J Comp Physiol B (2013) 183:947–957
DOI 10.1007/s00360-013-0755-2

ORIGINAL PAPER

Chronic hypoxic incubation blunts thermally dependent cholinergic tone on the cardiovascular system in embryonic American alligator (*Alligator mississippiensis*)

Chris Marks · John Eme · Ruth M. Elsey · Dane A. Crossley II

Received: 9 February 2013 / Revised: 4 March 2013 / Accepted: 15 March 2013 / Published online: 30 April 2013

Abstract Environmental conditions play a major role in shaping reptilian embryonic development, but studies addressing the impact of interactions between chronic and acute environmental stressors on embryonic systems are lacking. In the present study, we investigated thermal dependence of cholinergic and adrenergic cardiovascular tone in embryonic American alligators (*Alligator mississippiensis*) and assessed possible phenotypic plasticity in a chronic hypoxic incubation treatment. We compared changes in heart rate (f_H) and mean arterial blood pressure (P_M) for chronically hypoxic and normoxic-incubated embryos after cholinergic and adrenergic blockade following three different acute temperature treatments: (1) 30 °C (control incubation temperature), (2) acute, progressive decrease 30–24 °C then held at 24 °C, and (3) acute, progressive increase 30–36 °C then held at 36 °C. f_H progressively fell in response to decreasing temperature and rose in response to increasing temperature. P_M did not significantly change with decreasing temperature, but was lowered significantly with increasing acute temperature in the normoxic group at 90 % of development only. Propranolol administration (β adrenergic antagonist) produced a significant f_H decrease at 24, 30, and 36 °C that was similar at all temperatures for all groups. For normoxic-incubated embryos at 90 % of development, atropine administration (cholinergic antagonist) significantly increased f_H in both 24 and 36 °C treatments, but not in the 30 °C control treatment. This atropine response at 24 and 36 °C demonstrated acute thermally dependent cholinergic tone on f_H late in development for normoxic-incubated, but not chronically hypoxic-incubated embryos. Collectively, data indicated that cardiovascular control mechanisms in embryonic alligators may be activated by thermal extremes, and the maturation of control mechanisms was delayed by chronic hypoxia.

Keywords Atropine · Embryo · Hypoxia · Propranolol · Reptile · Temperature

Abbreviations

CAM	Chorioallantoic membrane
f_H	Heart rate
P_M	Mean arterial pressure
T_E	Temperature of embryo, from thermocouple probe inserted into allantoic fluid
N70 or N90	Alligator embryos incubated in chronic normoxia, measured at 70 or 90 % of embryonic development, respectively
H70 or H90	Alligator embryos incubated in chronic hypoxia (10 % O_2) beginning at 20 % of development, measured at 70 or 90 % of embryonic development, respectively

Introduction

Heat has pervasive effects on animal physiology, and heat fluctuations have been of central focus in studies of ectothermic or poikilothermic species, such as reptiles (Seebacher and Franklin 2005). The interactive effects of environmental oxygen and temperature on reptile

Communicated by H.V. Carey.

C. Marks · J. Eme · D. A. Crossley II (✉)
Department of Biological Sciences, University of North Texas, 1155 Union Circle #305220, Denton, TX 76203-5017, USA
e-mail: Dane.Crossley@unt.edu

R. M. Elsey
Louisiana Department of Wildlife and Fisheries, Rockefeller Wildlife Refuge, Grand Chenier, LA 70643, USA

physiology have been of long-standing interest to comparative physiology (Boyer 1967; Jackson 1973; Branco et al. 1993). Interactions between ambient temperature and other abiotic stressors such as hypoxia have been investigated in adult reptiles, specifically in relation to oxygen transport (Glass et al. 1979; Wood et al. 1987) and thermoregulation (Hicks and Wood 1985; Petersen et al. 2003), but the impact of these combined stressors on functional embryonic cardiovascular development is unknown. Embryos of the most temperate crocodilian species, American alligator (*Alligator mississippiensis*), are subjected to wide ranges of nesting temperatures between 23 and 38 °C (reviewed in Coulson and Hernandez 1983). These thermal variations are combined with the potential for alligator embryos, like other reptile embryos, to encounter hypoxia in the nest that could impact embryonic organ system development, such as the cardiovascular system (McIlhenny 1934; Joanen 1969; Chabreck 1973; Lutz and Dunbar-Cooper 1984; Booth 2000; Crossley and Altimiras 2005; Eme et al. 2011a, b). The cardiovascular system is the first functional embryonic organ system (Burggren and Keller 1997), and its responses to acute temperature change (Birchard 2000; Nechaeva et al. 2005, 2007) as well as chronic/acute hypoxic stresses have been individually explored during development of some reptiles (Crossley and Altimiras 2005; Du et al. 2010a; Eme et al. 2011a). However, combined effects of chronic hypoxic incubation and acute thermal changes on the developing cardiovascular system have not been explored.

For the majority of American alligator embryonic development, baseline cardiovascular function appears to be maintained by circulating catecholamines (Crossley et al. 2003; Crossley and Altimiras 2005; Eme et al. 2011a). Chronic hypoxic (10 % O_2) development caused a suite of phenotypic modifications of the alligator cardiovascular system including, reduced arterial pressure and heart rate, altered adrenoreceptor function, blunted acute hypoxic response and delayed neural reflexive control (Crossley and Altimiras 2005; Eme et al. 2011a, b). Cardiovascular effects of changing temperature have been investigated in juvenile crocodilians (Seebacher and Franklin 2004), but its effect on embryonic cardiovascular function is unknown. However, effects of both acute and chronic thermal stress have been studied in other embryonic reptiles (Birchard and Reiber 1996; Birchard 2000; Nechaeva et al. 2005, 2007; Du et al. 2010a; Nechaeva 2011). For example in veiled chameleon (*Chamaeleo calyptratus*) and pond turtle embryos (*Emys orbicularis*), heart rate (f_H) increased and decreased in response to changes in ambient temperature as would be predicted for ectothermic vertebrates (Nechaeva et al. 2005, 2007). However, neural regulation of f_H with change in temperature has not been investigated in embryonic reptiles.

The purpose of this study was to investigate how chronic hypoxic incubation in alligator embryos altered thermal dependency of cholinergic and adrenergic receptor-mediated cardiovascular regulation to acute thermal extremes. We have previously reported that chronic hypoxic incubation modified cardiovascular development in embryos of the American alligator, altering heart rate regulation (Eme et al. 2011a, b). In addition, we have shown that alligator embryos rely primarily on high, constant adrenergic tone during incubation. During periods of high temperature exposure, cholinergic tone may be active, limiting the increase in f_H associated with increased heat. Conversely cholinergic tone could be activated during periods of low temperature reducing heart rate in periods of reduced metabolic demand. We hypothesized that changes in embryonic temperature would activate cholinergic tone on the heart in American alligators while β-adrenergic tone on the cardiovascular system would be unaltered due to its importance during incubation. Further, we hypothesized that chronic hypoxic incubation would blunt temperature dependent cholinergic tone similar to prior investigations that reported blunted pharmacological responses in chronically hypoxic-incubated alligator embryos (Eme et al. 2011a, b).

Materials and methods

Embryos and incubation

American alligator eggs (*Alligator mississippiensis*) were obtained from the Rockefeller Wildlife Refuge in Grand Chenier, LA, USA and transported to the Department of Biological Sciences at the University of North Texas (UNT) in Denton, TX, USA ($N = 79$). Upon arrival, two eggs from each clutch were randomly selected to determine embryonic stage for estimations of age and total incubation length (Ferguson 1985; Crossley et al. 2003), and remaining eggs were distributed randomly across identical plastic containers containing a 1:1 vermiculite:water mixture (7–10 eggs per container). Water content of the vermiculite was maintained by weighing containers weekly and adding water as needed. Egg containers were sealed in large Ziploc® bags with two holes allowing for inflow and outflow of humidified gas mixtures, and containers were placed in a temperature controlled room maintained at 30 °C to ensure all embryos were developing as females. Hypoxic (10 % O_2) and normoxic (21 % O_2) conditions were maintained by passing N_2 and air or air alone, respectively, through rotameters and a H_2O bubbler into each bag's inflow. Chronic hypoxic incubation began at ~20 % of embryonic development (~day 14 post-laying) based on estimated egg age upon collection and embryonic

stage established upon arrival at UNT. Humidity was maintained at ≥80 %. Gas composition in bags was continuously monitored with an oxygen analyzer (S-3AI, Ametek Applied Electrochemistry, IL, USA). Two embryonic incubation points (70 and 90 %), reflective of in ovo developmental stages 24/25 and 27/28, were selected for study (Ferguson 1985; stage 26 absent in *A. mississippiensis*). Therefore, we studied our two chronic oxygen incubation treatments at the same developmental stages, and hypoxic incubation does not affect hatching time in alligator embryos (Eme et al. 2011a, b).

Developmental and chronic oxygen incubation ages are abbreviated N70 (normoxic, 70 % embryonic development), H70 (hypoxic, 70 % embryonic development), H90 (hypoxic, 90 % embryonic development), and N90 (normoxic incubation, 90 % embryonic development).

Surgical procedures

Eggs were removed from their incubation conditions and candled to locate a tertiary chorioallantoic membrane (CAM) artery. Eggs were placed in a temperature controlled surgical chamber (30 °C, 21 % O_2), and a portion of the eggshell removed under a dissection microscope (Leica MZ6; Leica Microsystems, Waukegan, IL, USA). An occlusive catheter was placed in a tertiary CAM artery using heat-pulled, heparinized saline-filled PE 50 tubing for measurements of arterial pressure (P_M) and drug injections (Crossley and Altimiras 2005). A thermocouple probe (RET-4, Physitemp Instruments, Clifton, NJ, USA) was immersed 1.5 cm into the allantois fluid next to the embryo and anchored to the shell with cyanoacrylate to monitor 'embryo temperature' (T_E). The probe was attached to a microprobe thermometer that continuously monitored T_E (BAT-12, Physitemp Instruments, Clifton, NJ, USA). No anesthesia/analgesia was used, until after experimentation to euthanize the embryo.

Following catheterization and thermocouple probe placement, embryos were placed on cotton wool in a water-jacketed, aluminum apparatus containing six chambers—700 cm^3 per chamber (7 × 7 × 10 cm), one embryo per chamber. Chambers were surrounded by a 2.5 cm. water-jacketed space, and the entire apparatus was made of aluminum. Temperature in the chambers was maintained at a starting point of 30 °C and altered by continuously recirculating water in the water-jacketed space via a Polestar® temperature-controlled circulator (Cole Parmer, Court Vernon Hills, IL, USA). Air was continuously pumped into each chamber at ~0.2 l min^{-1} after passing through a H_2O bubbler connected to a 2-m heating coil (PE-50) lining the chambers. Each chamber was sealed using a lid with three small ports allowing the catheter, thermocouple, and airline to enter. Ports were covered during experimentation with duct tape. Each catheter was attached to a pressure transducer (ADInstruments model MLT0699) 4–5 cm above the egg via saline-filled PE 50 tubing. Pressure signals were acquired at 40 Hz via a Powerlab® data recording system (ADInstruments, CO, USA) connected to a computer running ChartPro® software (v 7.2 ADInstruments). Pressure transducers were calibrated before each experiment with a vertical column of saline, and heart rate f_H determined with a software tachograph based on the arterial pressure pulse. Absolute blood pressure was corrected for a pressure transducer's distance above the egg by adding the distance from the transducer to the catheter (±0.1 cm) to recorded pressure (kPa).

Pharmacological treatments

Drugs were administered via a T connector in the arterial line, and total individual injection volumes were normalized according to embryonic age and did not surpass 5 % of total blood volume (100 µl for 70 % and 150 µl for 90 %; Tate et al. 2012). Each injection consisted of 1/3 drug (30 and 50 µl for 70 and 90 % embryos, respectively) and 2/3 saline to ensure drug entered the CAM artery (Crossley et al. 2003). Prior to drug injections, each embryo received a control injection of heparinized saline equal to the total drug plus saline injection volume.

Cardiovascular parameters were allowed to stabilize for 1 h prior to the control saline injection, and intervals between subsequent drug injections were 30–60 min. The project was divided into five separate Series, conducted on different sets of embryonic alligators.

Series I: cardiovascular responses to high and low temperatures for N70, H70, N90, and H90 embryos

T_E was decreased to 24 °C or increased to 36 °C by adjusting chamber temperature from a starting point of 30 °C using the circulator while f_H and P_M were continuously monitored. This Series was performed on normoxic and hypoxic-incubated embryos at 70 and 90 % of development (to 24 °C—N70 $N = 5$ and N90 $N = 6$; to 36 °C—H70 $N = 5$, N70 $N = 5$, H90 $N = 5$ and N90 $N = 6$). Our approximate upper and lower acute thermal limits, 36 and 24 °C, closely approached the upper and lower bounds found in field alligator nests (McIlhenny 1934; Joanen 1969; Chabreck 1973).

Series II: effects of 24 °C on cholinergic and β-adrenergic receptor tone for N70 and N90 embryos

This study assessed cholinergic and β-adrenergic receptor tone on f_H and P_M at 24 °C. T_E was decreased to 24 °C from a starting point of 30 °C by lowering chamber

temperature using the circulator. Following 30 min of T_E at 24 °C, the cholinergic antagonist atropine (3 mg kg^{-1}, SIGMA; a cholinergic receptor antagonist) and then the β-adrenergic antagonist propranolol (3 mg kg^{-1}, SIGMA; a non-specific β-adrenoreceptor antagonist) were administered sequentially. This Series was performed on normoxic-incubated embryos at 70 and 90 % of development (N70 $N = 5$, N90 $N = 6$).

Series III: effects of 36 °C on cholinergic and β-adrenergic receptor tone for N70, H70, N90, and H90 embryos

This study assessed cholinergic and β-adrenergic receptor tone on f_H and P_M at 36 °C. T_E was increased to 36 °C from a starting point of 30 °C by raising chamber temperature using the circulator. Following 30 min of T_E at 36 °C, the cholinergic antagonist atropine (3 mg kg^{-1}) and then the β-adrenergic antagonist propranolol (3 mg kg^{-1}) were administered. This Series was performed on normoxic and hypoxic-incubated embryos at 70 and 90 % of development (H70 $N = 5$, N70 $N = 5$, H90 $N = 5$, N90 $N = 6$).

Series IV: effects of zatebradine at 36 °C for N90 and H90 embryos

This study assessed the effect of high temperature on cholinergic tone on f_H and P_M independent of the thermally dependent increases in f_H, by decreasing the depolarization rate of the sinoatrial node function using zatebradine (3 mg kg^{-1}; inhibits HCN channels in sinoatrial node cells and Purkinje fibers). T_E was increased to 36 °C from a starting point of 30 °C by raising chamber temperature using the circulator. Following 30 min of T_E at 36 °C, zatebradine was administered. Subsequently, the cholinergic antagonist atropine (3 mg kg^{-1}) was administered. This Series was performed on normoxic and hypoxic-developed embryos at 90 % of development (H90 $N = 4$, N90 $N = 6$).

Series V: cholinergic and β-adrenergic tone at control temperature, 30 °C, for normoxic embryos

This study assessed difference in cholinergic and β-adrenergic tone on f_H and P_M at the control incubating temperature (30 °C) to other thermal studies. Following ≥60 min of T_E at 30 °C, the cholinergic antagonist atropine (3 mg kg^{-1}) and then the β-adrenergic antagonist propranolol (3 mg kg^{-1}) were administered. This Series was performed on normoxic-incubated embryos at 90 % of development (N90 $N = 5$).

Data analyses and presentation

In all cases, sample size differences are due to loss of arterial pressure pulse attributed to inadequate instrumentation. Absolute mean arterial pressure (P_M; kPa) and mean heart rate (f_H; beats min^{-1}) represent the grand mean of individual mean values from sets of embryos within each experimental protocol. For cardiovascular responses to increasing or decreasing temperature, mean f_H (beats min^{-1}) and mean CAM P_M (kPa) were taken for 5 min periods at ±0.5 °C around each 2 °C temperature interval between 30 and 36 °C or 30 and 24 °C. For example, to determine f_H and P_M at 32 °C, data between 31.5 and 32.5 °C was selected, and to determine f_H and P_M at 26 °C, data between 26.5 and 25.5 °C was selected. For values at 24, 30, and 36 °C, a stable 5 min period of data was selected. Embryos from the same oxygen treatment/developmental stage were pooled across experimental Series for this analysis.

For cholinergic and adrenergic tone responses, f_H and P_M were taken for approximately 30 min post injection after both parameters had been constant for 15 min. Cardiovascular responses to temperature change were assessed using one- or two-way repeated measures (RM) ANOVA and ANCOVA with oxygen treatment, developmental stage, and their interaction as independent variables, and embryo mass as the covariate for the ANCOVA. Pharmacological responses at 24, 30, and 36 °C were determined using paired t tests (pre-injection vs. post-injection). Where injection responses were significant across all treatments, the magnitude of the injection response was calculated as a fraction of the pre-injection value (Eme et al. 2011a). For comparisons of the magnitude of a change in f_H or P_M between oxygen incubation conditions and percentage of embryonic developmental, arcsine square root transformed fractions were used. The fraction of the effect of a drug was calculated in relative terms as the absolute change divided by the absolute control level. Data were transformed (arcsine square root) and then compared using a two-sample t test (for two group comparisons) or ANOVA (for more than two group comparisons; Eme et al. 2011b). Because the differences between absolute control level and the change following pharmacology is a ratio or percentage, data were transformed (arcsine square root) and then compared using ANOVA (Eme et al. 2011a, b). Rate of temperature change between our high and low acute temperature trials was compared with a two-tailed t test. For ANOVA tests, the SAS GLM procedure followed by Tukey's post hoc with $\alpha = 0.05$ was used (SAS Institute Inc., Cary, NC). Throughout the text, means are given ± SEM.

Results

Body mass and heart mass for normoxic and hypoxic incubated alligator embryos were similar to previous data (Eme et al. 2011a, b). Hypoxic-incubated embryos were smaller, with relatively enlarged hearts (Table 1).

Rate of temperature change

The overall rate of temperature change (3.7 °C h^{-1}) was consistent across both high and low temperature experiments. For the low temperature experiment, embryo temperature (T_E) of 24 °C was achieved in 95.9 ± 8.3 min. For the high temperature experiment, T_E of 36 °C was achieved in 93.0 ± 6.1 min. These times were not different. Therefore, the duration of the experiments and temperature rate change were similar for both high and low temperature studies and were not a confounding factor in this study. RM-ANCOVA showed no significant effect with embryo mass of percent incubation or oxygen condition on temperature change.

Series I: cardiovascular responses to high and low temperatures for N70, H70, N90 and H90 embryos

Resting P_M at 30 °C for 70 and 90 % of incubation alligator embryos was similar to previously reported data for both hypoxic and normoxic embryos, while f_H was slightly, but not significantly, decreased in the H90 compared to N90 embryos (Fig. 1a; Crossley and Altimiras 2005; Eme et al. 2011a, b). Decreasing T_E caused a significant f_H reduction in both N70 and N90 embryos (Fig. 1a; $P < 0.001$). Embryos at 70 % of development significantly decreased f_H between 30–28 and 26–24 °C, and 90 % embryos significantly decreased f_H between 28–26 and 26–24 °C ($P < 0.01$). P_M also significantly declined with increasing T_E (Fig. 1b; $P < 0.05$). Compared to 70 % of embryonic development, embryos at 90 % of development maintained significantly higher P_M throughout high and low temperature experiments ($P < 0.0001$), and decreasing temperature from 28 to 26 °C produced the maximal reduction (Fig. 1).

Increasing T_E caused a significant tachycardia (Fig. 1a; $P < 0.001$). Increasing temperature from 30 to 36 °C elevated f_H, with a significant increase compared to 30 °C at 34 °C ($P < 0.05$). There were no significant f_H increases between 34 and 36 °C in any groups. While increasing T_E was accompanied by a reduction in overall P_M for N90 embryos (Fig. 1b), P_M for the other groups were not different. The N90 embryos showed a higher P_M relative to H90, N70 and H70 (Fig. 1b; $P < 0.0001$).

Series II: effects of 24 °C on cholinergic and adrenergic tone in N70 and N90 embryos

Saline injections had no significant effect on f_H or P_M. At 24 °C, cholinergic receptor blockade with atropine significantly increased f_H in N90 embryos without affecting P_M, indicating active cholinergic tone at 24 °C (Fig. 2a; $P < 0.05$). Subsequent β-adrenergic receptor blockade with propranolol significantly lowered f_H at 24 °C for N70 and N90 embryos (Fig. 2b; $P < 0.05$). The magnitude of the f_H response to propranolol was similar between N70 and N90 embryos, and propranolol had no significant effect on P_M.

Series III: effects of 36 °C on cholinergic and adrenergic tone for N70, H70, N90, and H90 embryos

Saline injections had no significant effect on f_H or P_M. Atropine injection significantly increased f_H only in N90 embryos (Fig. 3a; $P < 0.01$). P_M was not significantly altered in any group by atropine. Propranolol injection significantly lowered f_H in all groups ($P < 0.05$), and the magnitude of the f_H response was similar across oxygen treatments, developmental ages, with no significant interaction effects after ANOVA. Propranolol had no significant effect of P_M on any group (Fig. 3b).

Series IV: effects of zatebradine at 36 °C for N90 and H90 embryos

Saline injections had no significant effect on f_H or P_M. Injection of zatebradine significantly lowered f_H in both H90 and N90 embryos (Fig. 4; $P < 0.01$). The reduction in f_H following zatebradine treatment was similar in intensity between N90 (−19.2 %) and H90 embryos (−10.9 %; Fig. 4). Similar to Series II and III results, subsequent atropine injection increased f_H only for N90 embryos ($P < 0.01$) but it had no affect on f_H in the H90 embryos (Fig. 4). P_M was not significantly altered by zatebradine in either H90 or N90 embryos (data not presented).

Series V: cholinergic and adrenergic tone at control temperature, 30 °C, for normoxic embryos

Saline injections had no significant effect on f_H or P_M. Atropine injection had no significant effect on f_H or P_M, and propranolol injection significantly lowered f_H at 30 °C (Fig. 5; $P < 0.01$). Propranolol injection significantly lowered f_H in all N90 embryos at 24, 30, and 36 °C; however, one-way RM ANOVA showed the magnitude of propranolol's effect did not significantly vary with T_E across Series (Fig. 5).

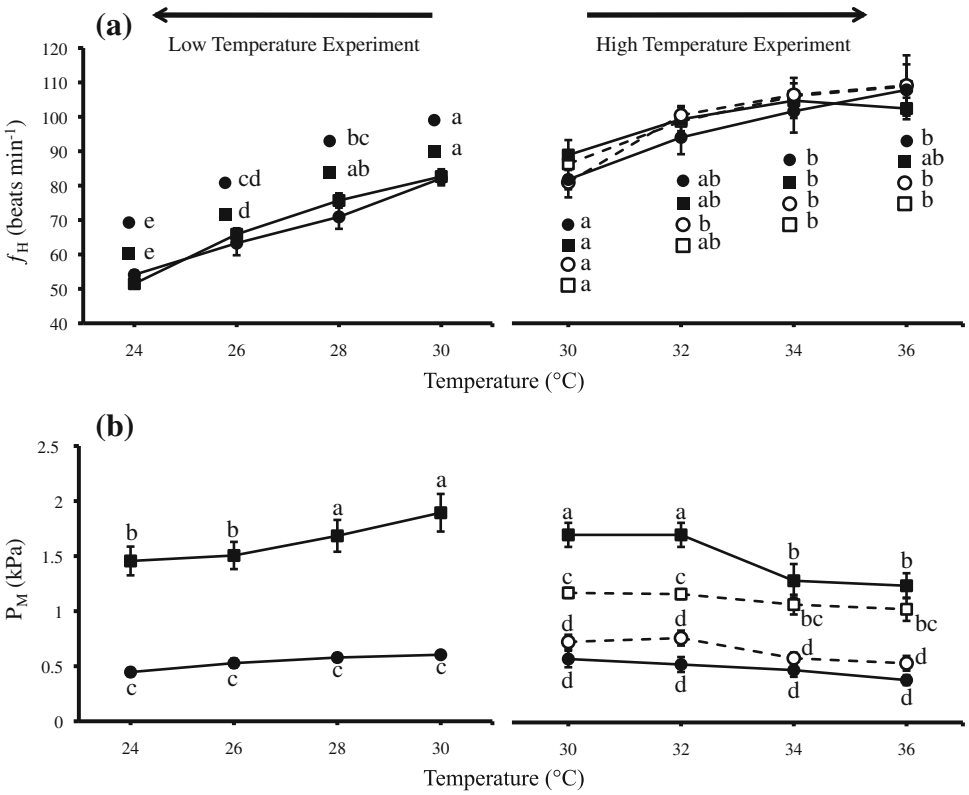

Fig. 1 For normoxic- (*filled symbols*) and hypoxic-incubated alligator embryos (*unfilled symbols*), mean heart rate (**a**) and CAM arterial pressure (**b**) responses to dynamically decreasing or increasing temperatures. *Circles* represent 70 % of development embryos, and *squares* represent 90 % of development embryos. *Letters* represent post hoc comparisons among groups at each temperature. *Similar letters* indicate responses were not significantly different, and *dissimilar letters* indicate responses were significantly different. *Error bars* are SEM

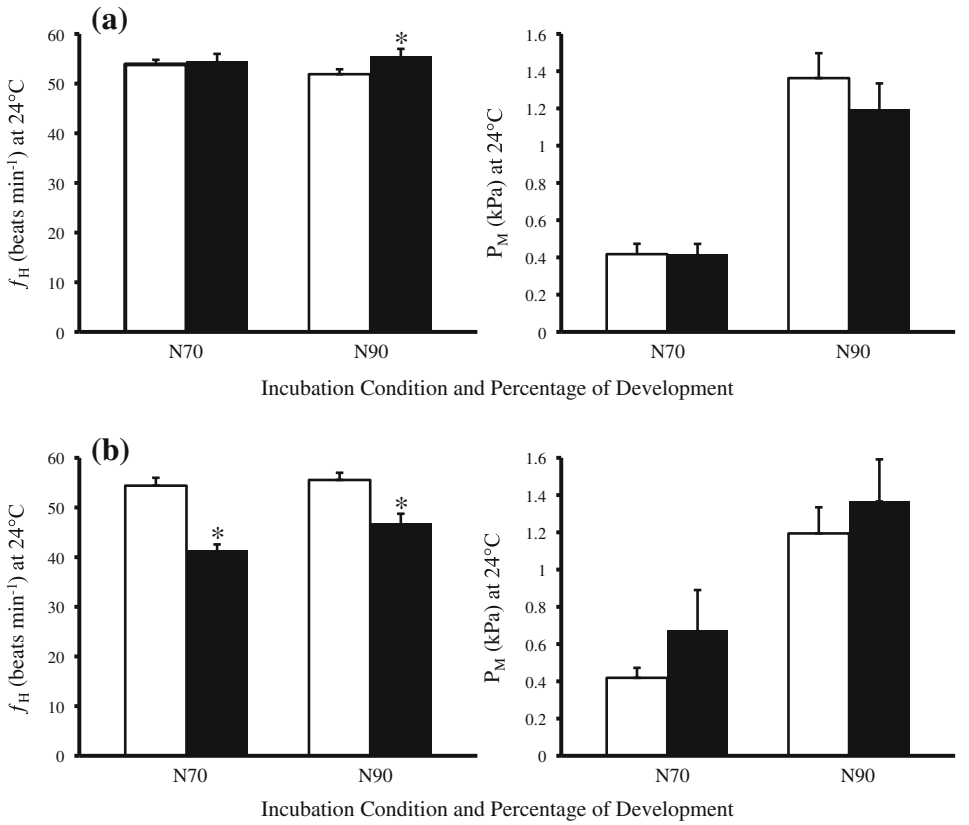

Fig. 2 For normoxic incubated alligator embryos at 70 and 90 % of development measured at 24 °C, mean heart rate (f_H) and mean CAM arterial pressure (P_M) before injection (*white bars*) and following atropine (**a**) or propranolol (**b**) injection (*black bars*). An *asterisk* indicates significant responses from paired *t* test. *Error bars* are SEM

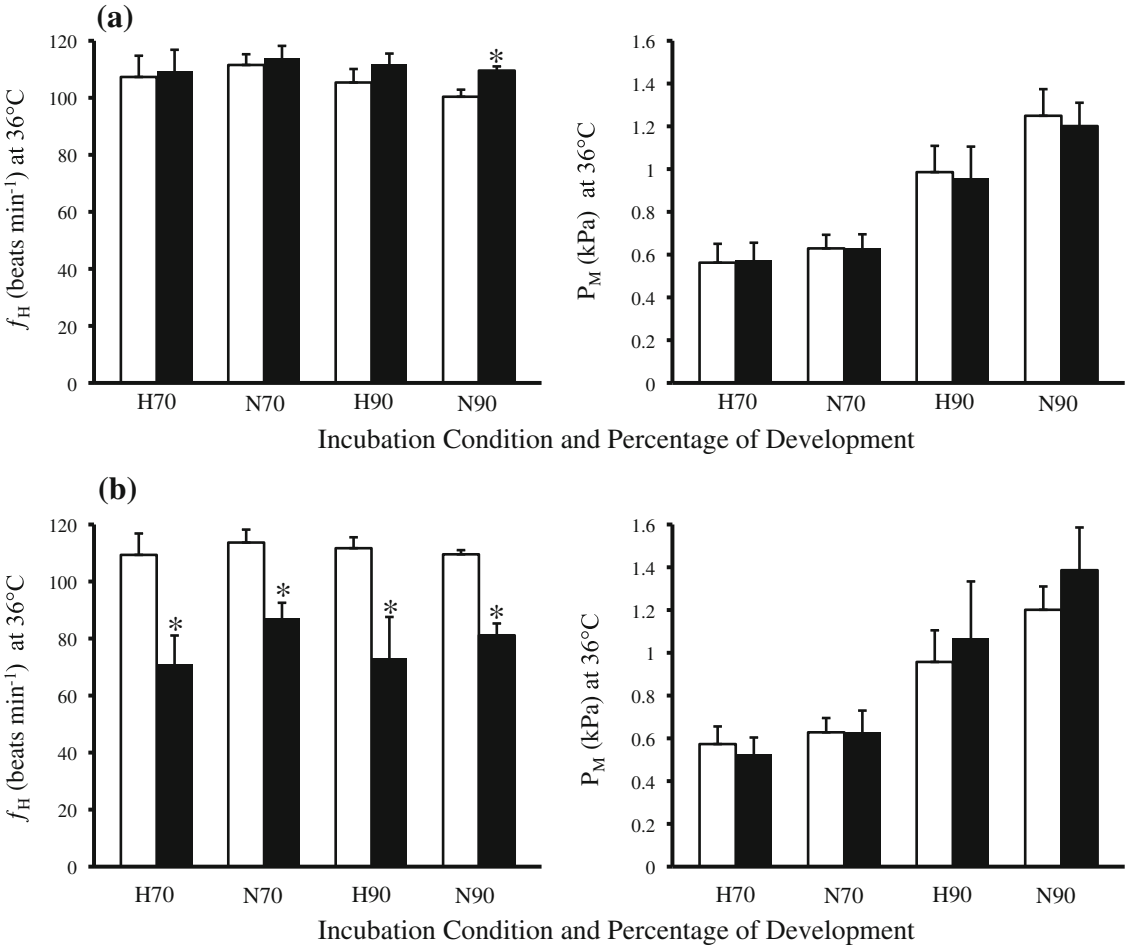

Fig. 3 For normoxic and hypoxic incubated alligator embryos at 70 and 90 % of development measured at 36 °C, mean heart rate (f_H) and mean CAM arterial pressure (P_M) before injection (*white bars*) and following atropine (**a**) or propranolol (**b**) injection (*black bars*). An *asterisk* indicates significant responses from paired *t* test. *Error bars* are SEM

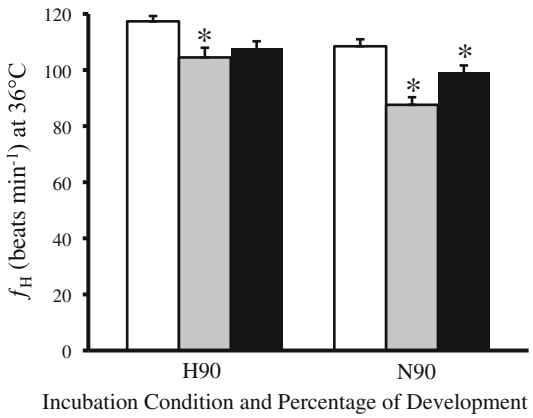

Fig. 4 For hypoxic and normoxic incubated alligator embryos at 90 % of development measured at 36 °C, mean heart rate (f_H) before injection (*white bars*), followed by sequential zatebradine (*gray bars*), and then atropine injection (*black bars*). An *asterisk* indicates significant injection responses compared to the previous value from paired *t* test. *Error bars* are SEM

Discussion

Thermal dependence of heart rate (f_H) has been extensively investigated in adult reptilian vertebrates (Seebacher 2000; Seebacher and Franklin 2003; Clark et al. 2005). Here, we demonstrated that embryonic American alligator responses to increasing and decreasing temperatures were similar to previously studied embryonic birds and reptiles (Oppenheim and Levin 1975; Nechaeva et al. 2005, 2007; Du et al. 2010b). Changing embryonic temperature from 30 to 24 °C and from 30 to 36 °C decreased and increased f_H, respectively (Fig. 1). In addition, P_M fell in response to increasing temperature from 30 to 36 °C in the N90 group. Our findings supported our hypothesis that temperature would activate tonic cholinergic stimulation on the heart, and data suggested that a functional limit to temperature-induced f_H increase may be cholinergic stimulation at thermal extremes. Tonic cholinergic receptor stimulation

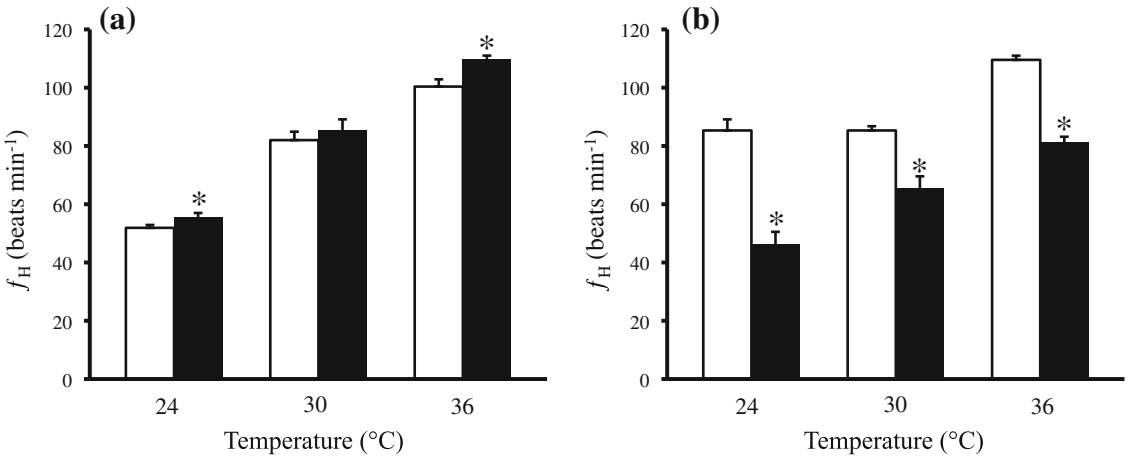

Fig. 5 For normoxic incubated alligator embryos at 90 % of development measured at 24, 30, or 36 °C, mean heart rate (f_H) and mean CAM arterial pressure (P_M) before injection (*white bars*) and following atropine (**a**) or propranolol (**b**) injection (*black bars*). An *asterisk* indicates significant responses from paired *t* test. Data represent combined results from three separate temperature experiments. *Error bars* are SEM

appeared to be mediated through thermal effects on thermoregulatory centers in the central nervous system, not in response to a temperature-dependent change in f_H. Cholinergic receptor tone was absent in chronic hypoxic embryos, which supported our hypothesis that hypoxic incubation attenuated cardiovascular regulatory capacity. This change occurred without differences in β-adrenergic tone, suggesting this regulatory mechanism may be maximized over this final third of embryonic alligator development. Therefore, while β-adrenergic tone was a dominant regulator of cardiac function across developmental ages and oxygen treatments, it was temperature independent, and cholinergic tone was dependent on thermal conditions, late in development.

This is the first study that examined in vivo thermal dependence of cardiovascular regulation in reptilian embryos. While cholinergic tone was absent in all alligator embryos measured at 30 °C, similar to previous results (Eme et al. 2011a, b), cholinergic tone was present at N90 embryos measured at 36 and 24 °C (Figs. 2a, 3a). The capacity for vagal actions on the heart of embryonic alligators has been previously reported and indicated the functional capacity of this cardiovascular regulator was present in embryos but not active at 30 °C (Crossley et al. 2003; Eme et al. 2011a, b). Increased cholinergic tone following acute temperature increase has been reported in other 'lower vertebrates', including Dogfish (*Scyliorhinus canicula*), Bald notothen (*Pagothenia borchgrevinki*), and African clawed frog (*Xenopus laevis*) (Taylor et al. 1977; Taylor and Ihmied 1995). Warm acclimated Common sole (*Solea vulgaris*) and European eel (*Anguilla anguilla*) also demonstrated greater cholinergic tone than their cold-acclimated counterparts (Seibert 1979; Sureau et al. 1989). Rainbow trout (*Salmo gairdneri*) exhibited increased cholinergic tone following cold acclimation (Priede 1974; Wood et al. 1979), and Cane toads (*Bufo marinus*) also showed greater vagal activity at lower temperatures (Courtice 1990). These collective findings demonstrated temperature-dependent cholinergic tone on f_H in fish and amphibians. This study demonstrated a similar response for American alligator embryos, and it appears that cholinergic tone can be altered with temperature across ectothermic vertebrate taxa, including in ovo.

Chronic hypoxic incubation delayed temperature-induced cholinergic tone on the heart of embryonic alligators (Fig. 3a), similar to findings previously reported for embryonic rainbow trout (*Oncorhynchus mykiss*) (Miller et al. 2011). Embryonic alligator development in low oxygen may favor maturation of mechanisms that increase cardiac function to ensure the scope for positive regulation (Miller et al. 2011). In this context, the lack of temperature activated cholinergic tone on the heart of hypoxic animals may be a consequence of preferential maturation or selective maturation of the capacity to increase function via adrenergic stimulation. While this speculation is plausible, the precise mechanism that accounts for this difference should be the context of future studies.

β-adrenergic tone on cardiovascular function of alligator embryos was constant and independent of thermal stress or incubation conditions. The overall response to β-adrenergic blockade was similar to that reported earlier (Eme et al. 2011a), and this consistent finding demonstrated that β-adrenergic tone was a major regulator of f_H under a wide variety of environmental conditions in embryonic alligators. Interestingly, while f_H responses to both acute temperature change and pharmacological manipulations could be elicited, P_M was relatively constant and only changed significantly in response to increased temperature (Figs. 1,

Table 1 Mean ± SEM body mass, heart mass and percentage heart mass for normoxic and hypoxic incubated alligator embryos at 70 and 90 % of embryonic development

	70 %		90 %	
	H	*N*	*H*	*N*
Body mass (g)	10.61 ± 0.29[a]	14.52 ± 0.84[a]	26.42 ± 1.66[b]	36.18 ± 1.01[c]
Heart mass (mg)	59.5 ± 2.5	67.8 ± 4.5	127.8 ± 10.3	153.3 ± 3.9
Heart mass/body mass (%)	0.56 ± 0.02[a]	0.47 ± 0.01[bc]	0.48 ± 0.03[b]	0.43 ± 0.00[c]

Similar letters indicate responses were not significantly different, and dissimilar letters indicate responses were significantly different

2, 3). These findings, coupled with the constant f_H response to β-adrenergic blockade, suggest adrenergic tone may be maximized across the final third of embryonic alligator development.

Heat could have exerted a direct effect on vagal activity in a number of ways, including by altering the number of pulses arriving at the heart or altered enzyme activity. Pulse frequency typically decreases at lower temperatures (Gasser 1931; Takeuchi 1958), but decreasing cholinesterase activity could counterbalance this effect, which in turn may have increased the efficacy of vagal input to the heart (Nakahara et al. 1998). During bouts of temperature reductions, cholinesterase activity may be depressed in alligator embryos, reducing the catabolism of acetylcholine and enhancing vagal control. The importance of enzyme kinetics would be amplified under a scenario where pulse frequency did not decrease with lower temperatures. Pockett and Macdonald (1986) demonstrated that pulse frequency could increase with lower temperatures, and the role of temperature in regulating vagal pulse frequency as well as cholinesterase activity in embryonic alligators warrants further investigation.

In addition, increased cholinergic tone at the upper and lower temperatures may be attributed to a differential stimulation of central thermoreceptors resulting in increased vagal output. To determine if the presence of cholinergic tone on f_H was due to direct effects of temperature on the heart, we used zatebradine to decrease f_H at 36 °C (Fig. 4). Zatebradine is a bradycardic agent that acts primarily by prolonging sinoatrial pacemaker conduction in mammals (Kalman et al. 1995). Zatebradine had previously been used as a bradycardic agent in fish (Altimiras and Axelsson 2004; Gamperl et al. 2011), and the decrease in f_H reported here suggested that zatebradine acted in a similar fashion in American alligators to that reported for mammals and fish. Since temperature changes affect pacemaker depolarization, heat could directly mediate changes in f_H based on thermodynamics, which likely occurred in our study. The elevation in f_H may have secondarily activated cholinergic tone on the heart due to barostatic mechanisms to limit f_H increases, which were active in embryonic alligators during the period of incubation we investigated (Crossley et al. 2003). Zatebradine injection uncoupled temperature-induced increases in f_H, and reduced f_H to near resting (30 °C) levels in embryos exposed to high temperature (36 °C). Subsequent cholinergic blockade still revealed a tone on f_H at 36 °C following zatebradine (Fig. 4), indicating that thermoreceptors may be stimulated as embryo temperature (T_E) varies. This may cause increased activity of the vagal dorsal motor nucleus and result in increased vagal output.

Interactions between hypoxia and temperature on embryonic alligator physiology have physiological, ecological, and evolutionary implications. Percentage oxygen values in reptile nests can drop to as low as 11–15 % (Ackerman 1980; Lutz and Dunbar-Cooper 1984), and temperatures around alligator nests can fluctuate by as much as 15 °C (McIlhenny 1934; Joanen 1969; Chabreck 1973). Understanding physiological responses to hypoxia and temperature in ovo could have direct consequences for uncovering proximate mechanisms underlying the long-term behavioral, morphological, and physiological consequences of developmental conditions (Gutzke and Packard 1987; Burger 1990; Janzen 1993; Elphick and Shine 1998; Burggren and Reyna 2011). Our results demonstrated that cholinergic tone appeared at temperatures significantly above and below the 'ideal incubation temperature', and that chronic hypoxia delayed the reliance upon cholinergic tone in developing systems. Hypoxia is a ubiquitous environmental stressor that slows growth and metabolism in numerous oviparous species (Ackerman 1981; Snyder et al. 1982; Kam 1993), and hypoxia may have slowed the maturation of thermally induced regulatory vagal function in this study. Given that cholinergic tone could be induced by both high and low temperatures, and its absence in hypoxic-incubated embryos, our findings suggest the American alligator makes a promising model to explore maturation of the interaction between thermoreception, oxygen level, and autonomic outflow from the central nervous system during embryonic development.

Acknowledgments Our work would not have been possible without help from Phillip "Scooter" Trosclair III and Dwayne LeJeune. We thank Kevin Tate and Zachary Kohl for assistance with embryo care and data collection. The National Science Foundation CAREER award IBN IOS-0845741 to DAC supported this work.

References

Ackerman RA (1980) Physiological and ecological aspects of gas exchange by sea turtle eggs. Am Zool 20:575–583

Ackerman RA (1981) Growth and gas exchange of embryonic sea turtles (*Chelonia caretta*). Copeia 1981:757–765

Altimiras J, Axelsson M (2004) Intrinsic autoregulation of cardiac output in rainbow trout (*Oncorhynchus mykiss*) at different heart rates. J Exp Biol 207:195–201

Birchard GF (2000) An ontogenetic shift in the response of heart rates to temperature in the developing snapping turtle (*Chelydra serpentina*). J Therm Biol 25:287–291

Birchard GF, Reiber CL (1996) Heart rate during development in the turtle embryo: effect of temperature. J Comp Physiol B 166:461–466

Booth DT (2000) The effect of hypoxia on oxygen consumption of embryonic estuarine crocodiles (*Crocodylus porosus*). J Herpetol 34:478–481

Boyer DR (1967) Interaction of temperature and hypoxia on respiratory and cardiac responses in the lizard, *Sauromalus obesus*. Comp Biochem Physiol 20:437–447

Branco LG, Portner HO, Wood SC (1993) Interaction between temperature and hypoxia in the alligator. Am J Physiol Reg Integ Comp 265:R1339–R1343

Burger J (1990) Effects of incubation temperature on behavior of young black racers (*Coluber constrictor*) and kingsnakes (*Lampropeltis getulus*). J Herpetol 24:158–163

Burggren WW, Keller BB (1997) Development of cardiovascular systems: molecules to organisms. In: Burggren WW, Keller BB (eds) Cambridge University Press, Cambridge, UK, pp 360

Burggren WW, Reyna KS (2011) Developmental trajectories, critical windows and phenotypic alteration during cardio-respiratory development. Respir Physiol Neurobiol 178:13–21

Chabreck RH (1973) Temperature variation in nests of the American alligator. Herpetologica 29:48–51

Clark TD, Butler PJ, Frappell PB (2005) Digestive state influences the heart rate hysteresis and rates of heat exchange in the varanid lizard *Varanus rosenbergi*. J Exp Biol 208:2269–2276

Coulson RA, Hernandez T (1983) Alligator metabolism. Studies on chemical reactions in vivo. Comp Biochem Physiol B 741:1–182

Courtice GP (1990) Effect of temperature on cardiac vagal action in the toad (*Bufo marinus*). J Exp Biol 149:439–447

Crossley DA II, Altimiras J (2005) Cardiovascular development in embryos of the American alligator *Alligator mississippiensis*: effects of chronic and acute hypoxia. J Exp Biol 208:31–39

Crossley DA II, Hicks JW, Altimiras J (2003) Ontogeny of baroreflex control in the American alligator *Alligator mississippiensis*. J Exp Biol 206:2895–2902

Du WG, Thompson MB, Shine R (2010a) Facultative cardiac responses to regional hypoxia in lizard embryos. Comp Biochem Physiol A 156:491–494

Du WG, Ye H, Zhao B, Warner DA, Shine R (2010b) Thermal acclimation of heart rates in reptilian embryos. PLoS One 5:e15308

Elphick MJ, Shine R (1998) Longterm effects of incubation temperatures on the morphology and locomotor performance of hatchling lizards (*Bassiana duperreyi*, Scincidae). Biol J Linn Soc 63:429–447

Eme J, Altimiras J, Hicks JW, Crossley DA II (2011a) Hypoxic alligator embryos: chronic hypoxia, catecholamine levels and autonomic responses of in ovo alligators. Comp Biochem Physiol A 160:412–420

Eme J, Hicks JW, Crossley DA II (2011b) Chronic hypoxic incubation blunts a cardiovascular reflex loop in embryonic American alligator (*Alligator mississippiensis*). J Comp Physiol B 181:981–990

Ferguson MWJ (1985) Reproductive biology and embryology of the crocodilians. In: Gans C, Billet F, Maderson P (eds) Biology of the reptilia, 14A edn. Wiley, New York, pp 329–349

Gamperl KA, Swafford BL, Rodnick KJ (2011) Elevated temperature, per se, does not limit the ability of rainbow trout to increase stroke volume. J Therm Biol 36:7–14

Gasser HS (1931) Nerve activity as modified by temperature changes. Am J Physiol 97:254–276

Glass ML, Hicks JW, Riedesel ML (1979) Respiratory responses to long-term temperature exposure in the box turtle, *Terrapene ornata*. J Comp Physiol B 131:353–359

Gutzke WHN, Packard GC (1987) Influence of the hydric and thermal environments on eggs and hatchlings of bull snakes (*Pituophis melanoleucus*). Physiol Zool 60:9–17

Hicks JW, Wood SC (1985) Temperature regulation in lizards: effects of hypoxia. Am J Physiol Regul Integr Comp 248:R595–R600

Jackson DC (1973) Ventilatory response to hypoxia in turtles at various temperatures. Respir Physiol 18:178–187

Janzen FJ (1993) The influences of incubation temperature and family on eggs, embryos, and hatchlings of the smooth softshell turtle (*Apalone mutica*). Physiol Zool 66:349–373

Joanen T (1969) Nesting ecology of alligators in Louisiana. In: Proceedings of the annual conference Southeast association game and fish communication 24:141–151

Kalman JM, Tonkin AM, Power JM (1995) Specific effects of zatebradine on sinus node function: suppression of automaticity, prolongation of sinoatrial conduction and pacemaker shift in the denervated canine heart. J Pharmacol Exp Ther 272: 85–93

Kam YC (1993) Physiological effects of hypoxia on metabolism and growth of turtle embryos. Respir Physiol 92:127–138

Lutz PL, Dunbar-Cooper A (1984) The nest environment of the American crocodile (*Crocodylus acutus*). Copeia 1:153–161

McIlhenny EA (1934) Notes on incubation and growth of alligators. Copeia 1934:80–88

Miller SC, Gillis TE, Wright PA (2011) The ontogeny of regulatory control of the rainbow trout (*Oncorhynchus mykiss*) heart and how this is influenced by chronic hypoxia exposure. J Exp Biol 214:2065–2072

Nakahara T, Kawada T, Sugimachi M, Miyano H, Sato T, Shishido T, Yoshimura R et al (1998) Cholinesterase affects dynamic transduction properties from vagal stimulation to heart rate. Am J Physiol Regul Integr Comp 275:R541–R547

Nechaeva MV (2011) Physiological responses to acute changes in temperature and oxygenation in bird and reptile embryos. Respir Physiol Neurobiol 178:108–117

Nechaeva MV, Makarenko IG, Tsitrin EB, Zhdanova NP (2005) Physiological and morphological characteristics of the rhythmic contractions of the amnion in veiled chameleon (*Chamaeleo calyptratus*) embryogenesis. Comp Biochem Physiol A 140: 19–28

Nechaeva MV, Vladimirova IG, Alekseeva TA (2007) Oxygen consumption as related to the development of the extraembryonic membranes and cardiovascular system in the European pond turtle (*Emys orbicularis*) embryogenesis. Comp Biochem Physiol A 148:599–606

Nielsen B (1962) On the regulation of respiration in reptiles II. The effect of hypoxia with and without moderate hypercapnia on the respiration and metabolism of lizards. J Exp Biol 39:107–117

Oppenheim RW, Levin HL (1975) Short-term changes in incubation temperature: behavioral and physiological effects in the chick embryo from 6 to 20 days. Dev Psychobiol 8:103–115

Petersen AM, Gleeson TT, Scholnick DA (2003) The effect of oxygen and adenosine on lizard thermoregulation. Physiol Biochem Zool 76:339–347

Pockett S, Macdonald JA (1986) Temperature dependence of neurotransmitter release in the Antarctic fish (*Pagothenia borchgrevinki*). Experientia 42:414–415

Priede IG (1974) The effect of swimming activity and section of the vagus nerves on heart rate in rainbow trout. J Exp Biol 60:305–319

Seebacher F (2000) Heat transfer in a microvascular network: the effect of heart rate on heating and cooling in reptiles (*Pogona barbata* and *Varanus varius*). J Theor Biol 203:97–109

Seebacher F, Franklin CE (2003) Prostaglandins are important in thermoregulation of a reptile (*Pogona vitticeps*). Proc Biol Sci 270:S50–S53

Seebacher F, Franklin CE (2004) Integration of autonomic and local mechanisms in regulating cardiovascular responses to heating and cooling in a reptile (*Crocodylus porosus*). J Comp Physiol B 174:577–585

Seebacher F, Franklin CE (2005) Physiological mechanisms of thermoregulation in reptiles: a review. J Comp Physiol B 175:533–541

Seibert H (1979) Thermal adaptation of heart rate and its parasympathetic control in the European eel *Anguilla anguilla* (L.). Comp Biochem Physiol C 64:275–278

Snyder GK, Black CP, Birchard GF (1982) Development and metabolism during hypoxia in embryos of high altitude *Anser indicus* versus sea level *Branta canadensis* geese. Physiol Zool 55:113–123

Sureau D, Lagardere JP, Pennec JP (1989) Heart rate and its cholinergic control in the sole (*Solea vulgaris*), acclimatized to different temperatures. Comp Biochem Physiol A 92:49–51

Takeuchi N (1958) The effect of temperature on the neuromuscular junction of the frog. Jpn J Physiol 8:391–404

Tate KB, Eme J, Swart J, Conlon JM, Crossley DA II (2012) Effects of dehydration on cardiovascular development in the embryonic American alligator (*Alligator mississippiensis*). Comp Biochem Physiol A 162:252–258

Taylor EW, Ihmied YM (1995) Vagal and adrenergic tone on the heart of *Xenopus laevis* at different temperatures. J Therm Biol 20:55–59

Taylor EW, Short S, Butler PJ (1977) The role of the cardiac vagus in the response of the dogfish (*Scyliorhinus canicula*) to hypoxia. J Exp Biol 70:57–75

Wood CM, Pieprzak P, Trott JN (1979) The influence of temperature and anaemia on the adrenergic and cholinergic mechanisms controlling heart rate in the rainbow trout. Can J Zool 57: 2440–2447

Wood SC, Hicks JW, Dupré RK (1987) Hypoxic reptilians: blood gases, body temperature, and control of breathing. Am Zool 27:21–29