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RESEARCH ARTICLE

Temperature-dependent sex determination modulates cardiovascular maturation in embryonic snapping turtles *Chelydra serpentina*

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SUMMARY

We investigated sex differences in cardiovascular maturation in embryos of the snapping turtle *Chelydra serpentina*, a species with temperature-dependent sex determination. One group of eggs was incubated at 26.5°C to produce males. Another group of eggs was incubated at 26.5°C until embryos reached stage 17; eggs were then shifted to 31°C for 6 days to produce females, and returned to 26.5°C for the rest of embryogenesis. Thus, males and females were at the same temperature when autonomic tone was determined and for most of development. Cholinergic blockade increased resting blood pressure (P_m) and heart rate (f_H) in both sexes at 75% and 90% of incubation. However, the magnitude of the f_H response was enhanced in males compared with females at 90% of incubation. β-adrenergic blockade increased P_m at 75% of incubation in both sexes but had no effect at 90% of incubation. β-adrenergic blockade reduced f_H at both time points but produced a stronger response at 90% *versus* 75% of incubation. We found that α-adrenergic blockade decreased P_m in both sexes at 75% and 90% of incubation and decreased f_H at 75% of incubation in both sexes. At 90% of incubation, f_H decreased in females but not males. Although these data clearly demonstrate sexual dimorphism in the autonomic regulation of cardiovascular physiology in embryos, further studies are needed to test whether differences are caused by endocrine signals from gonads or by a hormone-independent temperature effect.

Key words: development, sexual differentiation, cardiovascular, cholinergic, adrenergic, snapping turtle.

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INTRODUCTION

Temperature-dependent sex determination (TSD) has been documented in four of five living orders of reptiles, including crocodilians, sphenodontians, squamates and testudines (Bull, 1980; Crews and Bull, 2008). Our understanding of the mechanistic basis and adaptive significance of TSD has increased significantly since Charnier first reported the phenomenon in 1966 (Charnier, 1966). Work in several TSD species indicates that temperature determines sex by influencing steroidogenesis in the bipotential gonads of embryos (reviewed in Lance, 2009; Ramsey and Crews, 2009). Studies in two TSD species, a fish and a lizard, have demonstrated that incubation temperature has differential effects on male and female reproductive success as predicted by evolutionary theory (Conover and Heins, 1987; Warner and Shine, 2008). Research in other reptiles also reveals long-term temperature effects on various traits (reviewed in Rhen and Lang, 2004). Yet the link between the proximate mechanisms and the ultimate causes of TSD remains murky. In particular, we need more information on the developmental and physiological mechanisms that underlie temperature and sex effects on the whole organism.

Although incubation temperature and sex are confounded in TSD species, it is possible to tease apart their effects using various experimental approaches (reviewed in Rhen and Lang, 2004). Studies of this sort reveal sex effects on physiology and development that are independent of incubation temperature, as observed for fat metabolism and weight loss in snapping turtle hatchlings before they start feeding (Rhen and Lang, 1999). Incubation temperature can also influence physiology and development separately from its role in sex determination, as observed for post-hatching growth and body size in snapping turtles after they start feeding (Rhen and Lang, 1995). Finally, incubation temperature and sex may interact to influence phenotype, as reported for adult body size and shape in leopard geckos (reviewed in Crews et al., 1998). Given that incubation temperature and gonadal sex independently influence growth and metabolism in snapping turtle hatchlings, we sought to identify developmental and physiological mechanisms that might explain these effects.

In mammals, placental insufficiency, maternal undernutrition and fetal cardiovascular defects influence prenatal growth and metabolism. These early experiences also have developmental effects that alter postnatal growth and increase the risk for metabolic and cardiovascular disease later in life (Gagnon, 2003; Wu et al., 2004; Warner and Ozanne, 2010). Interestingly, incubation temperature has been shown to influence heart rate in embryonic snapping turtles: temperature has direct effects on heart rate (i.e. a Q_{10} effect) and induces thermal acclimation of heart rate (i.e. changes in Q_{10}) (Birchard and Reiber, 1996; Birchard, 2000; Du et al., 2010). Temperature-induced variation (or sex differences) in heart rate (f_H) and arterial blood pressure (P_m) could influence growth and metabolism through changes in tissue perfusion, gas exchange and nutrient/waste transport. Yet no one has examined temperature or sex effects on the physiological

mechanisms that regulate cardiovascular function (i.e. f_H and P_m) in snapping turtle embryos.

As mentioned above, steroidogenesis in the bipotential gonads plays a pivotal role in sex determination in TSD species. More specifically, estrogen signaling is required for ovary development in the snapping turtle (Rhen and Lang, 1994). Administration of estrogens to eggs at male-producing temperatures induces ovary formation. Conversely, inhibition of aromatase (the enzyme that converts androgens into estrogens) increases the number of males at a temperature that normally produces a female-biased sex ratio. In accord with these findings, aromatase expression and estrogen synthesis is higher in gonads from embryos at female-producing temperatures than in embryos at male-producing temperatures (Place et al., 2001; Rhen et al., 2007). There are also sex differences in circulating testosterone and estradiol levels in snapping turtle hatchlings (Rhen et al., 1996) and adults (Mahmoud and Licht, 1997). Substantial evidence indicates that estrogens cause sex differences in cardiovascular function in other vertebrates (Altimiras et al., 1996; Evans et al., 2001; Kuo et al., 2001; Bubb et al., 2012). Thus, sex may have effects on cardiovascular physiology that are independent of incubation temperature.

Here we characterize sex/temperature effects and developmental changes in autonomic tone in snapping turtle embryos. In this species, brief exposure to 31°C induces ovary development in embryos otherwise incubated at 26.5°C, a male-producing temperature (Rhen et al., 2007). By comparing females and males incubated at the same temperature for ~58 days (>90% of embryogenesis), we reduce the potential for confounding sex and temperature effects to a 6 day period (~9% of embryogenesis). We hypothesized that male and female embryos would exhibit differences in maturation of vagal and sympathetic regulatory mechanisms. We examined f_H and P_m at 75% and 90% of the total incubation period. Cholinergic and adrenergic receptor antagonists were applied at 75% and 90% of the total incubation period to assess the onset of cholinergic and adrenergic control of the cardiovascular system.

MATERIALS AND METHODS

Study subjects

Minnesota Department of Natural Resources special permit no. 14424 allowed collection of snapping turtle *Chelydra serpentina* (Linnaeus 1758) eggs in northwestern Minnesota and transportation of eggs to the University of North Dakota. We collected snapping turtle eggs from four newly constructed nests in June 2007. Nests were found on the edge of gravel roads along the Mississippi River north of Lake Itasca State Park, MN, USA. Eggs were transported to the Biology Department at the University of North Dakota. Upon arrival, eggs were numbered and weighed, and 20 eggs from each clutch were randomly assigned to one of two groups. Eggs were placed in one of three plastic boxes (volume ≈3 l) containing vermiculite mixed with water in a 1:1 ratio by mass. Boxes were put in incubators set to 26.5°C. Water content of vermiculite was maintained by weighing boxes twice weekly and adding water as needed.

Incubation procedures

One egg from each clutch was dissected for embryo staging at ~9 days of an estimated 64 day incubation period. Embryos were staged based on anatomical features described by Yntema (Yntema, 1968). Tables with temperature-specific developmental rates were used to estimate when embryos at 26.5°C would reach the thermosensitive period for sex determination (at 35% of the 64 day incubation period), as well as two time points well after sex determination (at 75% and 90% of the 64 day incubation period) (Yntema, 1968).

Two groups of eggs were exposed to thermal regimes that produce only male or female hatchlings (Rhen et al., 2007; Rhen et al., 2009). One group of eggs was incubated at 26.5±0.5°C for the entire duration of the experiment. This temperature produces exclusively males in our study population (Rhen and Lang, 1994; Rhen and Lang, 1998; Ewert et al., 2005; Rhen et al., 2007). The other group of eggs was incubated at 26.5°C until embryos reached stage 17 of development (Yntema, 1968). Eggs in this group were shifted to a female-producing temperature (31°C) for 6 days and then transferred back to 26.5°C for the remainder of the study. Prior research has shown that this temperature shift produces exclusively female hatchlings (Yntema, 1979; Rhen et al., 2007; Rhen et al., 2009). The sex of embryos at 90% of incubation was verified by inspection of gonads and reproductive tracts, which are visually dimorphic under a dissecting scope. Embryos incubated at 26.5°C throughout development had testes (shorter, opaque, yellowish-white gonads with a smooth surface) and visible Wolffian ducts, but lacked Müllerian ducts. Embryos briefly exposed to 31°C had ovaries (longer, translucent gonads with prominent ovarian follicles) and well-developed Müllerian ducts.

Surgical procedures

Eggs were taken from incubators at 75% and 90% of development, weighed and candled to locate a chorioallantoic artery for cannulation. Eggs were placed in cotton-lined 4 cm Pyrex bottle caps and set in temperature-controlled surgical chambers at 26.5±0.5°C. A portion of the eggshell was removed and the chorioallantoic artery was occlusively cannulated under a dissection microscope (Leica MZ6, Leica Microsystems, Waukegan, IL, USA) using a heat-pulled, 0.9% NaCl saline filled polyethylene tube (PE-50) as previously described (Crossley and Altimiras, 2000). The cannula was fixed to the eggshell with cyanoacrylic glue, and the egg was placed in an experimental chamber. Each chamber consisted of a water jacket stainless steel box and a steel lid. Each lid had three 5 mm holes, which provided an exit for the arterial cannula as well as routes for the inflow of humidified air. Eggs were maintained at 26.5±0.5°C during the physiological studies described below.

Signal recording and calibration

The cannula was attached to a pressure transducer powered by an Octal bridge amplifier (Colorado Springs, CO, USA). Data were collected at 40 Hz using a PowerLab® data recording system connected to a Macintosh computer running the Chart® 5.5 acquisition program (ADInstruments, Colorado Springs, CO, USA). The zero pressure reference was set at the top of the experimental bath and all values were corrected after the experiment (Altimiras and Crossley, 2000).

Physiological procedures

Embryos were allowed to recover for 45 min prior to experimental treatments. During this period, P_m and f_H stabilized. Embryos that failed to recover were removed from the study. We also excluded embryos that experienced cardiovascular failure or premature hatching during the course of the physiological experiments. Final sample sizes for these experiments are presented in the Results.

The experimental protocol included serial injections of 0.9% saline, a cholinergic antagonist in 0.9% saline and adrenergic antagonists in 0.9% saline. Male and female embryos were each injected with ~40 µl of saline *via* a T-connector in the arterial catheter line. This volume was equivalent to the total volume used

for drug delivery and a saline flush. Following a stabilization period for P_m and f_H, each embryo was injected with the cholinergic receptor antagonist atropine (3 mg kg^{-1}). Cardiovascular parameters were allowed to stabilize (for an average of 45 min) before injection of the β-adrenergic blocker propranolol (3 mg kg^{-1}). This protocol of stabilization and injection was repeated for the α-adrenergic antagonist phentolamine (3 mg kg^{-1}). All drug doses were calculated based on published data for embryonic mass in eggs at the same temperature (Birchard and Reiber, 1995). Injection volumes were ~20 µl of the drug in saline followed by 20 µl of saline as previously described (Crossley et al., 2003b). This protocol resulted in a total injection volume of less than 5% of the estimated maximal blood volume of an animal at this stage of development.

After completion of the physiological experiments, embryos were euthanized with an arterial injection of sodium pentobarbital (Socumb, 50 mg kg^{-1}) and saturated potassium chloride. Embryos were then removed from the egg for staging and to measure whole embryo mass, heart mass and liver mass. Organs that could not be completely extracted were eliminated from this analysis. Another set of male and female embryos was euthanized at 90% of incubation for mass determinations only. Embryo staging was performed by one of the authors (D.A.C.) in accordance with Yntema (Yntema, 1968).

Statistical analysis

Statistical analyses were conducted as described for similar parameters in American alligator embryos (Eme et al., 2011a; Eme et al., 2011b). Briefly, a two-way ANOVA was used to test for developmental changes (75% and 90% of incubation period) and sex differences in egg, embryo, liver and heart masses and resting P_m and f_H. We also used a two-way ANOVA to analyze developmental changes and sex differences in cardiovascular response to receptor antagonists. Changes in P_m and f_H were analyzed relative to resting parameters for each individual embryo. Thus, the dependent variable was the arcsine square root of change in P_m (or f_H) after receptor blockade divided by resting P_m (or f_H) for each individual. Paired *t*-tests were used to compare resting values for P_m and f_H with the response to saline or drug injections (atropine, propranolol or phentolamine). A Fisher's least significant difference test was used to correct for multiple comparisons. Throughout the text, means are given ±s.e.m. Test statistics were deemed significant when *P*-values were less than or equal to 0.05 (Statistica v9.0, StatSoft, Tulsa, OK, USA).

RESULTS

Estimated *versus* actual age

We based our sampling protocol on staging of embryos at ~9 days post-oviposition and the incubation period at 26.5°C reported in prior studies (~64 days from oviposition to hatching). Our method of estimating when embryos would reach 75% and 90% of incubation, based on Yntema (Yntema, 1976; Yntema, 1978), produced relatively accurate predictions for both time points (Table 1). There were only slight differences between predicted and actual percentage of development under the current experimental conditions.

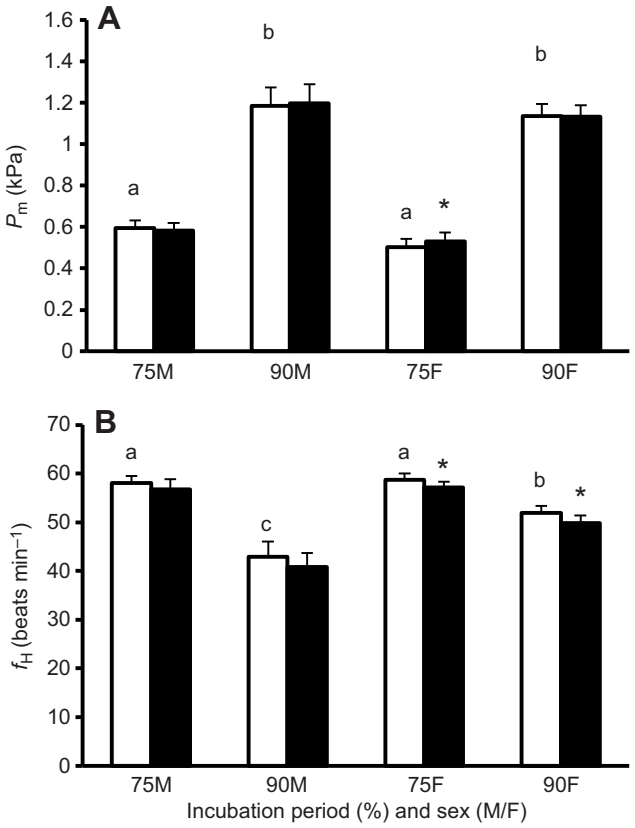

Fig. 1. (A) Mean arterial blood pressure (P_m) and (B) heart rate (f_H) from male and female embryonic snapping turtles *Chelydra serpentina* prior to (open columns) and following (filled columns) an injection of control heparinized 0.9% NaCl saline at each point of incubation studied. Asterisks represent a significant (P<0.05) response to the saline injection. Different letters indicate a significant (P<0.05) difference in the pre-injection values between incubation period and sex. Data are presented as means ± s.e.m.

Saline response

In both experimental groups resting pressure (P_m) was lower and resting heart rate (f_H) was elevated in 75% developed embryos relative to the 90% embryos (Fig. 1). P_m increased significantly during development, roughly doubling between measurements at 75% and 90% of incubation in male and female embryos (Fig. 1A). Resting P_m did not differ between the sexes at either time point. In contrast, there was a significant developmental difference in resting f_H between males and females (Fig. 1B). Resting f_H was very similar in males and females at 75% of incubation, but was sexually dimorphic at 90% of incubation. Resting f_H decreased 15 beats min^{-1} in males, but only decreased 6 beats min^{-1} in females (Fig. 1B). Thus, resting f_H was 21% faster in female than in male embryos (52±1 *versus* 43±3 beats min^{-1}, respectively). Saline was injected to quantify changes in P_m or f_H due to the injection volume alone. Neither parameter was affected by the injection in males.

Table 1. Estimated and actual percentage of incubation based on initial staging of *Chelydra serpentina* embryos and pipping of eggs from each clutch for the male and female embryos

	75% incubation		90% incubation	
	Estimated (%)	Actual (%)	Estimated (%)	Actual (%)
Male	75	76	90	92
Female	75	76	90	90

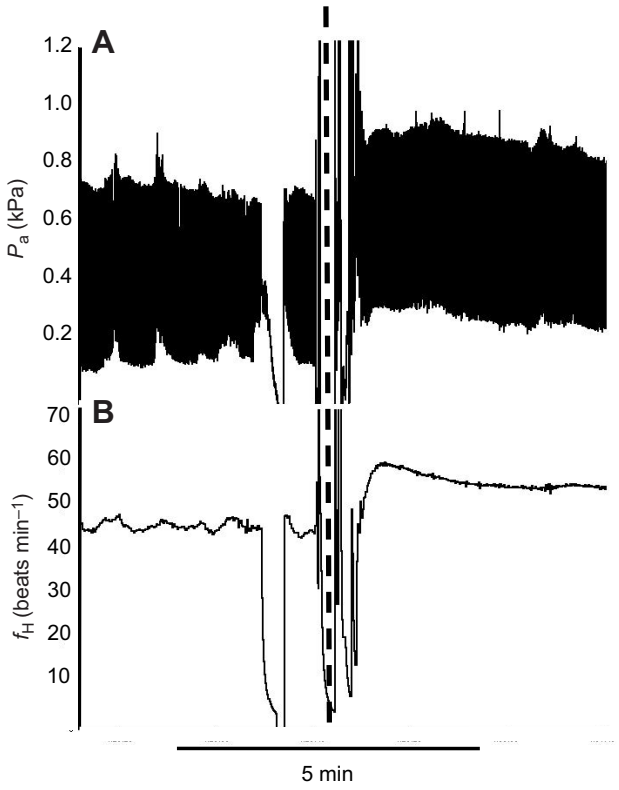

Fig. 2. (A) P_a (absolute arterial pressure) and (B) f_H response of a male embryonic snapping turtle to an injection of atropine (3 mg kg^{-1}) at 75% of incubation. The dashed line indicates the point of drug injection and the bar represents a 5 min time period.

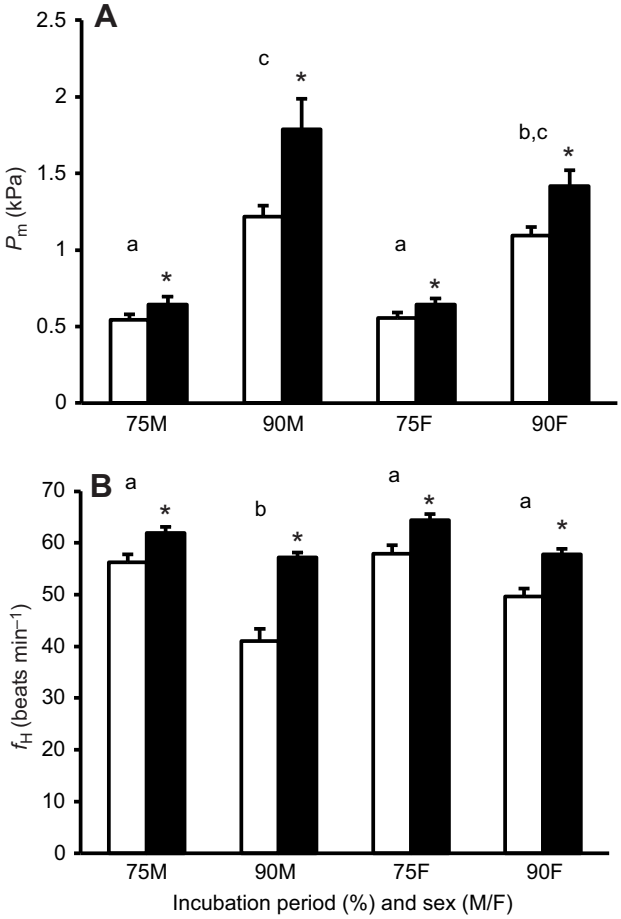

Fig. 3. (A) P_m and (B) and f_H from male and female embryonic snapping turtles prior to (open columns) and following (filled columns) an injection of atropine (3 mg kg^{-1}) at each point of incubation studied. Asterisks represent a significant (P<0.05) response to the injection. Different letters indicate a significant (P<0.05) difference in the atropine response intensity between incubation period and sex. Data are presented as means ± s.e.m.

However, saline injection did result in a slight but significant increase in P_m at 75% of incubation and a slight decrease in f_H at both points in females (Fig. 1). Sample sizes were 8 (75%) and 6 (90%) in the male group and 10 (75%) and 8 (90%) in the female group.

Cholinergic blockade

Injection of atropine (3 mg kg^{-1}) increased P_m and f_H as indicated in the representative trace (Fig. 2) at both points of incubation and in both experimental groups of embryonic turtles (Fig. 3). This injection significantly increased P_m, by 28%, in both sexes at 75% of incubation (Fig. 3A). This hypertensive response to atropine was significantly stronger at 90% of incubation, with an increase of 50% in males and 36% in females (Fig. 3A). Cholinergic blockade also increased f_H in both sexes at both time points (Fig. 3B). However, this effect was sexually dimorphic and significantly stronger at 90% of incubation, with an increase of 39% in males compared with an increase of 20% in females (Fig. 3B). Sample sizes were 12 (75%) and 6 (90%) for males and 9 (75%) and 8 (90%) for females.

β-adrenergic blockade

Propranolol (3 mg kg^{-1}) significantly increased P_m, by 25–35%, at 75% of incubation in both sexes (Fig. 4A). This response was absent at 90% of incubation (Fig. 4A). Propranolol significantly reduced f_H to a similar degree in both sexes (Fig. 4B). However, the reduction in f_H following β-blockade was developmentally dependent, increasing in intensity from 75% of incubation (–26%) to 90% of incubation (–38 to –41%). Sample sizes were 12 (75%) and 5 (90%) for males and 9 (75%) and 4 (90%) for females.

α-adrenergic blockade

Phentolamine (3 mg kg^{-1}) significantly decreased P_m to a similar degree, by 44%, in both sexes at both time points (Fig. 5A). Resting f_H was also significantly depressed, by 11%, following α-adrenergic blockade in both sexes at 75% of incubation (Fig. 5B). Although this depression in f_H was evident at 90% of incubation in females, depression in f_H was not significant in males at 90% of incubation. Sample sizes were 11 (75%) and 4 (90%) for males and 9 (75%) and 4 (90%) for females.

Embryonic and organ mass

Total egg mass decreased significantly over time in both sexes (Table 2). Total embryonic mass, heart mass and liver mass were significantly greater at 90% of incubation than at 75% of development (Table 2). However, these masses did not differ between males and females (Table 2). The total number of embryonic turtles used for mass determinations was 14 (75%) and 28 (90%) for males. Sample sizes were 10 (75%) and 26 (90%) for females. Sample sizes for heart and liver mass were reduced because some organs were not removed intact.

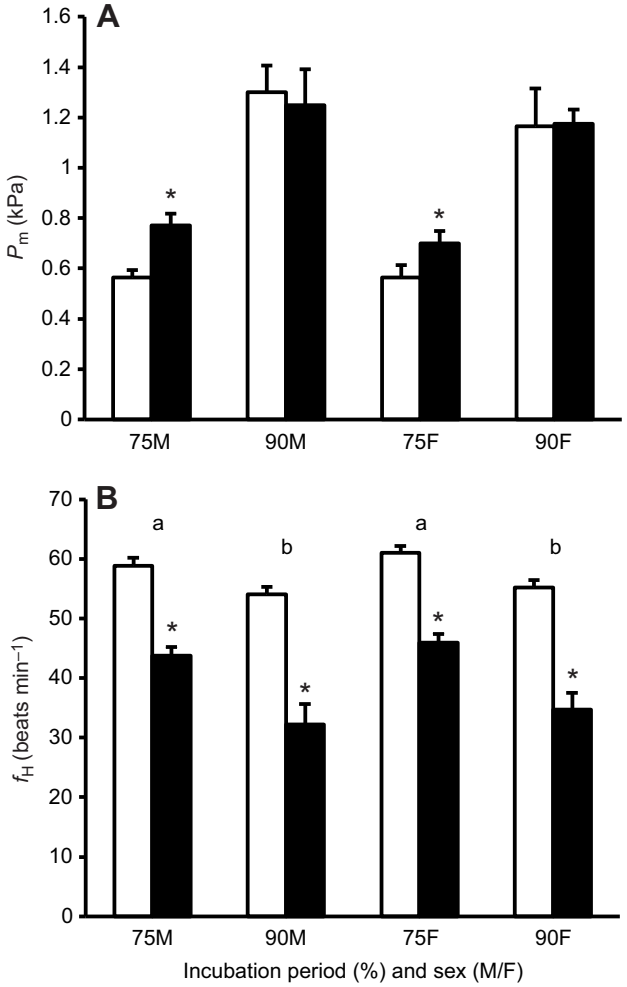

Fig. 4. (A) P_m and (B) f_H from male and female embryonic snapping turtles prior to (open columns) and following (filled columns) an injection of propranolol (3 mg kg^{-1}) at each point of incubation studied. Asterisks represent a significant (P<0.05) response to the injection. Different letters indicate a significant (P<0.05) difference in the propranolol response intensity between incubation periods. Data are presented as means ± s.e.m.

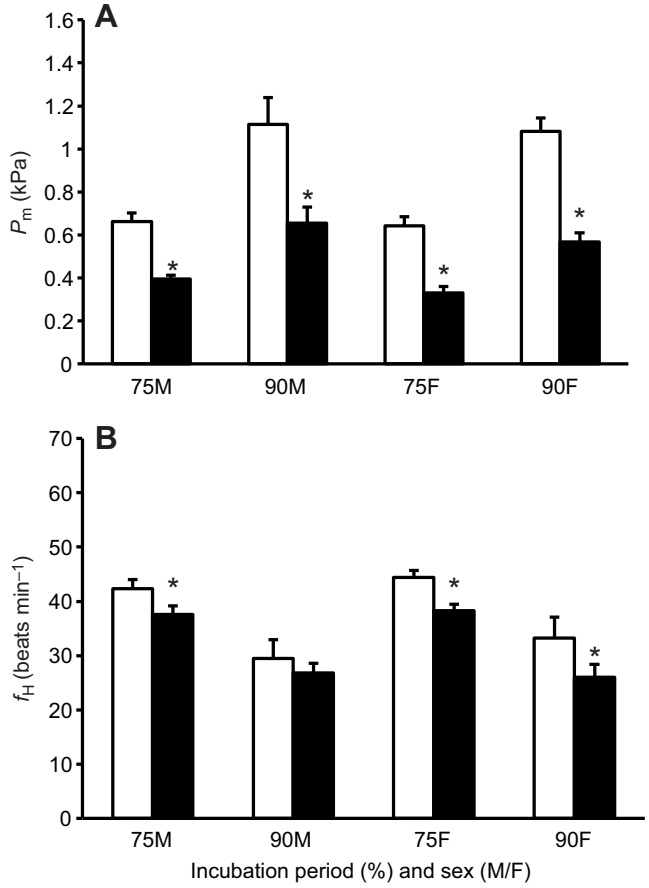

Fig. 5. (A) P_m and (B) f_H from male and female embryonic snapping turtles prior to (open columns) and following (filled column) an injection of phentolamine (3 mg kg^{-1}) at each point of incubation studied. Asterisks represent a significant (P<0.05) response to the injection. Data are presented as means ± s.e.m.

DISCUSSION

This study is the first to document sexual dimorphism in cardiovascular function at any stage of development in the snapping turtle, a TSD reptile. Specifically, male embryos are bradycardic compared with female embryos at the end of incubation, which can be directly attributed to higher cholinergic tone in males *versus* females. Female turtles were briefly exposed to a higher incubation temperature than males, so it is possible that temperature rather than sex influenced development of the parasympathetic system and cholinergic tone. Yet several observations indicate that the difference we observed is not a temperature effect *per se*.

Males and females were at the same temperature when autonomic tone was determined, so the sex difference cannot be a direct temperature effect (i.e. a Q_{10} effect). Several papers have reported thermal acclimation for resting heart rate in snapping turtle embryos (Birchard and Reiber, 1996; Birchard, 2000; Du et al., 2010). However, thermal acclimation of heart rate is not a permanent developmental effect, but can be reset by shifting embryos to the opposite temperature for 3–5 days (Birchard, 2000). Male and female embryos in our study were at the same temperature for approximately 16 days before the first measurements of heart rate and blood pressure. Our finding that males and females did not differ in autonomic tone at 75% of incubation is consistent with the idea of reversible thermal acclimation. Moreover, even though cholinergic tone was present at 75% of incubation, the sex difference did not emerge until 90% of incubation. This coincides more closely with the chronology of gonad differentiation and steroidogenesis (T.R., unpublished results). Finally, the effect of thermal acclimation in prior studies was in the opposite direction from the sex difference we observed: embryos acclimated to lower (male-producing) temperatures had faster heart rates than embryos acclimated to higher (female-producing) temperatures when measured at the same ambient temperature. Taken together, these observations strongly suggest that the effect we observed is indeed a sex difference and that temperature can have independent effects *via* physiological acclimation.

Embryonic snapping turtles possess a cholinergic receptor tone on P_m and f_H in the latter third of incubation, which is absent in the other reptiles studied to date. In addition, a clear β-adrenergic tone was evident on f_H in the latter third of incubation. This was accompanied by a significant α-adrenergic tone on P_m and f_H. Resting P_m increased similarly in both male and female snapping turtle embryos between measurements at 75% and 90% of incubation. An increase in resting P_m during embryonic development has previously been documented in the desert tortoise (*Gopherus*

Table 2. Snapping turtle egg, embryonic, heart and liver wet mass at 75% and 90% of incubation for males and females

	75%		90%	
	Male	Female	Male	Female
Egg (g)	14.3±0.3 (14)[a]	14.5±0.3 (10)[a]	13.8±0.3 (28)[b]	14.1±0.2 (26)[b]
Embryo (g)	5.7±0.2 (14)[a]	5.5±0.2 (10)[a]	7.8±0.2 (28)[b]	7.6±0.2 (26)[b]
Heart (mg)	16.0±1.0 (14)[a]	16.4±1.0 (8)[a]	25.5±1.0 (28)[b]	24.8±1.0 (25)[b]
Liver (mg)	190.0±21.0 (6)[a]	186.0±18.0 (6)[a]	241.0±9.0 (10)[b]	249.0±8.0 (10)[b]

Different superscripted letters indicate significant (P<0.05) differences between embryonic incubation periods for each mass parameter. Data are presented as means ± s.e.m. In all cases sample size is indicated in parentheses.

agassizii) (Crossley and Burggren, 2009), the American alligator (*Alligator mississippiensis*) (Crossley et al., 2003b; Crossley and Altimiras, 2005; Eme et al., 2011a), the emu (*Dromiceius novaehollandiae*) (Crossley et al., 2003a) and the domestic chicken (*Gallus gallus*) (Crossley and Altimiras, 2000). These findings suggest that an increase in blood pressure during ontogeny is a fundamental requirement for maintaining convective transport in embryos that are increasing in body mass.

The decrease in f_H for embryonic snapping turtles during this span of incubation is similar to that documented in the emu (Crossley et al., 2003a) and the desert tortoise (Crossley and Burggren, 2009), but it differs from that in the American alligator (Crossley et al., 2003b; Crossley and Altimiras, 2005; Eme et al., 2011a; Eme et al., 2011b), the embryonic chicken (Crossley and Altimiras, 2000; Altimiras and Crossley, 2000) and the African brown house snake (*Lamprophis fuliginosus*) (Crossley and Burggren, 2009). Resting f_H differed significantly (~9 beats min^{-1}) in male and female snapping turtles at 90% of incubation. Thus, sex strongly influences f_H just prior to hatching in this species.

There were also small but significant sex differences in the response to saline injection (Fig. 1). Females were bradycardic in response to saline injection at both time points, but males were not. Whether responsiveness to blood volume changes (i.e. baroreflex) is sexually dimorphic could be tested using injections of greater volumes of saline than those used in this study. Notably, despite these differences, there were no differences in developmental stage or mass between sexes.

Embryonic snapping turtles have a clear cholinergic tone on f_H during the final 25% of embryonic incubation (Fig. 3B). These data suggest that central nervous system regulatory capacity in snapping turtles is functional relatively early in development compared with that of other embryonic reptiles (Crossley et al., 2003b; Crossley and Burggren, 2009; Eme et al., 2011a). Cholinergic receptor tone was present in snapping turtles at both time points, but the intensity was sexually dimorphic at 90% of incubation. Studies in humans and other animals show that autonomic tone on basal cardiovascular function differs between males and females (Shechtman and Katovich, 1993; Liao et al., 1995; Evans et al., 2001). More specifically, androgens and estrogens contribute to the difference in baroreflex between adult males and females, an effect that appears to be mediated by hormone effects on vagal cholinergic function (Du et al., 1994; Abdel-Rahman, 1999; El-Mas et al., 2001; Dart et al., 2002; Thompson and Khalil, 2003; Orshal and Khalil, 2004; Mendelsohn and Karas, 2005; Semyachkina-Gluchkovskaya et al., 2008).

Although we did not measure sex steroid concentrations in this study, there are sex differences in circulating sex steroid levels in hatchlings (Rhen et al., 1996). There is also strong evidence that estrogen synthesis and signaling in the gonads plays a key role in TSD in the common snapping turtle (Rhen and Lang, 1994). Several genes involved in steroidogenesis are induced by the brief shift to a female-producing temperature: steroidogenic acute regulatory protein increases threefold, cytochrome P450 17A1 increases twofold and aromatase increases 11-fold (Rhen et al., 2007) (T.R., unpublished results). Thus, sex differences in circulating estrogens at later stages of embryonic development could cause differences in cholinergic tone. This is a plausible hypothesis given estrogen effects on cholinergic physiology in other tissues. For instance, estradiol regulates expression of choline acetyltransferase in the central nervous system (Spencer et al., 2008). Estrogens also regulate expression of muscarinic M2 acetylcholine receptors in the hippocampus and the lung (Daniel et al., 2005; Carey et al., 2007). Additional experiments are required to directly test the hypothesis that sex steroids influence autonomic control of P_m and f_H in snapping turtle embryos. One could administer estrogens, estrogen receptor antagonists, androgens, androgen receptor antagonists and/or aromatase inhibitors to determine which hormone(s) are responsible for the sex difference in cholinergic tone documented here. Although cholinergic tone on heart rate was altered by gonadal sex, adrenergic tone was mostly unaffected.

Males and females have similar β-adrenergic tone on P_m or f_H at both points of incubation investigated (Fig. 4). Although β-adrenergic blockade increased P_m equally in both groups of snapping turtle embryos at 75% of incubation, it had no effect at 90% of incubation (Fig. 4A). The f_H response to β-adrenergic blockade was similar in both sexes at 75% and 90% of incubation and increased in intensity as incubation progressed (Fig. 4B). The β-adrenergic receptor tone we observed in snapping turtle embryos in this study is generally similar to that documented in other egg-laying amniotic vertebrates, although the changes in intensity are species-specific (Crossley et al., 2003a; Crossley and Altimiras, 2005; Lindgren et al., 2011). The American alligator, emu and chicken all exhibit an increase in P_m following β-adrenergic receptor blockade, indicating a β-adrenergic depressor tone during the last third of embryogenesis (Eme et al., 2011a; Crossley et al., 2003a; Crossley and Altimiras, 2000). However, the snapping turtle is unique in that the P_m response is absent at 90% of embryonic development. In contrast, the bradycardic response to β-adrenergic blockade reported here in common snapping turtles has been previously reported in egg-laying amniotes: it either increases in intensity or is constant during the final third of incubation (Crossley and Altimiras, 2000; Crossley et al., 2003a; Crossley and Burggren, 2009; Eme et al., 2011a). This suggests that dependence on β-adrenergic tone to maintain cardiovascular function is a common feature of amniotic development.

A clear β-adrenergic tone on f_H is present in snapping turtle embryos, but the source of this tone was not investigated. Prior studies suggest that the majority of β-adrenergic tone is derived from receptor stimulation *via* circulating catecholamines (Dragon et al., 1996; Mulder et al., 2000; Crossley and Altimiras, 2000; Eme et

al., 2011a). If this is also the case for embryonic snapping turtles, it implies that sex hormone profile does not alter catecholamine release from chromaffin tissue.

As observed for β-adrenergic blockade, α-adrenergic blockade altered P_m in embryonic snapping turtles. This depressor response was the same in males and females and did not change from 75% to 90% of incubation (Fig. 5A). This has been documented in numerous studies, suggesting that α-adrenergic pressor tone is a conserved homeostatic mechanism in embryonic vertebrates (Crossley and Altimiras, 2000; Crossley et al., 2003a; Eme et al., 2011a). Like β-adrenergic tone on f_H, α-adrenergic tone on f_H was similar in both sexes at 75% of incubation (Fig. 5B). However, there was a slight but significant sex difference in α-adrenergic tone on f_H at 90% of incubation: female embryos responded to phentolamine but male embryos did not (Fig. 5B). A sex difference in adrenergic action has been observed in rat aortic smooth muscle, suggesting again that this difference may be attributed to embryonic turtle sex (Shechtman and Katovich, 1993).

Conclusions

In summary, we demonstrate that sexual dimorphism in cardiovascular physiology manifests prior to hatching. Male and female embryos differ in basal f_H and autonomic regulation of f_H and P_m. This sex difference was primarily due to higher cholinergic tone at 90% of incubation in male embryos. Although the effect was smaller in magnitude, higher α-adrenergic tone in female embryos also contributed to the sex difference at 90% of incubation. These differences in autonomic tone between sexes are potentially due to differences in circulating levels of sex steroids. In addition, we highlight differences in development of the embryonic snapping turtle cardiovascular system compared with cardiovascular development in other vertebrates.

LIST OF SYMBOLS AND ABBREVIATIONS

f_H	heart rate (beats min^{-1})
P_m	resting blood pressure (kPa)
TSD	temperature-dependent sex determination

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