Does control of the heart hold the key to survival in a warming world in a diving ectotherm?

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Abstract

The ability to remain submerged for prolonged periods offers a significant advantage for air-breathing diving vertebrates. Dive bradycardia is believed to be a key physiological adaptation that aids in extending dive duration by reducing oxygen consumption during diving. Dive bradycardia has been observed across all phyla of air-breathing vertebrates and is controlled chiefly by the interaction between the cholinergic and adrenergic nervous systems acting on the pacemaker cells of the heart. In ectothermic vertebrates, another major factor that influences diving capacity is environmental temperature through its effect on the metabolic rate. In this study we investigated the control and role of dive bradycardia and the effect of elevated temperatures on the diving capacity of an ectothermic vertebrate, the saltwater crocodile, *Crocodylus porosus*. We acclimated juvenile *C. porosus*, to three water temperatures selected based on predicted future climate change; 28°C (*N* = 7), 31.5°C (*N* = 6) and 35°C (*N* = 7). We then experimentally manipulated dive bradycardia using pharmacological antagonists acting on the cholinergic and adrenergic systems. We observed a significant bradycardia, ranging between 33 – 46 %, during fright dives (where animals dived in response to disturbance) across all temperatures. However, we found no significant differences in heart rate or magnitude of dive bradycardia between temperatures. Diving bradycardia in *C. porosus* was controlled entirely by the action of the cholinergic system, with no significant difference in adrenergic tone when at the surface or during diving. Surprisingly, we found no significant effect of temperature or dive bradycardia on fright dive duration. Our findings highlight the dominant role of the cholinergic system in controlling heart rate during diving. They also further support that dive bradycardia may only influence prolonged submergence during dives well beyond an animal's aerobic dive limit. Possibly, the most significant result was that *C. porosus* was capable of maintaining diving performance even when exposed to relatively high temperatures. This suggests that *C. porosus* has the capacity to maintain performance under predicted future increases in global temperatures. Whether, this is due to thermal acclimation or *C. porosus* maintaining a broad plateau of thermal independence of performance remains inconclusive and further study testing heart rate and diving capacity over a range of temperatures in addition to the acclimation temperatures would help elucidate this.

Contents

Introduction

For many air-breathing vertebrates diving plays a key ecological role in feeding and predator avoidance (Butler 1982; Boyd 1997). Being able to dive for prolonged periods offers a significant advantage and the evolution of key physiological adaptations appear to be important in extending submergence times (Andersen 1966). One of the most widely conserved physiological mechanisms believed to help prolong dive duration is the dive bradycardia (Andersen 1966; Butler 1982; Butler & Jones 1997). When air-breathing vertebrates dive they typically exhibit a dive response involving a reduction in heart rate (bradycardia) which contributes to a decrease in oxygen consumption and is believed to aid in extending dive duration (Butler & Jones 1997; Davis *et al.* 2004; Alboni *et al.* 2011). This response has been observed across all phyla of air-breathing vertebrates (Andersen 1966; Butler & Jones 1997). Although the response of heart rate to diving is highly conserved across taxa, the magnitude of the bradycardia varies significantly not only between species but also between dives. Diving can be loosely classified into two types; a voluntary dive, which occurs during routine behaviours, and a 'fright dive', which occurs in response to disturbance (Gaunt & Gans 1969). Voluntary dives typically result in only a slight bradycardia, whilst 'fright' dives produce a marked decrease in heart rate resulting in a 'fright' bradycardia (Belkin 1968; Gaunt & Gans 1969; Smith *et al.* 1974; Butler 1982; Smith & Jr 1985). These differences in the magnitude of bradycardia exhibited during diving are well documented; however, experimental studies quantifying how bradycardia influences dive duration is limited.

In vertebrates, heart rate is primarily controlled by the cholinergic and adrenergic nervous systems working in parallel, with heart rate depending on the balance (tone) between the two systems. Dive bradycardia is almost entirely driven by a significant increase in cholinergic activity via the release of acetylcholine acting on muscarinic receptors (Smith & Jr 1985; Signore & Jones 1995; Elliott *et al.* 2002). Cholinergic blockade with the administration of atropine either abolished or greatly reduced dive bradycardia in study animals (Smith & Jr 1985; Signore & Jones 1995; Elliott *et al.* 2002). Conversely, adrenergic blockade via treatment with β-adrenergic antagonists, sotalol or propranolol, had no significant effect on the magnitude of the dive bradycardia (Smith & Jr 1985; Signore & Jones

1995; Elliott *et al.* 2002). Surprisingly, abolishing the dive bradycardia pharmacologically has no significant effect on voluntary diving behaviour in the Harbour seal, *Phoca vituline,* and the muskrat, *Ondatra zibethicus.* However, when observing forced dives in muskrats, maximum forced dive duration was significantly reduced when bradycardia was abolished (Signore & Jones 1995). This suggests that bradycardia plays a significant role in prolonging dive duration during extended forced dives (Signore & Jones 1995). These findings highlight that the significance of bradycardia in prolonging dive duration depends on dive type and it may also relate to dive duration with forced dives lasting significantly longer than voluntary dives. No equivalent research into the role of bradycardia during diving has been conducted on air-breathing diving ectotherms.

One of the challenges faced when studying diving behaviour is that dive duration is not only influenced by intrinsic physiological factors but also extrinsic factors, e.g. temperature, presence of predators or prey, water depth, etc. (Costa 1991; Costa *et al.* 2004). Early studies into the physiology of diving often used forced dives to determine an animal's maximum dive duration (Andersen 1966). Recent studies have relied more on examining unrestrained voluntary and fright diving behaviour either in a laboratory or using remote sensing in the field (Wright *et al.* 1992; Signore & Jones 1995; Elliott *et al.* 2002; Campbell *et al.* 2010b; Pratt *et al.* 2010). These approaches are far more ecologically relevant but can be limited when trying to develop a causal relationship between a physiological trait and diving capacity due to the large influence of extrinsic factors.

Temperature has a major role in influencing diving capacity, especially in ectotherms. The close association in ectotherms between body and environmental temperature means that physiological function is strongly influenced by ambient temperature (Pörtner & Farrell 2008). Animal performance would be expected to be optimised for a limited thermal window and as temperature moves outside of this, performance would be negatively impacted (Pörtner 2002; Pörtner & Farrell 2008). In relation to diving performance, as temperature rises above the optimum there is a proportional increase in metabolic rate and, therefore, a decrease in diving performance, which is dictated by oxygen stores and consumption rate (Kooyman *et al.* 1980; Costa *et al.* 2004; Jackson 2007). This has been demonstrated in diving ectotherms with acute increases in temperature leading to a reduction in diving performance (Priest & Franklin 2002; Clark *et al.* 2008; Šamajová & Gvoždík 2009; Bruton *et al.* 2012). Of more ecological relevance in the face of global climate

change and rising temperatures, is the impact of chronic temperature increases and whether ectotherms have the ability to thermally acclimate (IPCC 2007).

In diving air-breathing ectotherms the ability to acclimate diving performance to elevated temperatures is still uncertain and appears to vary between genera. In the freshwater Mary River turtle, *Elusor macrurus*, there was no evidence of thermal acclimation of diving performance after long term exposure to elevated temperatures (Clark *et al.* 2008). A field study on the freshwater crocodile, *Crocodylus johnstoni*, that compared dive performance over summer and winter, also found no indication of thermal acclimatisation to warmer temperatures, with dive duration during summer being significantly shorter than during winter (Campbell *et al.* 2010a). Contrary to these findings, a significant effect of thermal acclimation on diving performance was found for the Arafura filesnake, *Acrochordus arafurae,* with warm acclimated animals having significantly longer maximum and modal dive duration at cold and warm temperatures (Bruton *et al.* 2012). From these studies it's clear that variation exists between species in their ability to thermally acclimate dive capacity. Presently, there has been limited study looking into the ability of ectotherms to acclimate dive capacity to the higher temperatures predicted with future climate change. Given that conservative predictions set global surface temperatures to increase by 1.8°C over the next 100 years (based on a low emissions scenario, SRES B1), furthering our understanding of the effects of increased temperature on animal physiology and performance is of increasing importance (IPCC 2007).

To this end we looked at the effect of chronic increases in temperature on the control and role of heart rate and dive bradycardia during diving in the saltwater crocodile, *Crocodylus porosus*. *Crocodylus porosus* has been shown to thermally acclimate swimming performance to cold (winter) and warm (summer) temperatures (Glanville & Seebacher 2006). Additionally, *C.* porosus are capable of prolonged dives and previous studies have observed a small (14 %) and large (65 %) bradycardia during voluntary and fright dives, respectively (Wright 1987; Wright *et al.* 1992). The first aim of this study was to investigate how heart rate and bradycardia are influenced by chronic increases to elevated water temperature. We hypothesised that heart rate would increase and dive bradycardia magnitude would decrease as temperature increased. Secondly, we investigated the cholinergic and adrenergic control of heart rate during diving and whether this was affected by increasing temperature. Here we expected to find dive bradycardia controlled primarily

by changes in cholinergic tone acting on the heart and that as the magnitude of bradycardia decreased with increasing temperature we would see a decrease in cholinergic tone with increasing temperature during diving. Finally, we looked at the effect of temperature and dive bradycardia on dive duration. We hypothesised that dive duration would decrease with increasing temperature and also with decreasing magnitude of bradycardia (cholinergic and complete blockade treatments).

Materials and Methods

Study species and housing

Juvenile estuarine crocodiles (*Crocodylus porosus,* Schneider 1801; *N* = 11) were collected as eggs from David Fleay's Wildlife Park (Gold Coast, QLD, Australia) and hatched and raised at The University of Queensland. Crocodiles were approximately 18 months old and had mean body mass of 426 ± 126 g and a mean snout-tail length of 529 ± 57 mm (mean ± SD). Crocodiles were housed in three plastic tanks (1200 X 600 X 600 mm, 3-4 animals per tank) within an animal holding facility that was sheltered but still exposed to ambient temperatures. Tanks contained water to a depth of 150 mm which was heated to the experimental temperatures using submersible heaters (Jäger, Germany, Aqua One, United Kingdom, Aqua Zonic, Singapore). A dry area was provided at both ends of the tanks via suspended plywood platforms (300 X 600 mm). Within each tank above one of the platforms a 150 W ceramic infra-red heat lamp (Exo-Terra, Montreal, Canada) was provided for basking and a full-spectrum light source (Sylvania Reptistar, Germany) was suspended above the other. A constant light cycle (14 h: 10 h L: D), based on the summer conditions of northern Queensland, Australia, was maintained for all treatments. Animals were fed chopped beef and mackerel mixed with supplementary vitamins (Repti-Vite, Aristopet**,** Brisbane, Australia), at least twice a week and were fed between 15 - 20 % of their body mass per week. Holding tanks were cleaned twice a week. Ethics approval was granted by The University of Queensland Animal Ethics committee for animal collection, housing and experimental procedures (SBS/018/14/ARC/AUSTZOO and Ecoaccess permit no. WISP14243214).

Thermal acclimation treatments

Crocodiles were randomly allocated into one of three acclimation treatments with different water temperatures; 28 (25± 2°C, *N* = 7), 31.5 (29 ± 3°C, *N* = 6*)* and 35 °C (33 ± 3, *N* = 7) (mean ± SD). These temperatures were chosen based on average summer sea surface temperatures around north Queensland, Australia as predicted by the Intergovernmental Panel on Climate Change (IPCC) Special Report Emissions Scenarios (SRES) (IPCC 2007). The 28°C treatment corresponds with the summer sea surface temperature prediction based on a low rate of global warming (the SRES B1 emission scenario). The 31.5°C treatment is based on a moderate rate of global warming (the SRES A1B scenario), while the 35°C is based on the maximums predicted in a high rate of global warming scenario (SRES A1Fl scenario), essentially the worst case scenario for global warming. All other conditions within the treatment tanks were the same across all temperatures treatments. Basking opportunity was provided for 8 h day^{-1}, to imitate the period the sun is available for thermoregulation, and mean temperature on the platform directly below the basking light for each tank was: 28 ± 7, 28 ± 6 and 29 ± 7°C (mean ± SD) for the 28, 31.5 and 35°C treatment tanks respectively. iButton data-loggers (Dallas Semiconductor, Dallas, TX, USA; accurate to ± 0.5°C) were used to record water and basking light temperature throughout the acclimation period. The large fluctuations in water and basking light temperatures during the acclimation period were due to the external location of the treatment tanks where they were in a sheltered enclosure yet were still exposed to environmental air temperatures. The acclimation period lasted a minimum of 30 days before any experiments commenced.

Experimental setup

Following the acclimation period experiments were conducted in a controlled temperature room with air temperature set to the acclimation treatment temperatures of the animals being tested. Two small plastic tanks (370 X 390 X 560 mm) containing water to a depth of 260 mm and enclosed with a metal-grate lid, so that animals could be observed but not escape, were set-up within the room. A small 55 W submersible heater (Aqua one, Australia) was placed in each tank to heat water to the acclimation treatment temperature. Two crocodiles could be tested simultaneously, one per tank.

Instrumentation

Prior to being placed into experimental tanks, crocodiles had their jaws secured using rubber bands and were weighed. They were then restrained and two electrocardiogram (ECG) wires (MLA1203 Needle electrode, AD Instruments, Sydney, Australia) were inserted subcutaneously on the ventral surface anterior and posterior to the heart. Wires were then anchored to the dorsal surface via waterproof surgical tape (2.5 cm Leukoplast Waterproof, Rigid, Zinc Oxide tape, Smith & Nephew, Australia). A conductivity sensor (insulated wire, Bambach, Australia) was then anchored along the dorsal surface and nuchal shield using surgical tape. Instrumentation and relocation of animals into the experimental tanks was conducted the evening before any experimental measurements were recorded (at least 12 h prior) to allow animals to recover overnight.

Physiological recordings

A video camera (Microsoft LifeCam Studio, Microsoft, USA) was setup above the experimental tanks and used to record dive behaviour. ECG wires, conductivity sensors and video camera connectors were run into an adjacent room via a custom built port in the wall. Electrocardiogram wires were connected to a Bioamp (ML132, AD Instruments) and the conductivity sensor was connected to a Conductivity pod (ML307, AD Instruments,). The Bioamp, Conductivity pod and video camera were connected to a PowerLab (4/30 series ML866, AD Instruments) and recording was visualised on a computer running Lab Chart software (AD Instruments) with a sample rate of 100 Hz. Using Lab Chart software ECG recordings were converted to heart rate and recordings from the conductivity sensor, along with video footage, were used to determine when crocodiles dived and surfaced. With this setup it was possible to remotely monitor animal behaviour and heart rate throughout the experiment.

Experimental treatments

Three different treatments were used to examine the role and control of the dive bradycardia in *C. porosus*. Pharmacological doses were based on the study by Altimiras *et al.* (1998):

i) *Control* - involving no pharmacological blockade of heart rate and a 0.9 % saline injection, of an equivalent volume (0.5 ml kg^{-1} body mass) to other treatments, to remove any confounding effects of injection procedure and volume.

ii) *Cholinergic blockade* – involving the blockade of the muscarinic receptors using the antagonist atropine which acts to inhibit any reductions in heart rate due to increased cholinergic activity. Dose used was 1.2 mg kg^{-1} body mass.

iii) *Complete blockade* – involving blockade of both muscarinic and β-adrenergic receptors using an injection of sotalol which, in conjunction with the previous atropine injection from the cholinergic blockade treatment, acts to restore the heart to its intrinsic rate where we expect to observe no changes in heart rate. Dose used was 3 mg kg^{-1} body mass.

Experimental procedure

Acclimation treatment and animal test order were randomised and animals were fasted for at least 48 h before instrumentation. Following instrumentation, animals were allowed to recover overnight in test tanks. Following the overnight rest period the control treatment was administered between 0700 and 0800 to the test animal using an insulin syringe (1.0 mL 29 gauge x ½", Terumo, Elkton, USA). The animal was then left alone and allowed a recovery period of 30 min followed by a 30 min voluntary recording period during which the heart rate of the animals were recorded when they were at the water's surface and during voluntary diving. A dive was defined as when an animal fully submerged and lowered to the bottom of the tank. Shallow dips of the head below the surface between breathing episodes were not counted as dives. Following the voluntary recording period the experimenter would elicit a fright dive by banging on the side of the tank, if this failed to produce a dive the crocodile was lightly touched on the tail until a fright dive was produced (Wright *et al.* 1992). Once the animal dived the experimenter would depart the room. This procedure was conducted a total of three times. If animals were highly active during the voluntary recording period or prior to a fright dive, recordings were excluded until heart rate was observed to have returned to resting levels. Following the end of the third fright dive the next pharmacological treatment was administered and the procedure was repeated again; 30 min recovery, 30 min voluntary recording period and then three fright dives. The

treatment order was control first, cholinergic blockade second and then complete blockade third. At the end of the trial, instruments were removed and test crocodiles were returned to acclimation treatment tanks.

Data analysis

Using Lab Chart software heart rate measurements were extracted along with voluntary and fright dive durations. For surface and diving heart rates, multiple recordings of between 45 to 60 seconds were extracted and then compiled using Microsoft Excel software (Microsoft, USA) where recordings were averaged for each treatment and animal. Bradycardia was also calculated for each fright dive by calculating the percentage drop in heart rate between mean surface and fright dive heart rates. Cholinergic and adrenergic tone were also calculated for surface and fright dive heart rates using the calculation described by Seebacher and Franklin (2001).

Using the statistical software RStudio (Version 0.98.1062, RStudio, Inc.), running the lmerTest package (Alexandra Kuznetsova *et al.* 2013), random linear mixed effects models were used to compare mean heart rate, bradycardia, tone and dive duration between temperatures, treatments and surface versus diving. Animal ID was included as a random effect within the models to account for repeated measures between treatments on each animal and also individual variation. Heart rate, bradycardia and cholinergic and adrenergic tone were compared in separate models using temperature, treatment and surface versus fright diving as fixed factors. Dive duration was analysed again using temperature, treatment and surface versus fright diving as fixed factors with mean fright diving heart rates included as a covariate. P-values were extracted from the model using ANOVAs and also the differences in least square means calculated within the lmerTest package. All visualisations were produced in Microsoft Excel using analysis output from the models run in RStudio. All means are presented ± S.E.

Results

Effect of submergence and temperature on the heart rate of *Crocodylus porosus*

There was no effect of water temperature on the heart rates of *C. porosus* resting at the surface or during fright dives ($F_{2, 28}$ = 1.7654, p > 0.1). Mean heart rates of *C. porosus* at the surface were 38.6 ± 2.8, 42.9 ± 3.0 and 44.5 ± 2.8 beats per minute (bpm) at 28, 31.5 and 35°C, respectively. There was however a significant effect of submergence during fright dives on the heart rate of *C. porosus* at all temperatures ($F_{1, 39}$ = 39.3299, p < 0.001) (Figure 1). During fright dives, heart rates decreased to 23.7 ± 2.8, 24.2 ± 3.0 and 29.7 ± 2.8 bpm at 28, 31.5 and 35°C, respectively. This represented diving bradycardias of 40.2 ± 5.4 %, 46.4 ± 5.8 % and 32.8 ± 5.4 % at 28, 31.5 and 35°C, respectively (Figure 2). There was no significant effect of temperature on the magnitude of the bradycardia during fright dives (Figure 2).

Effect of cholinergic and complete blockade on heart rate of *Crocodylus porosus*

The effect of pharmacological treatment did not significantly differ between temperatures ($F_{4,93}$ = 1.0035, p = 0.4099) (supplementary figure 8 & 9). We therefore pooled results from all temperature treatments when comparing the effect of pharmacological treatments on heart rate. There was a significant effect of pharmacological treatment on heart rate and this effect changed significantly between animals at the surface and during fright dives ($F_{2, 93}$ = 11.7093, p < 0.0001).

At the surface, heart rate was highest in the cholinergic blockade, followed by the control treatment and lowest in the complete blockade treatment (p < 0.005). Mean heart rates of *C. porosus* at the surface were 42.1 ± 2.3, 50.1 ± 2.3 and 35.6 ± 2.3 bpm following the control, cholinergic blockade and control blockade treatments, respectively (Figure 3a). Conversely, during fright dives heart rate was highest in the cholinergic blockade, followed by the complete blockade treatment and lowest in the control treatment (p < 0.005). Mean heart rates during fright dives following control, cholinergic blockade and complete blockade treatments were 26.1 ± 2.3, 46.1 ± 2.3 and 33.0 ± 2.3 bpm, respectively (Figure 3a).

Unlike the control treatment, there was no significant effect of submergence on heart rate in the two pharmacological blockade treatments ($p > 0.05$) (Figure 3b). Fright dive bradycardia was significantly reduced in the cholinergic and complete blockade treatments compared to the control treatment ($p < 0.0001$). Mean bradycardia was 39.6 ± 3.9 %, 7.7 ± 3.9 % and 8.0 ± 3.9 % for the control, cholinergic blockade and complete blockade treatments, respectively (Figure 4).

Cholinergic and adrenergic control of heart rate during diving in *Crocodylus porosus*

There was no significant effect of temperature on adrenergic ($F_{2,33} = 1.6508$, $p = 0.2069$) or cholinergic tone ($F_{2,30} = 0.4737$, $p = 0.6272$) and no significant interaction between temperature and diving on adrenergic ($F_{2,24} = 0.0195$, $p = 0.9807$) or cholinergic tone ($F_{2,24} = 0.4696$, $p = 0.6368$) (supplementary figure 10). We therefore pooled the results from the all temperatures for analysis of cholinergic and adrenergic tone during diving. When *C. porosus* were resting at the surface adrenergic tone was significantly higher than cholinergic tone (42.3 ± 7.3 % versus 22.8 ± 7.3 %, respectively; $p < 0.01$) (Figure 5). This was reversed during fright diving where cholinergic tone was significantly higher than adrenergic tone (64.3 ± 7.3 % versus 43.3 ± 7.3%, respectively; $p < 0.01$) (Figure 5). Submergence resulted in a significant increase in cholinergic tone ($p = <0.0001$) and had no effect on adrenergic tone ($p = 0.881$) (Figure 5).

Effects of temperature and dive bradycardia on dive duration in *Crocodylus porosus*

There was no significant difference in mean fright dive duration between temperatures ($F_{4,34} = 0.4891$, $p = 0.4891$). Dive duration for the control treatment was 296 ± 113 s, 247 ± 42 s and 245 ± 34 s at 28, 31.5 and 35°C temperatures, respectively (Figure 6). There was no significant interaction between pharmacological treatment and temperature ($F_{4,34} = 0.8746$, $p = 0.4891$). We therefore pooled results from all temperatures when comparing the effect of pharmacological treatments on heart rate. Even when data were pooled there was no significant effect of pharmacological treatment on dive duration ($F_{2,35} =$

1.2476, p = 0.2994). Mean fright dive duration was 263 ± 45 s, 303 ± 35 s and 305 ± 37 s for the control, cholinergic blockade and complete blockade treatments, respectively.

Discussion

We investigated the control and role of dive bradycardia and the effect of elevated temperatures on the diving capacity of an ectothermic vertebrate, the saltwater crocodile, *Crocodylus porosus*. As expected, a significant bradycardia, ranging between 33 – 46 %, was observed in *C.* porosus during fright dives. The control of heart rate and dive bradycardia in *C. porosus* was entirely driven by the cholinergic system, with no significant difference in adrenergic tone when at the surface or during diving. This did not change with increasing temperature and, contrary to our hypothesis; we found no effect of increased temperature on heart rate or magnitude of dive bradycardia. Similarly, neither temperature nor dive bradycardia had a significant effect on the diving capacity of *C. porosus*.

Interestingly, exposure to increased temperature between 28 - 35°C had no effect on heart rate of *C. porosus* resting at the surface or during fright dives. As a consequence, the magnitude of the dive bradycardia during fright dives was also thermally insensitive across the temperatures tested. In ectotherms physiological function is closely tied with environmental temperature and we would expect to see an increase in heart rate as temperature moves outside an animal's optimal temperature and more energy is required to maintain physiological function (Pörtner 2002; Pörtner & Farrell 2008). Our results suggest that *C. porosus* was either able to shift its optimal temperature to suit the treatment temperatures or the window over which *C. porosus* is able to maintain physiological function covers a wide range of temperatures (i.e. an increase in the thermal breadth). Oxygen consumption, and therefore metabolic rate, in in *C. porosus* are known to increase with increasing temperature. In crocodilians, routine oxygen consumption increases with acute temperature exposure between 10 and 35°C (Grigg 1978; Lewis & Gatten Jr 1985; Emshwiller & Gleeson 1997). Similarly, in *C. porosus* maintained at 28°C oxygen consumption increased 10-fold between 28 and 33°C following exercise in a swimming flume (Campbell *et al.* 2013). It is therefore unlikely that the insignificant differences in heart rate between temperature treatments observed in this study are due to a broad thermal plateau of independence of heart rate in *C. porosus.* It is more likely that *C. porosus* were able to

acclimate to increased temperatures up to 35°C. Thermal acclimation in *C. porosus* has previously only been observed for winter (20°C) and summer (29°C) temperatures (Glanville & Seebacher 2006). Our results suggest that *C. porosus* are capable of acclimating their physiological function to elevated temperatures between 28 - 35°C.

Similar to heart rate, there was no significant difference in mean fright dive duration of *C. porosus* between 28 - 35°C. A broad plateau of thermal independence has been previously shown for swimming performance in *C. porosus* (Elsworth *et al.* 2003; Campbell *et al.* 2013). In *C. porosus* maintained at 28°C, swimming performance remained stable between 23 and 33°C before declining at 35°C (Elsworth *et al.* 2003; Campbell *et al.* 2013). This would support the hypothesis that the observed patterns in diving performance in this study are due to *C. porosus* having a broad plateau of thermal independence. However, when we factor in that heart rate did not differ between any of the temperatures, including at 35°C which is when swimming performance declined in the previous studies, this suggests that thermal acclimation is the mechanism explaining these patterns. This is again supported by Glanville and Seebacher (2006), which found clear evidence of thermal acclimation of swimming performance to winter (20°C) and summer (29°C) temperatures, with maximum sustained swimming performance being highest at acclimation temperatures and before declining at temperatures above and below this. In order to clearly identify the mechanism explaining how *C. porosus* was able to maintain heart rate and diving performance at elevated temperatures, further study involving testing animals over a range temperatures, rather than solely at the acclimation temperatures, is needed.

Cholinergic tone varied significantly between when *C.* porosus were resting at the surface and during fright diving. This is compared to adrenergic tone which showed no significant difference between the surface and diving. Cholinergic withdrawal (reduction in tone) at the surface produced the higher surface heart rates while dramatic increase in cholinergic activity during fright dives caused the significant bradycardia observed. This finding is similar to those found in diving mammals, where dive bradycardia was primarily driven by increased cholinergic activity (Signore & Jones 1995; Elliott *et al.* 2002). This reliance on the cholinergic system for controlling heart rate during diving is most likely attributed to heart rate responding more rapidly to changes in parasympathetic (cholinergic) activity when compared to changes in sympathetic (adrenergic) activity (Furilla & Jones 1987; Japundzic *et al.* 1990). This would facilitate rapid response of heart rate between

bradycardia during diving, to conserve oxygen, and tachycardia at the surface following a dive, to aid in oxygen uptake (Furilla & Jones 1987).

C. porosus exhibited a large bradycardia during fright dives, and this was significantly reduced with cholinergic and adrenergic blockade (administration of atropine and sotalol). We hypothesised that dive bradycardia aids in extending mean fright dive duration; however, we found no significant reduction in dive duration when bradycardia was pharmacologically inhibited. Similar, was found in diving mammals, with no significant decrease in voluntary dive duration when dive bradycardia was abolished (Signore & Jones 1995; Elliott *et al.* 2002). Dive bradycardia has only been shown to extend dive duration during maximum forced dives in muskrats (Signore & Jones 1995). This suggests that dive bradycardia may only aid in extending dive duration during prolonged dives beyond an animal's aerobic dive limit (ADL; the limit beyond which an animal must start relying on anaerobic metabolism) (Kooyman *et al.* 1980; Butler & Jones 1997). Our results suggest that even during fright dive when a significant bradycardia is observed, there is no effect of dive bradycardia on dive duration. It is probable that this is due to *C. porosus* diving within their ADL. The longest fright dive we observed lasted less than 12 min, whereas juvenile *C. porosus* have been observed to undertake fright dives in excess of 19 min (Wright *et al.* 1992). Our findings further support that dive bradycardia may only influence prolonged submergence during dives well beyond an animal's aerobic dive limit and that this is comparable for diving mammals and ectotherms.

We aimed to determine the control and role of dive bradycardia and how it is influenced by chronic increases in temperature in a diving ectotherm, *C. porosus*. From our data it is evident that *C. porosus* is able to maintain physiological function and diving performance when chronically exposed to elevated temperatures. Whether, this is due to thermal acclimation or *C. porosus* maintaining a broad plateau of thermal independence on performance remains inconclusive. Further study testing heart rate and diving capacity over a range of temperatures in addition to acclimation temperatures would help elucidate this. We also determined that cholinergic and adrenergic control of heart rate during diving was unaffected by temperature and that the control of heart rate during diving is driven primarily by shifts in cholinergic activity. Additionally, bradycardia was shown to have no effect in increasing fright dive duration. Further experimental study looking at the role of heart rate in prolonging dive duration will need to identify a protocol that encourages animals to dive

beyond their aerobic dive limit in order to clearly determine if dive bradycardia plays a key role in prolonging dive duration.

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Figures

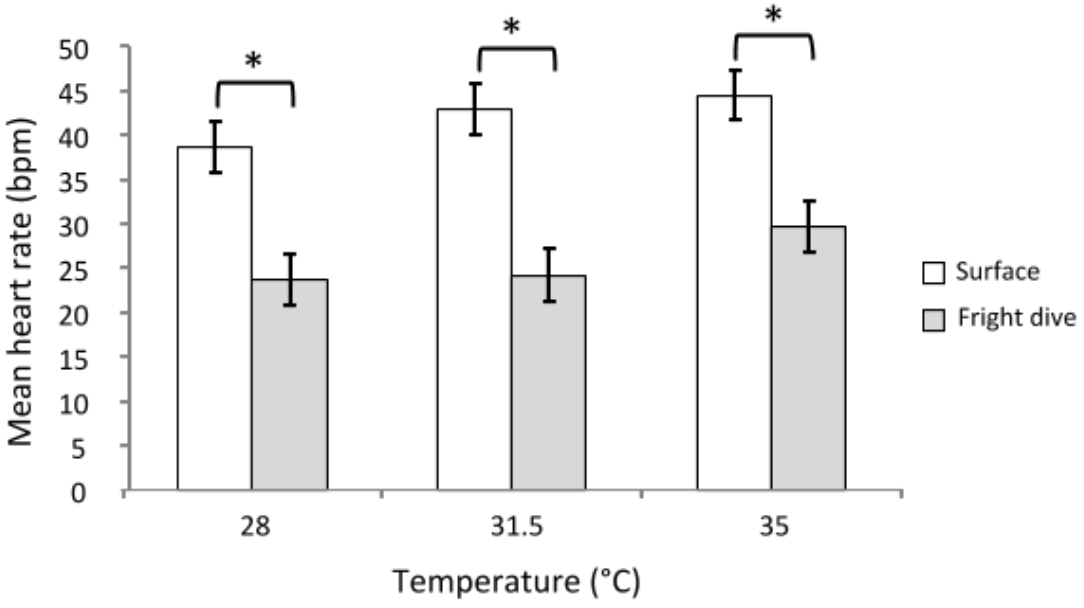


Figure 1: Mean heart rate (bpm ± SE) of *Crocodylus porosus* in control treated animals when resting at the surface (white bars) and during fright dives (light-grey bars) based on acclimation temperature. Brackets and * indicate a significant difference with $p < 0.05$.

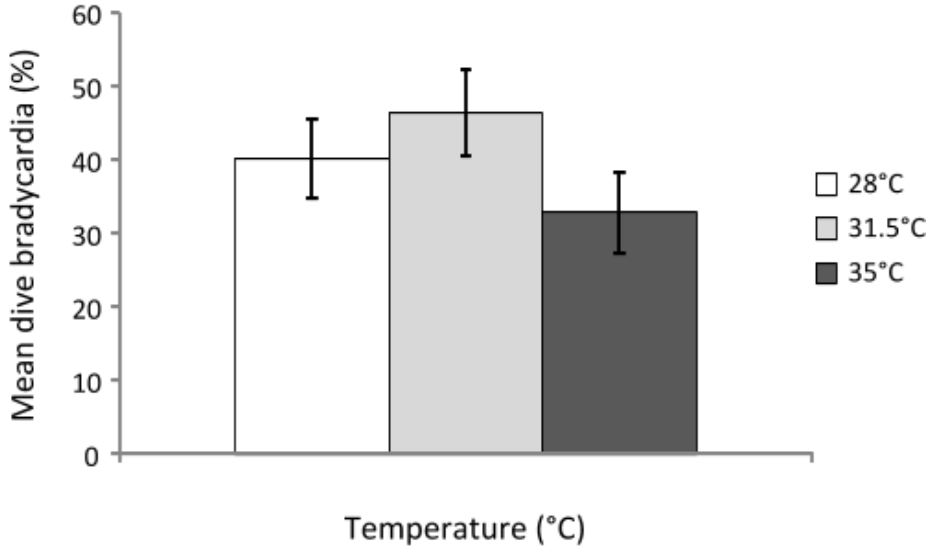


Figure 2: Mean percentage fright dive bradycardia (% ± SE) of *Crocodylus porosus*, based on the mean surface and fright dive heart rate in control animals, for the different acclimation temperatures; 28°C (white bars), 31.5°C (light-grey bars) and 35°C (dark-grey bars) .

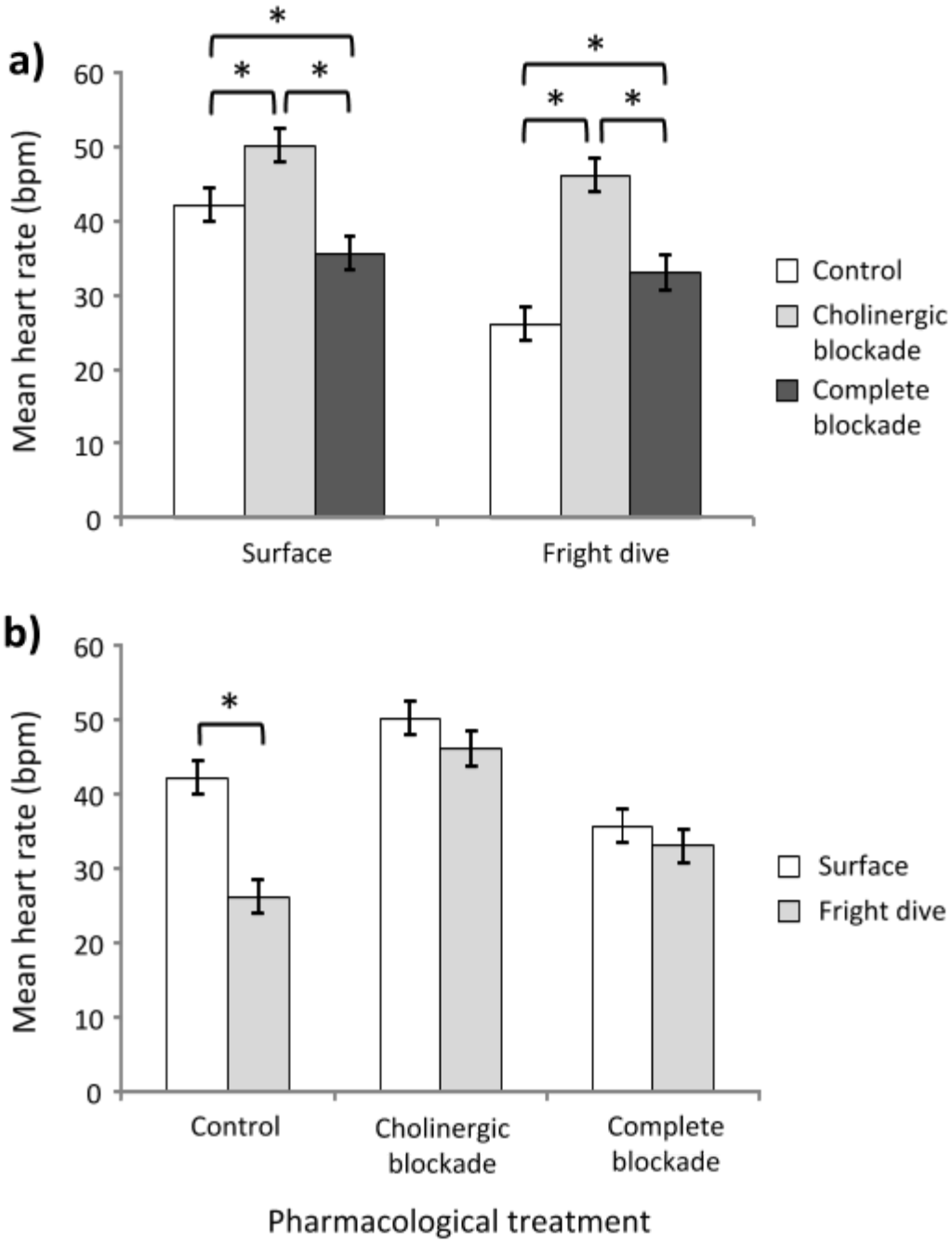


Figure 3: Mean heart rate (bpm ± SE) of *Crocodylus porosus* when following control, cholinergic blockade and complete blockade treatments at the surface and during fright diving, using pooled data from all acclimation temperatures. The top graph **(a)** compares mean heart rates between pharmacological treatments when crocodiles were at the surface and during fright dives. The bottom graph **(b)** shows the same information but compares mean heart rate between *C. porosus* at the surface (white bars) or during fright dives (light-grey bars) for each pharmacological treatment. Brackets and * indicate a significant difference with $p < 0.05$.

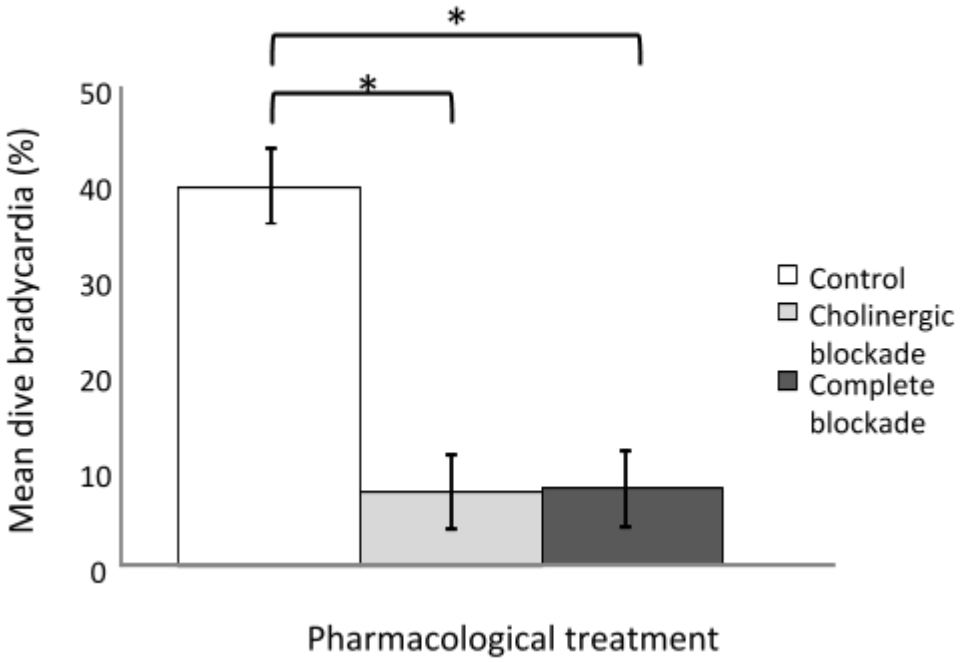


Figure 4: Mean fright dive bradycardia (% ± SE) of *Crocodylus porosus*, calculated as percentage drop in fright dive heart rate versus surface heart rate, following control (white bar), cholinergic blockade (light-grey bar) and complete blockade (dark-grey bar) pharmacological treatments. Using pooled data from all acclimation temperatures. Brackets and * indicate a significant difference with $p < 0.05$.

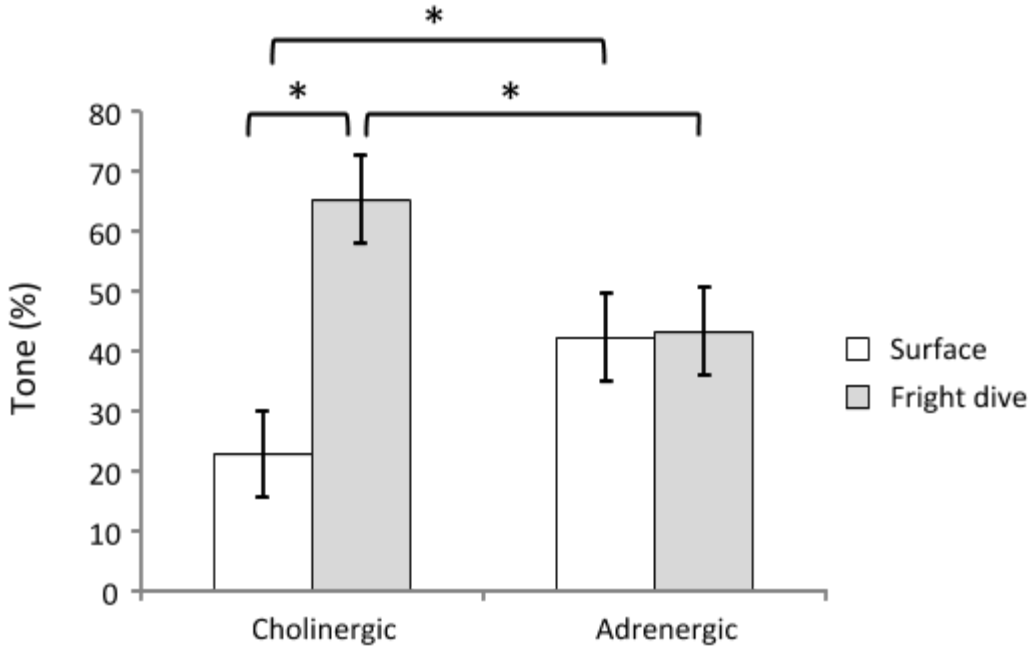


Figure 5: Mean cholinergic and adrenergic tone (% ± SE) for *Crocodylus porosus* when resting at the surface (white bars) and during fright dives (light-grey bars). Brackets and * indicate a significant difference with $p < 0.05$.

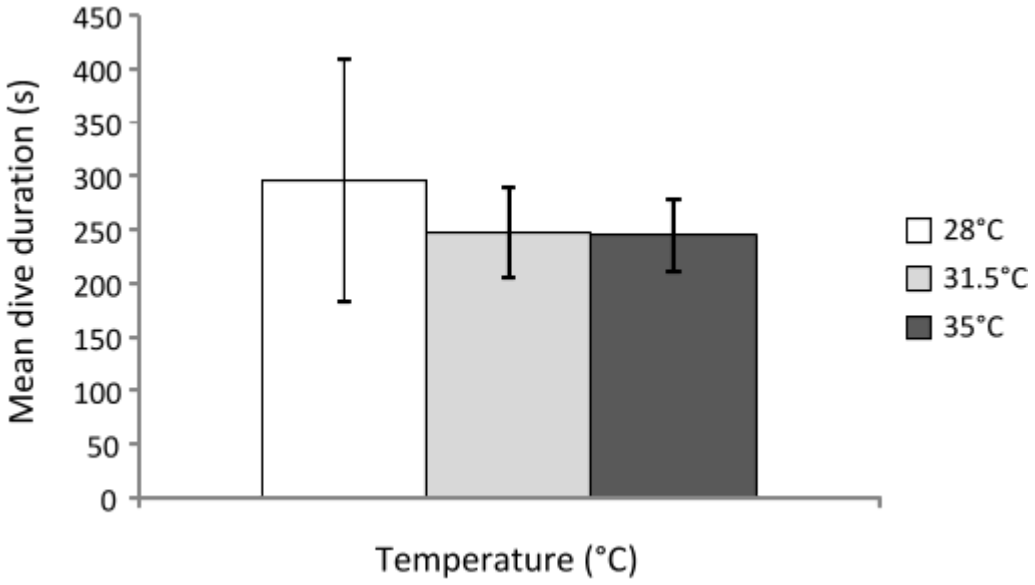


Figure 6: Mean fright dive duration (seconds ± SE) of *Crocodylus porosus* in control treated animals when acclimated at 28°C (white bar), 31.5°C (light-grey bar) and 35°C (dark-grey bar).

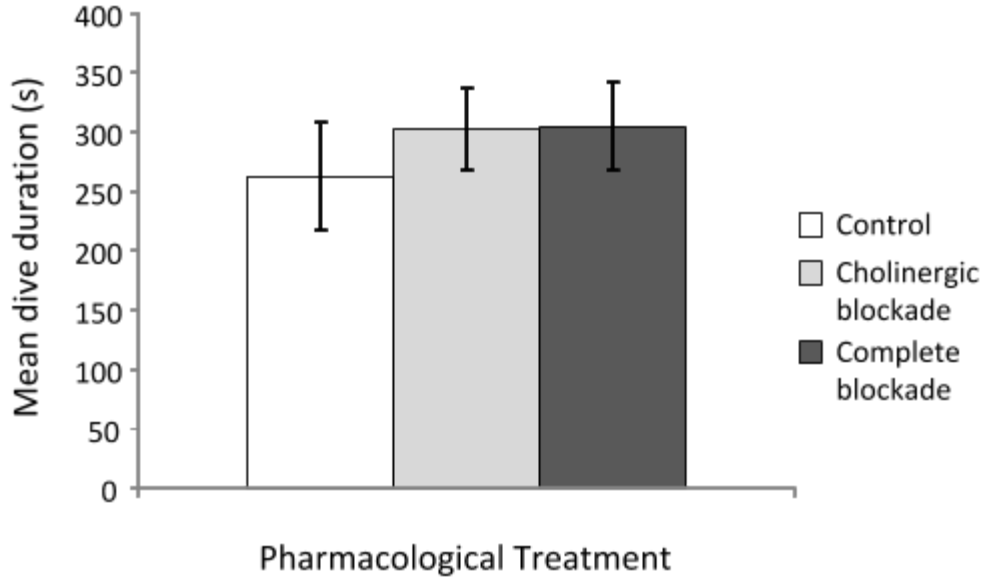
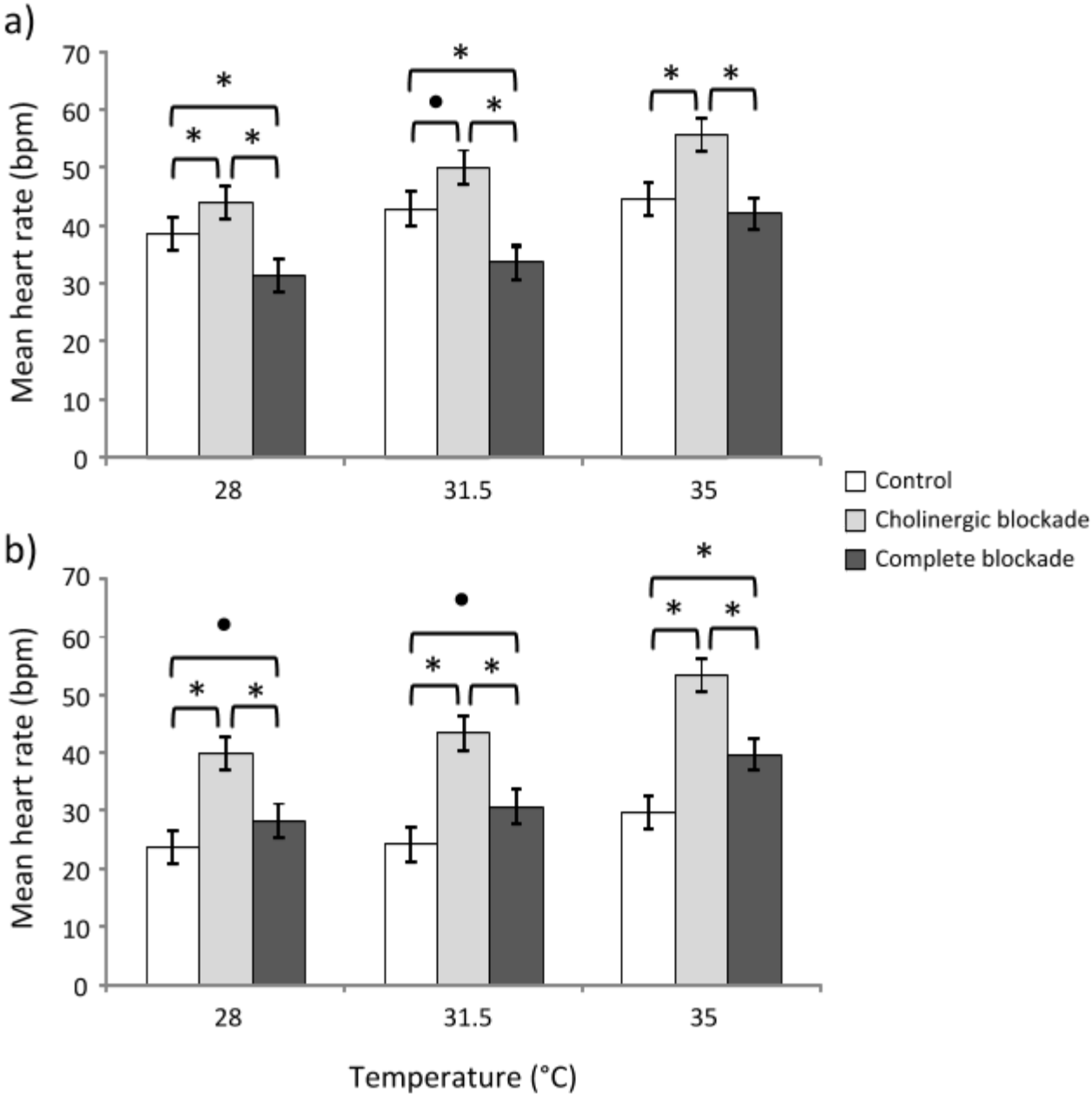
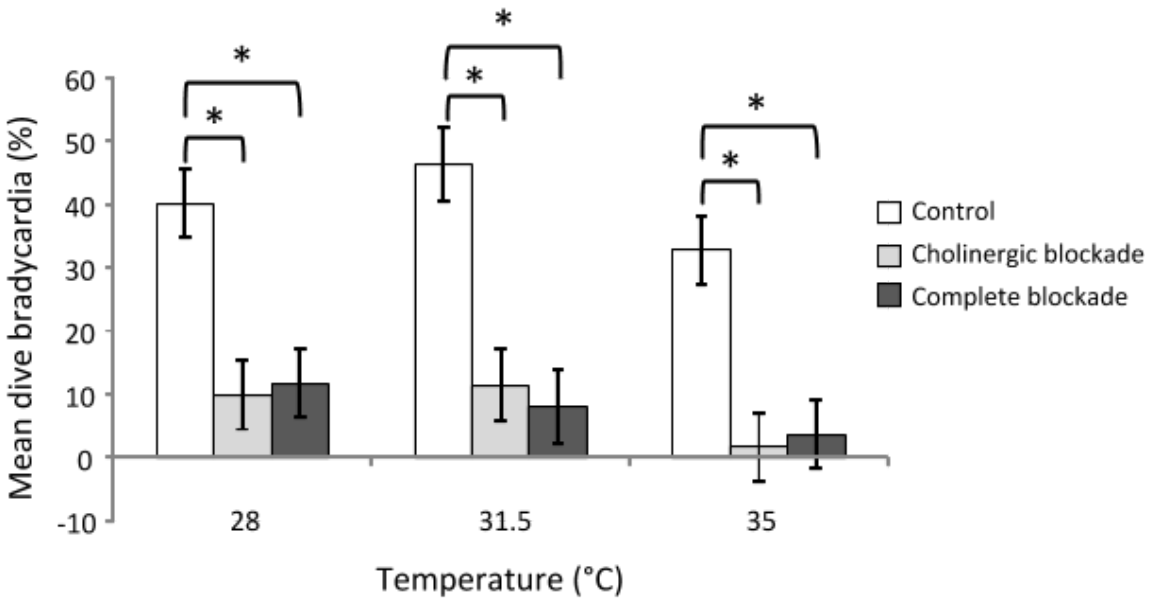


Figure 7: Mean fright dive duration (seconds ± SE) of *Crocodylus porosus* following control (white bar), cholinergic blockade (light-grey bar) and complete blockade (dark-grey bar) treatments. Using data pooled from all acclimation temperatures.

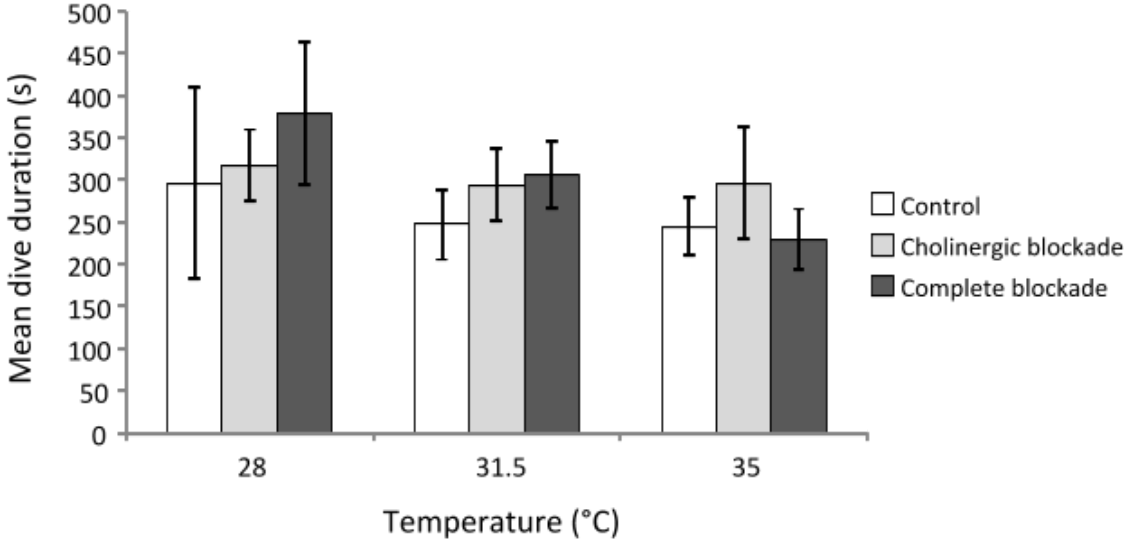
Supplementary Information



Supplementary figure 8: Mean heart rate (bpm ± SE) of *Crocodylus porosus* following control (white bar), cholinergic blockade (light-grey bar) and complete blockade (dark-grey bar) treatments for each acclimation temperature. The top graph **(a)** shows mean heart rate when crocodiles were resting at the surface. The bottom graph **(b)** shows mean heart rate of crocodiles during fright dives. There was no significant interaction between acclimation temperature and treatment ($F_{4,93}$ = 1.0035, p = 0.4099). Brackets with * indicate a significant difference with $p < 0.05$. Brackets with • indicate a difference where $p < 0.10$ and was included to help illustrate the similar response of heart rate across the different temperatures.



Supplementary figure 9: Mean fright dive bradycardia (% ± SE) of *Crocodylus porosus,* calculated as percentage drop in fright dive heart rate versus surface heart rate, following control (white bar), cholinergic blockade (light-grey bar) and complete blockade (dark-grey bar) treatments for each acclimation temperature. There was no significant interaction between acclimation temperature and treatment ($F_{4, 41} = 0.3438$, $p = 0.8461$)**.** Brackets with * indicate a significant difference with $p < 0.05$.



Supplementary figure 10: Mean fright dive duration (seconds ± SE) of *Crocodylus porosus* following control (white bar), cholinergic blockade (light-grey bar) and complete blockade (dark-grey bar) treatments for each acclimation temperature. There was no significant interaction between acclimation temperature and treatment ($F_{4, 34} = 0.8746$, $p = 0.4891$). There were no significant differences in mean fright dive duration between pharmacological treatments across all acclimation temperatures.