min^{-1} kg^{-1}.
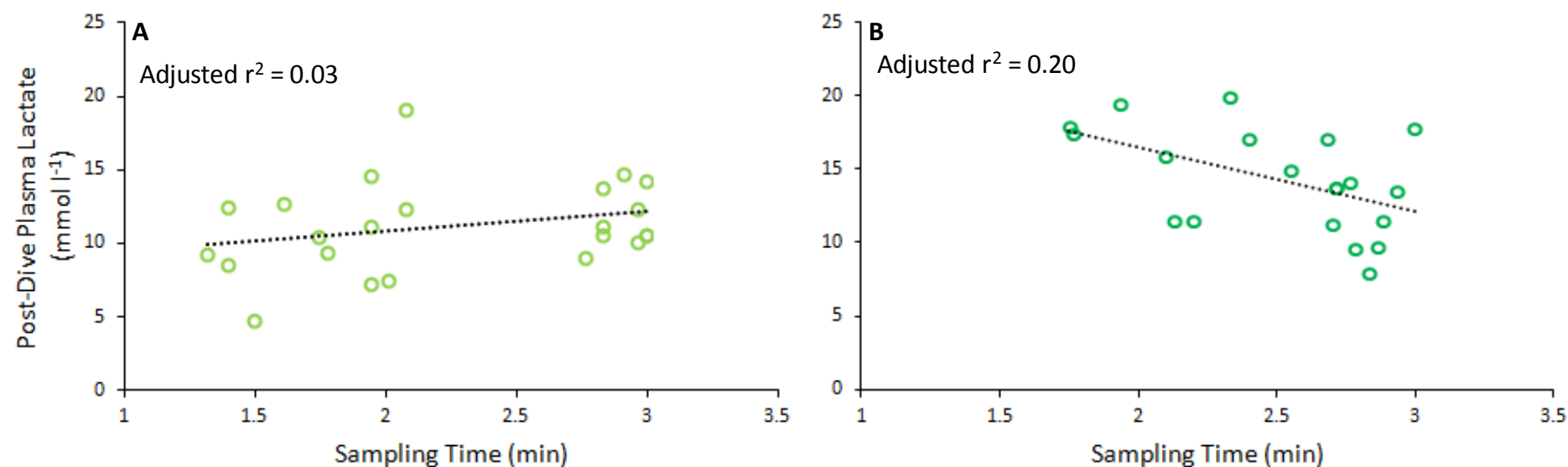
Figure A3.3: Relationship between blood sampling time (min) and post-dive plasma-lactate accumulation following single dive trials (**A**, light green circles) and sustained dive trials (**B**, dark green circles). Lactate concentrations were independent of blood sampling times ($p = 0.87$, adjusted $r^2 = 0.03$ [single]; $p = 0.98$, adjusted $r^2 = 0.20$ [sustained], linear regression).
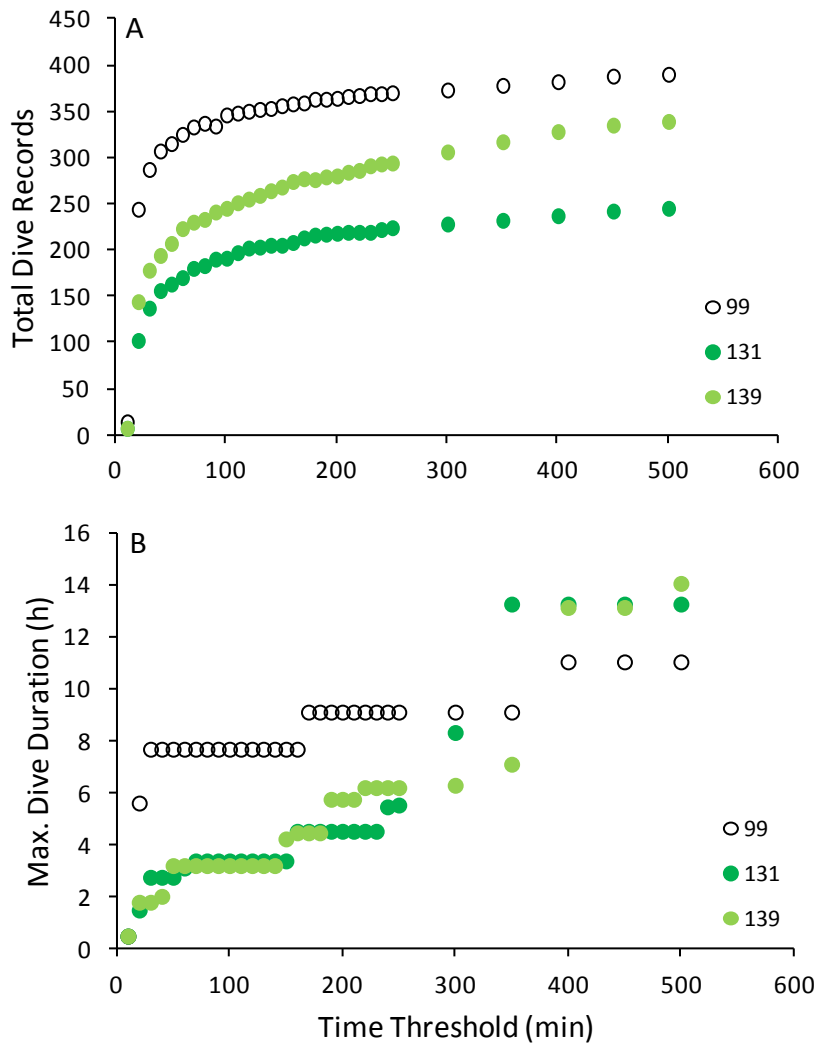
Figure A5.1: Sensitivity analyses used to parameterise the behavioural event qualifier in V-Track (Campbell et al., 2012). Total number of dives (A) and maximum dive duration (B) extracted from the acoustic data set across a range of time thresholds (i.e. time between detections). Different coloured circles represent crocodile identification numbers (i.e. 99, 131, 139). The total number of dives and maximum dive duration detected reached a plateau at 60 min, indicating that submergences were not excluded at this time interval. Time thresholds above this derived unrealistically long maximum dive durations when referenced to the dive data recorded by the wet-dry satellite tags.
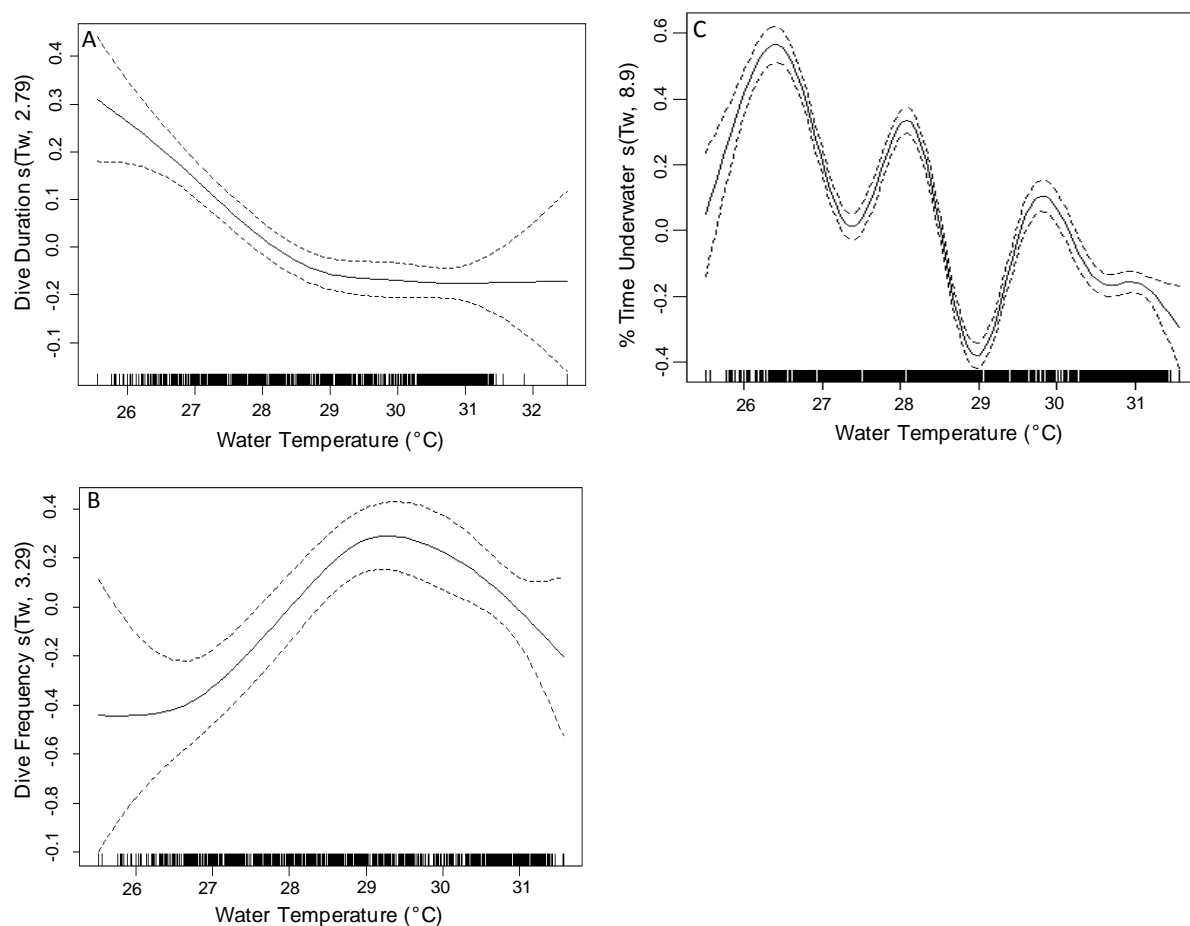
Figure A5.2: General additive mixed-effects model (GAMM) outputs showing estimated smoother for water temperature (Tw) as a function of (A) dive duration (*d.f.* = 2.79; F_1 = 22.88, *p* < 0.0001), (B) dive frequency (*d.f.* = 3.29; F_1 = 8.86, *p* < 0.001) and (C) percentage of time spent underwater (*d.f.* = 8.90; F_1 = 8.90, *p* < 0.001). Dotted lines mark 95% confidence limits and the black solid lines represent the mean population response (i.e. fixed effect). The black bars along the x-axis show the spread of raw data.