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Physiological mechanisms constraining ectotherm fright-dive performance at elevated temperatures

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ABSTRACT

Survival of air-breathing, diving ectotherms is dependent on their capacity to optimise the time available for obligate underwater activities, such as predator avoidance. 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 demand and a reduced capacity for metabolic depression at elevated temperatures. Here, we investigated how water temperature (both acute and chronic exposures) affected the physiology of juvenile estuarine crocodiles (*Crocodylus porosus*) performing predator avoidance dives (i.e. fright-dives). Diving oxygen consumption, 'fright' bradycardia, haematocrit and haemoglobin (indicators of 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 consumption rate increased threefold between 28 and 34°C (Q_{10}=7.4). The capacity to depress oxygen demand was reduced at elevated temperatures, with animals lowering oxygen demand from surface levels by 52.9±27.8% and 27.8±16.5% (means±s.e.m.) at 28°C and 34°C, respectively. Resting and post-fright-dive haematocrit and haemoglobin were thermally insensitive. Together these findings suggest decrements in fright-dive performance at elevated temperatures stem from increased oxygen demand coupled with a reduced capacity for metabolic depression.

KEY WORDS: Aerobic dive limit, Diving metabolism, Thermal sensitivity, Climate change, Bradycardia, Heart rate

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 probably 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 performance (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 demand exceeding oxygen supply capacity [i.e. cardio-respiratory system failure; oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis] (Pörtner, 2001, 2010; Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Eliason et al., 2011). Compromised aerobic performance may occur at high temperatures when maximal rates of oxygen consumption ($\dot{V}_{O_2,max}$) plateau or decrease but resting/standard metabolic rate ($\dot{V}_{O_2,standard}$) increases exponentially, reducing absolute aerobic scope (=$\dot{V}_{O_2,max}$−$\dot{V}_{O_2,standard}$). 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, 2015, 2016; Norin et al., 2014). Many ectotherms experience compromised performance at temperatures below those affecting aerobic scope (Norin et al., 2014), suggesting the mechanistic explanation may be multi-faceted and thus it 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 with winter months (Carr et al., 1980; Bentivegna et al., 2003; Gordos et al., 2003; Hochscheid et al., 2005; Bradshaw 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). 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), but physiological

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List of abbreviations

ADL	aerobic dive limit
f_H	heart rate
OCLTT	oxygen- and capacity-limited thermal tolerance
RQ	respiratory quotient
TBO	total body oxygen
$\dot{V}_{O_2}$	oxygen consumption rate
$\dot{V}_{O_2,dive}$	diving oxygen consumption rate
$\dot{V}_{O_2,post-dive}$	post-fright-dive oxygen consumption rate
$\dot{V}_{O_2,pre-dive}$	surface/pre-fright-dive oxygen consumption rate
$\dot{V}_{O_2,standard}$	standard oxygen consumption rate

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 consumption reflective of a shorter ADL (Butler, 2006). Oxygen can be stored in the lungs, blood and tissue, and the percentage contribution of stores varies between species (Kooyman, 1989). In estuarine crocodiles (*Crocodylus porosus*), for instance, pulmonary (i.e. lung) 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 duration is 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 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 at elevated temperatures have been associated with increased diving metabolism in two species of sea snake (spine-bellied sea snake, *Hydrophis curtus*, and elegant sea snake, *Hydrophis elegans*; Udyawer et al., 2016). Ectotherm divers routinely dive within ADLs but submergences can be extended beyond this limit with the use of anaerobic pathways (Butler and Jones, 1982; Seymour, 1982). Exceeding the ADL incurs the cost of longer post-dive surface intervals to clear accumulated lactate (Kooyman et al., 1980; Costa et al., 2004).

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 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 may facilitate prolonged submergence 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). A pronounced drop in heart rate during a predator avoidance dive is termed 'fright bradycardia' and can be initiated in a laboratory setting where an

animal escapes/dives underwater in response to a perceived threat (e.g. loud noise or the presence of an experimenter; Wright et al., 1992). Diving is a vital pre-condition for the onset of fright bradycardia and heart rate does not drop in *C. porosus* in response to a threat on land (Wright et al., 1992). The mechanism underlying the thermal sensitivity of fright-dive performance probably differs from OCLTT, which does not consider hypometabolic states. High temperatures may not only elevate fright-dive metabolic rate ($\dot{V}_{O_2,dive}$) but also compromise a diver's capacity to depress metabolic rate from resting/surface levels.

The aim of this study was to investigate how the fright-dive 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., 2005; Campbell et al., 2010b). Predator escape dives are vital in hatchling and juvenile *C. porosus*, which may fall prey to a range of species including: monitor lizards (*Varanus mertensi*, *Varanus panoptes*), barramundi (*Lates calcarifer*), whistling kites (*Haliastur sphenurus*), olive pythons (*Liasis olivasceus*), great egrets (*Ardea alba*) and larger *C. porosus* (Grigg and Kirshner, 2015). The fright-dive capacity of juvenile *C. porosus* is thermally sensitive, with marked reductions in dive duration at temperatures above 28°C (Rodgers et al., 2015), but the physiological mechanism underlying this pattern is unresolved. We assessed the effect of water temperature on $\dot{V}_{O_2,dive}$ and fright bradycardia. We hypothesised that $\dot{V}_{O_2,dive}$ would increase exponentially with temperature and align with compromised performance (i.e. shorter dive duration; hypothesis 1). Similarly, we predicted that *C. porosus*' relative capacity for metabolic depression (i.e. reductions in heart rate and oxygen consumption compared with surface rates) would be impaired at elevated temperatures (hypothesis 2). The assessment of fright-dive 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, QLD, Australia, −28.108901, 153.444175) and juveniles were obtained from three clutches at Cairns Crocodile Farm (Gordonvale, QLD, Australia, −17.039566, 145.792283; N=14 from 4 clutches: 6 reared from eggs, 8 obtained as juveniles). Eggs were transported to The University of Queensland (St Lucia, QLD, 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 (water temperature 31.5±0.5°C). The juveniles obtained from Cairns Crocodile Farm had been incubated at 32.5±0.5°C and kept in outdoor enclosures (i.e. March 2014 to June 2015) before we received them. 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

history. Animals were between 15 and 22 months old at the time of testing (total length, 56.6±9.6 cm; snout–vent length, 28.9±5.2 cm; body mass, 488.0±251.6 g; means±s.d.). 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 assigned to one of two thermal acclimation treatments, based on the following Intergovernmental Panel on Climate Change (Solomon et al., 2007) climate change scenarios. (1) Low rate of global warming (SRES B1 storyline; 10th percentile of IPPC global warming range)/current summer water temperature. This scenario reflects a low-emissions future. Summer water temperatures remain unchanged in this scenario. Experimental water temperature simulating this scenario was 28±0.5°C (N=7). (2) High rate of global warming (SRES A1F1 storyline; 90th percentile of IPCC global warming range). This scenario is based 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 range between 33 and 35°C. Experimental water temperature simulating this scenario was 34±0.5°C (N=7).

Treatment assignment was randomised using a random number generator (www.random.org; even number→28°C, odd number→34°C) but occurred separately for crocodiles from each source (i.e. Cairns Crocodile Farm and David Fleay Wildlife Park) to ensure animals from the two sources were equally distributed between treatments. 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, NSW, Australia). 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, SA, Australia; suspended 26 cm above the platform) and a UV-B light (25 W; Exo Terra®, Montreal, QC, Canada) and the other a relatively 'cool' platform with no lamps. Basking opportunity (i.e. the time the heat lamp was switched on) was 8 h day^{-1} (08.00 h–16.00 h) for all 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 h–19.00 h light) for all 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.

Fright-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 the 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.

Fright-dive performance was assessed in animals from both thermal acclimation treatments (i.e. 28°C acclimated and 34°C acclimated) at two test temperatures (28 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 full recovery 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 designed to simulate the presence of a predator where crocodiles dived underwater as an escape behaviour. Fright-dives were initiated by an experimenter lightly tapping a crocodile with a blunt wooden pole. The experimenter was in view of the crocodile throughout the dive trial to simulate the prolonged presence of a predator. Dive durations were directly observed and timed.

Blood sampling and analyses

Crocodiles were immediately captured upon surfacing at the end of a fright-dive trial for blood sampling. Blood samples (0.5–2 ml) were drawn from a branch of the jugular vein (venous blood) using ½ in 23 gauge needles attached to heparinised (lithium salt; Sigma-Aldrich Sydney, NSW, Australia) syringes. An aliquot (~5 µl) of whole blood was used to determine haemoglobin concentration ([Hb]) and two microcapillary tubes were filled to measure haematocrit (percentage packed red cell volume, %Hct). Microcapillary tubes were spun at 5000 g for 2 min (micro-haematocrit centrifuge, Hawksley, Lancing, Sussex, UK) and %Hct was measured. A colorimetric assay kit (Sigma-Aldrich; MAK115) was used to determine duplicate [Hb]. Blood samples were also taken from resting animals as a reference point. Resting blood samples were obtained by capturing animals in their holding tank and immediately sampling. Blood sampling times were recorded and all samples were obtained within 3 min (Finger et al., 2015a,b).

Fright bradycardia

Surface and fright-dive 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 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 temperature-controlled room. Two electrocardiogram (ECG) wires (MLA1203 Needle electrode, AD Instruments, Sydney, NSW, Australia) were inserted subcutaneously on the animal's ventral surface, anterior and posterior to the heart, and held in place using strapping tape (Elastoplast rigid sports strapping, Beiersdorf, Hamburg, Germany). The ECG wires were run into an adjacent room, attached to a BioAmp (ML132, ADInstruments Pty Ltd, Bella Vista, NSW, Australia) and the bioamp was connected to a PowerLab (4/30 series ML866, ADInstruments). f_H recordings were visualised on a laptop using LabChart software (ADInstruments) with a sampling rate of 100 Hz. A video camera (Microsoft LifeCam Studio, Microsoft, Redmond, WA, USA) was placed above the dive tank and recordings were synchronised with f_H 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 fright-dive f_H were determined by extracting and averaging multiple recordings (three per animal) between 60 and 300 s in duration. Surface f_H was recorded before each fright-dive.

Fright-dive and surface f_H were averaged for each animal at each test temperature. Relative (%) fright bradycardia was calculated as:

$$\% \text{ Bradycardia} = (f_{H,\text{surface}} - f_{H,\text{diving}})/f_{H,\text{surface}} \times 100, \quad (1)$$

where $f_{H,\text{surface}}$ is mean heart rate when the animal is at the surface of the water and $f_{H,\text{diving}}$ is mean heart rate when the animal is performing a fright-dive. Surface f_H measurements were not a measure of resting f_H but a measure of routine surface f_H. Animals were left to recover after instrumentation and equilibrate body temperature with water temperature for a minimum of 1 h before trials began, at which point f_H recordings had levelled out.

Fright-dive metabolism
Fright-dive metabolic rate ($\dot{V}_{O_2,\text{dive}}$) was determined for crocodiles from each thermal acclimation treatment at two test temperatures (28 and 34°C) using flow-through respirometry. Animals were fasted for a minimum of 5 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 1 h prior to testing to ensure body temperature had equilibrated with water temperature. $\dot{V}_{O_2,\text{dive}}$ 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. A pull-system was utilised with flow rate ranging between 590 and 1240 ml min^{-1}, depending on animal body mass, using a mass-flow controller (SS3, Sable Systems International, North Las Vegas, NV, USA; calibrated with a Bubble-O-Meter, Dublin, OH, USA). Outflowing air was scrubbed of water vapour by passing it through a drying column (Drierite, Sigma-Aldrich). Fractional concentrations of CO_2 and O_2 were measured by passing dry air into a CO_2 analyser (LI-820, LI-COR, Lincoln, NE, USA) and subsequently into an O_2 analyser (Oxzilla, Sable Systems International). 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 eqn 11.2 from Lighton (2008) and respiratory quotients (i.e. ratio of carbon dioxide produced to oxygen consumed at a given time point) were estimated for each test temperature using Grigg's (1978) eqn 2 derived from juvenile *C. porosus* (N=11; P<0.001):

$$\text{RQ} = 1.098 - 0.0203T, \quad (2)$$

where RQ is the respiratory quotient and T is test temperature (°C). Surface oxygen consumption rates ($\dot{V}_{O_2,\text{pre-dive}}$) were measured for 1 h, followed by a single fright-dive. Post-fright-dive oxygen consumption ($\dot{V}_{O_2,\text{post-dive}}$) was measured to estimate oxygen debt accumulated during submergence, and oxygen debt was assumed to be cleared once $\dot{V}_{O_2,\text{post-dive}}$ equalled $\dot{V}_{O_2,\text{pre-dive}}$. $\dot{V}_{O_2,\text{dive}}$ was calculated using Eqn 3 (Hurley and Costa, 2001):

$$\dot{V}_{O_2,\text{dive}} = \dot{V}_{O_2,\text{debt}}/\text{DD}, \quad (3)$$

where $\dot{V}_{O_2,\text{debt}}$ represents oxygen debt accumulated during submergence (ml min^{-1}) and DD is dive duration (i.e. total time submerged, min). 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 ambient fractional concentrations of O_2 and CO_2.

Statistical analyses
Data analyses were performed in R Studio (version 3.1.3; www.R-project.org/) using the nlme (linear and non-linear mixed effects models; https://CRAN.R-project.org/package=nlme) package. A series of linear mixed effects models were used to determine the effects of test temperature and thermal acclimation treatments on fright-dive performance (i.e. minutes submerged), fright-dive metabolism ($\dot{V}_{O_2,\text{dive}}$) and fright-dive f_H (% fright bradycardia). Test temperature (two-level factor), acclimation treatment (two-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 (ID) were included as random effects (ID nested within source) in all models. Statistical significance was accepted at P≤0.05.

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

Fright bradycardia
$f_{H,\text{surface}}$ was significantly higher at a test temperature of 34°C (65±4 beats min^{-1}) than at 28°C (46±3 beats min^{-1}; Fig. 1C; $P<0.001$, $F_{1,6}=43.7$, lme) but independent of thermal acclimation treatment ($P=0.54$, $F_{1,5}=0.4$, lme). Fright-dive f_H ($f_{H,\text{diving}}$) was independent of thermal acclimation treatment ($P=0.30$, $F_{5,1}=1.3$, lme) but test temperature had a borderline significant effect ($P=0.052$, $F_{1,6}=5.8$, lme), with elevated $f_{H,\text{diving}}$ at 34°C (24±2 beats min^{-1}) compared with 28°C (19±1 beats min^{-1}; Fig. 1C). All animals exhibited fright bradycardia, with f_H reduced by 12–55 beats min^{-1} from surface levels. Relative fright bradycardia (i.e. percentage reduction from $f_{H,\text{surface}}$) 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. 2A). Body mass had no significant effect on $f_{H,\text{surface}}$, $f_{H,\text{diving}}$ and relative fright bradycardia ($P>0.27$, lme).

Fright-dive metabolism
Surface ($\dot{V}_{O_2,\text{pre-dive}}$) and fright-dive ($\dot{V}_{O_2,\text{dive}}$) metabolic rates were significantly higher at a test temperature of 34°C than at 28°C (Fig. 1D; $\dot{V}_{O_2,\text{pre-dive}}$ $P<0.01$, $F_{1,5}=19.1$, $Q_{10}=2.32$, lme; $\dot{V}_{O_2,\text{dive}}$ $P<0.05$, $F_{1,4}=9.1$, $Q_{10}=7.4$, lme). Thermal acclimation treatment and body mass had no effect on $\dot{V}_{O_2,\text{pre-dive}}$ (thermal acclimation treatment $P=0.73$, $F_{1,5}=0.1$; body mass $P=0.16$, $F_{1,5}=0.2$, lme) and $\dot{V}_{O_2,\text{dive}}$ (thermal acclimation treatment $P=0.37$, $F_{1,5}=0.98$, body mass $P=0.13$, $F_{1,4}=3.5$ lme). $\dot{V}_{O_2,\text{dive}}$ was depressed from surface levels by 52.9±27.8% at 28°C and 27.8±16.5% at 34°C (pooled
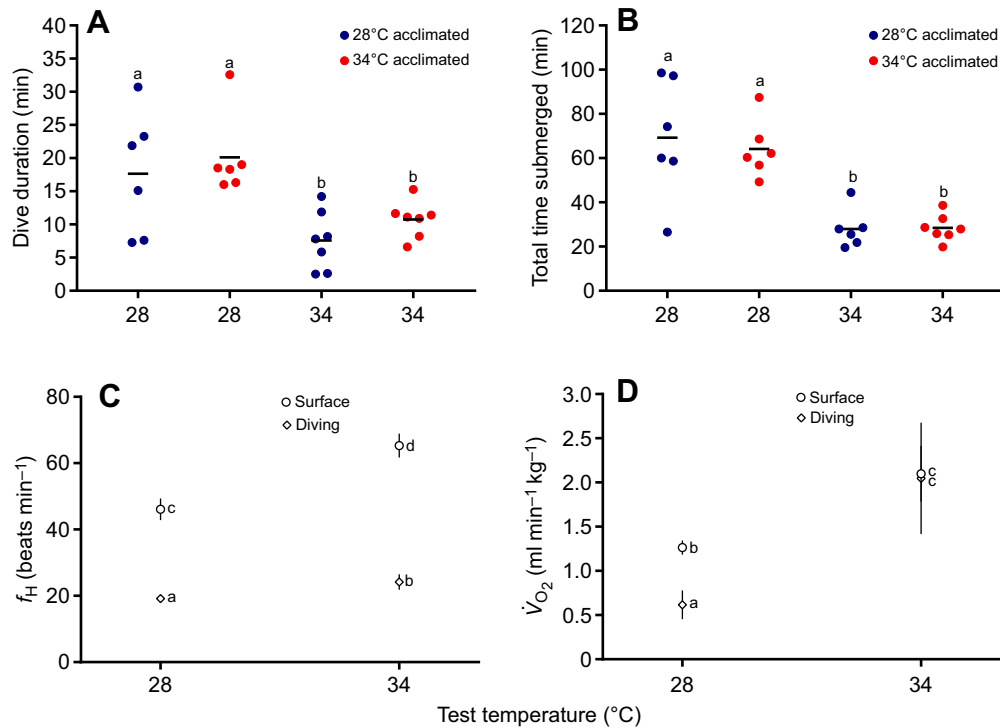
Fig. 1. Thermal sensitivity of fright-dive performance, heart rate (f_H) and oxygen consumption rate ($\dot{V}_{O_2}$) in juvenile estuarine crocodiles (*Crocodylus porosus*). Both single (A) and sustained (B) fright-dive performance, assessed as minutes submerged, was reduced at a test temperature of 34°C compared with 28°C (single $P<0.05$; sustained $P<0.0001$; linear mixed-effects model, lme; $N=6–7$ per acclimation treatment; Table S1), regardless of thermal acclimation treatment ($P\geq0.52$, lme). Surface and diving f_H ($N=4$ per acclimation treatment; C) and $\dot{V}_{O_2}$ ($N=3–4$ per acclimation treatment; D; Table S1) increased with rising temperature ($f_{H,surface}$ $P<0.001$, lme; $f_{H,diving}$ $P=0.052$, lme; $\dot{V}_{O_2,pre-dive}$ $P<0.01$, $Q_{10}=2.32$, lme; $\dot{V}_{O_2,diving}$ $P<0.05$, $Q_{10}=7.4$, lme). Values are shown as raw data points in A and B, and as means±s.e.m. in C and D. Different letters indicate significant differences between groups.

means±s.e.m.). Test temperature had a borderline significant effect on relative metabolic depression (Fig. 2B; $P=0.052$, $F_{1,4}=6.8$, 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-fright-dive oxygen debt and recovery duration were thermally insensitive, averaging 13.5 ± 2.6 ml kg^{-1} and 18.0 ± 2.0 min for both test temperatures (Fig. 3; 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).

Hct and Hb

Resting Hct (%) and [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 Hct averaging $18\pm1\%$ and [Hb] averaging 64 ± 6 g l^{-1} in all animals. Post-fright-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).
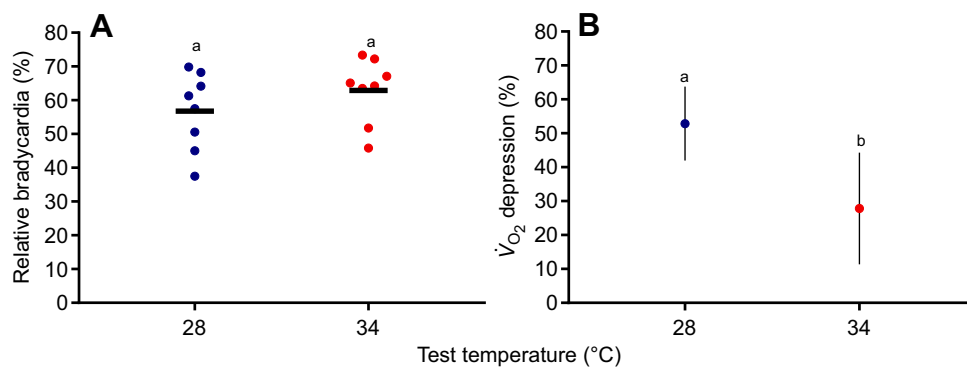

Fig. 2. Fright-dive bradycardia and metabolic depression in *C. porosus*. (A) Thermal sensitivity of relative 'fright' bradycardia (i.e. percentage reduction from $f_{H,surface}$) of juvenile estuarine crocodiles ($N=8$ per test temperature, 4 per acclimation treatment). Relative bradycardia was independent of test temperature ($P=0.44$, lme). (B) Effect of test temperature on diving metabolic depression (i.e. percentage reduction from $\dot{V}_{O_2,pre-dive}$) in juvenile estuarine crocodiles ($N=7–8$ per test temperature, 3–4 per acclimation treatment; Table S1). Values are shown as individual data points in A and as means±s.e.m. in B. Diving metabolic depression was reduced at a test temperature of 34°C compared with that at 28°C ($P=0.052$, lme). Different letters indicate significant differences between groups.
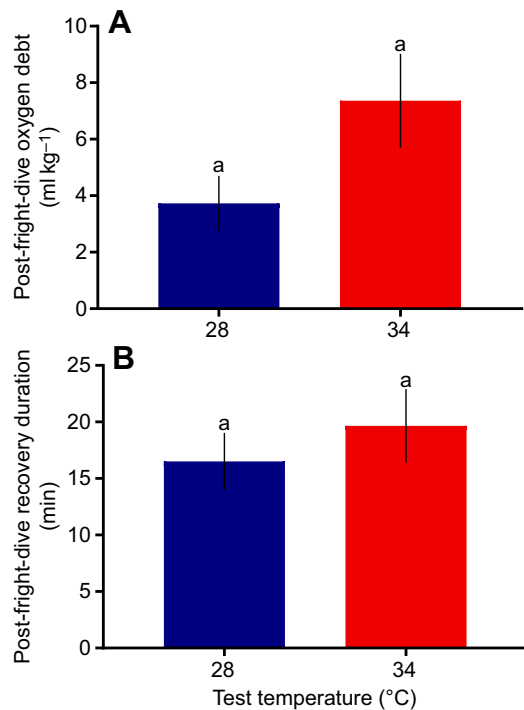
Fig. 3. Thermal sensitivity of post-fright-dive oxygen debt and recovery duration in juvenile estuarine crocodiles (*C. porosus*). N=8 per test temperature, pooled from thermal acclimation treatments. (A) Post-fright-dive oxygen debt (N=3–4, Table S1). (B) Recovery duration (i.e. time required for $\dot{V}_{O_2,\text{post-dive}}$= $\dot{V}_{O_2,\text{pre-dive}}$, N=3–4; Table S1). Test temperature had no effect on post-fright-dive oxygen debt (P=0.07, lme) and recovery duration (P=0.21, lme). Different letters indicate significant differences between groups.

DISCUSSION

Ectotherm functioning and performance are 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 fright-dive capacity at elevated temperatures in juvenile estuarine crocodiles. In line with previous findings (Rodgers et al., 2015), fright-dive performance of *C. porosus* was markedly compromised at elevated temperatures, with submergence times halving between 28 and 34°C. Performance decrements appear to be linked to reductions in ADLs at elevated temperatures, stemming from increased oxygen demand and a reduced capacity for metabolic depression (supporting hypothesis 1 and hypothesis 2). Cumulatively, these results suggest *C. porosus* terminated dives earlier at the elevated temperature because of a faster use of body oxygen stores.

Thermal sensitivity of fright-dive metabolism and metabolic depression

Body temperature has long been identified as the most influential abiotic factor governing aerobic metabolism in ectotherms (Brett, 1971). $\dot{V}_{O_2,\text{pre-dive}}$ increased markedly between 28 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 ($\dot{V}_{O_2,\text{standard}}$, Q_{10}=2.68, range=20–33°C; Grigg, 1978), suggesting metabolic thermal sensitivity does not differ between these contexts. In line with hypothesis 1, diving metabolic rate increased threefold between 28 and 34°C. This increase in oxygen demand translates into a faster use of TBO stores during

submergence. Rapid use of TBO stores probably 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. $\dot{V}_{O_2,\text{dive}}$ Q_{10}=7.4; $\dot{V}_{O_2,\text{pre-dive}}$ Q_{10}=2.32) and this is probably due to a compromised capacity for metabolic depression at the 34°C. Diving oxygen requirements were lowered from surface levels by 52% and 28% at 28 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 in reptiles is controlled by interactions between the cholinergic and adrenergic nervous systems governing pacemaker heart cells (Burggren, 1987; Hicks and Farrell, 2000; Hicks, 1994). 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 f_{H} 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 f_{H} during submergence (i.e. percentage bradycardia) at both test temperatures, suggesting other components of the dive response were compromised. For example, limited capacity for the 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.

Hct and Hb

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 of compensation in $\dot{V}_{O_2,\text{dive}}$ probably played a greater role in compromised fright-dive performance (Wright, 1985). [Hb] and Hct levels rise in some vertebrates during hypoxia and/or diving (Thornton and Hochachka, 2004), probably 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 $\dot{V}_{O_2,\text{standard}}$ measurements on the same species (i.e. *C. porosus*) with a similar body mass

(180–6200 g) and age (juveniles) range, making estimates comparable (Grigg, 1978). Using Grigg's (1978) RQ assumes equal thermal sensitivity of $\dot{V}_{O_2,standard}$, $\dot{V}_{O_2,pre\text{-}dive}$ and $\dot{V}_{O_2,post\text{-}dive}$. This assumption may be valid as animals are stationary when resting at the water surface, similar to resting conditions when measuring $\dot{V}_{O_2,standard}$. Estimating RQs for conditions where anaerobic metabolism is partially fuelling activity, as may be the case here with fright-dives, is often avoided as lactate accumulation can shift the bicarbonate–CO_2 equilibrium towards CO_2 (Lighton and Halsey, 2011). However, post-dive recovery durations did not differ between test temperatures, suggesting reliance on anaerobic metabolism (if any) was equal. Therefore, any error introduced from anaerobiosis would be the same and have no overall impact on the relative effect of test temperature on $\dot{V}_{O_2,dive}$. Further reassuring is that the RQs calculated when the CO_2 meter was functioning fall within the range measured by Grigg (1978; $\dot{V}_{O_2,pre\text{-}dive}$ RQ=0.65± 0.02, $\dot{V}_{O_2,post\text{-}dive}$ RQ=0.55±0.0, means±s.d.).

Ecological consequences of reduced fright-dive times

Fright-dive durations were reduced by ~50% at a water temperature reflective of predicted climate change (i.e. 34°C), compared with present-day summer temperatures (28°C). This finding suggests predator avoidance dives 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 (within 30 days) at elevated temperatures has been demonstrated previously in *C. porosus* (Rodgers et al., 2015). Similarly, many other diving ectotherms have limited thermal acclimation capacity (Graham et al., 1971; Clark et al., 2008; Heatwole et al., 2012) and some species are unable to compensate for seasonal thermal increases (Carr et al., 1980; Bentivegna et al., 2003; Gordos et al., 2003; Hochscheid et al., 2005; Campbell et al., 2010a). Underlying this lack of acclimation capacity in the diving performance of *C. porosus* is probably the inability to adjust rates of oxygen consumption ($\dot{V}_{O_2,dive}$ and $\dot{V}_{O_2,pre\text{-}dive}$), f_H, Hb and Hct 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 probably 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 (Šamajová and Gvoždík, 2009) and freshwater crocodiles (Campbell et al., 2010a). Unless behavioural compensation is employed (e.g. diving in deep, cool water pockets or migrating polewards to cooler climates), juvenile *C. porosus* may need to frequently replenish oxygen stores at the potential cost of becoming increasingly conspicuous to predators.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.M.R., C.E.F.; Methodology: E.M.R., C.E.F.; Validation: E.M.R.; Formal analysis: E.M.R.; Investigation: E.M.R.; Resources: C.E.F.; Data curation: E.M.R.; Writing - original draft: E.M.R.; Writing - review & editing: E.M.R., C.E.F.; Visualization: E.M.R.; Supervision: C.E.F.; Project administration: C.E.F.; Funding acquisition: C.E.F.

Supplementary information

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