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# Development of sympathetic cardiovascular control in embryonic, hatchling, and yearling female American alligator (*Alligator mississippiensis*)

John Eme [a,*], Ruth M. Elsey [b], Dane A. Crossley II [a]

[a] Department of Biological Sciences, University of North Texas, Denton, TX, USA
[b] Rockefeller Wildlife Refuge, Louisiana Department of Wildlife and Fisheries, Grand Chenier, LA, USA

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ABSTRACT

We used arterial tyramine injections to study development of sympathetic actions on *in vivo* heart rate and blood pressure in embryonic, hatching and yearling female American alligators. Tyramine is a pharmacological tool for understanding comparative and developmental sympathetic regulation of cardiovascular function, and this indirect sympathomimetic agent causes endogenous neuronal catecholamine release, increasing blood pressure and heart rate. Arterial tyramine injection in hatchling and yearling alligators caused the typical vertebrate response — rise in heart rate and blood pressure. However, in embryonic alligators, tyramine caused a substantial and immediate bradycardia at both 70% and 90% of embryonic development. This embryonic bradycardia was accompanied by hypotension, followed by a sustained hypertension similar to the hatchling and juvenile responses. Pretreatment with atropine injection (cholinergic receptor blocker) eliminated the embryonic hypotensive bradycardia, and phentolamine pretreatment (α-adrenergic receptor blocker) eliminated the embryonic hypotensive and hypertensive responses but not the bradycardia. In addition, hexamethonium pretreatment (nicotinic receptor blocker) significantly blunted embryos' bradycardic tyramine response. However, pretreatment with 6-hydroxydopamine, a neurotoxin that destroys catecholaminergic terminals, did not eliminate the embryonic bradycardia. Tyramine likely stimulated a unique embryonic response — neurotransmitter release from preganglionic nerve terminals (blocked with hexamethonium) and an acetylcholine mediated bradycardia with a secondary norepinephrine-dependent sustained hypertension. In addition, tyramine appears to stimulate sympathetic nerve terminals directly, which contributed to the overall hypertension in the embryonic, hatchling and yearling animals. Data demonstrated that humoral catecholamine control of cardiovascular function was dominant over the immature parasympathetic nervous system in developing alligator embryos, and suggested that sympathetic and parasympathetic nerve terminals were present and developing *in ovo* but were not tonically active.



## 1. Introduction

We used the biologically active amine, tyramine, as a pharmacological tool to study sympathetic control of the cardiovascular system in developing alligator embryos, hatchling alligators, and yearling alligators. The autonomic nervous system of embryos and fetuses are immature prior to hatching/birth, and reptile embryos represent the basal condition for the common ancestor of endothermic vertebrates. Investigations of sympathetic neural regulation of cardiovascular function have included treatment with tyramine, which is a biologically active amine that along with β-phenylethylamine, tryptamine and octopamine, occurs in trace amounts in vertebrates (Schafers et al., 1997; Branchek and Blackburn, 2003). Injecting tyramine, an indirect sympathomimetic agent, into the venous or arterial circulation causes release of endogenous catecholamines from intraneuronal presynaptic terminals *in vivo*, and intracellular injections cause catecholamine release from chromaffin cells of the adrenal medulla *in vitro* (Mundorf et al., 1999; Khwanchuea et al., 2008). Unlike amphetamine, tyramine has not been shown to cross the blood–brain barrier in adult animals (McCulloch et al., 1978). Chicken embryo hearts responded to tyramine in a positive chronotropic fashion, from ca. 50% of embryonic incubation through hatching (Pappano, 1976; Crossley et al., 2003a), but embryonic effects on the closest relative to birds, crocodilians, have not been studied.

*Abbreviations:* CAM, Chorioallantoic Membrane; CNS, central nervous system; Hatchling, alligator ca. 2 months old; Yearling, alligator ca. 9–12 months old; $f_H$, heart rate (beats $min^{-1}$); $P_m$, Mean Arterial Pressure (kPa) measured in kPa through a CAM artery in embryos or through a femoral artery in hatchling and yearling animals; 70%, 70% of embryonic incubation/development for alligator embryos (ca. 50 days of 72 day total incubation); 90%, 90% of embryonic incubation/development for alligator embryos (ca. 65 days of 72 day total incubation).

* Corresponding author at: University of North Texas, Department of Biological Sciences, 1155 Union Circle, #305220, Denton, TX76203-5017, USA. Tel.: +1 940 580 8725.
*E-mail address:* johneme23@gmail.com (J. Eme).
*URL:* http://www.biol.unt.edu/~jme0150 (J. Eme).

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American alligator embryos (*Alligator mississippiensis*) have a pronounced β-adrenergic tone on heart rate, as well as an α-adrenergic tone on the vasculature to maintain cardiovascular function (Eme et al., 2011a). Unlike some strains of late-term bird embryos, alligator embryos lacked cholinergic or vagal tone prior to hatching, thus adrenergic receptor mediated control was dominant in the embryonic phase of life (Crossley and Altimiras, 2000; Altimiras et al., 2009; Eme et al., 2011b). While tonic vagal output was not present, the capacity for efferent vagal motor output has been shown for alligator embryos. Stimulation of 5-$HT_3$ receptor vagal pulmonary C-fiber afferents with phenylbiguanide injection caused a massive and immediate bradycardia, a response that was blunted by administration of hexamethonium (Eme et al., 2011b). In addition, the cardiac baroreflex has been documented as early as 70% of incubation for alligator embryos (Crossley et al., 2003b). Therefore, cholinergic receptors (muscarinic and nicotinic) were likely present but not tonically active at 30 °C in alligator embryos by 2/3 of embryonic incubation. Like some fetal mammals (Thornburg and Morton, 1986; Reller et al., 1989; Koos et al., 1993), embryonic alligator's cardiovascular regulation is 'immature' and provides a vertebrate model without maternal influences for understanding cardiovascular function prior to full onset of autonomic tone. In addition, crocodilians represent an independent lineage of vertebrate evolution, and embryonic data allow for comparisons of commonalities or differences in cardiovascular development across vertebrate taxa.

In response to tyramine-induced catecholamine release from sympathetic neurons, mammalian animal models experience a hypertensive tachycardia (Broadley, 2010). The 'pressor response' has been suggested to be both a $\beta_1$-adrenoreceptor mediated inotropic effect on the myocardium, and an α-adrenoreceptor receptor mediated vascular hypertension (Schafers et al., 1997; Khwanchuea et al., 2008). In addition to these catecholamine-mediated responses, a class of novel trace amine receptors (TAR; G-protein-coupled receptors) exists in the peripheral vasculature, and stimulation of these receptors directly by amines, such as tyramine, could cause vasoconstriction and increased blood pressure (Borowsky et al., 2001; Zucchi et al., 2006). Due to these documented cardiovascular responses, tyramine injection has been used to study the cardiovascular system of a wide range of vertebrates, including fish, invertebrates, birds and mammals (e.g. Fange and Ostlund, 1954; Pappano, 1976; Schafers et al., 1997; Crossley et al., 2003a).

The purpose of this study was to examine adrenergic cardiovascular control in embryonic, hatchling, and yearling female alligators. We hypothesized that all ages of alligators studied would exhibit the typical vertebrate response to tyramine injection — increasing heart rate and blood pressure. We also hypothesized that embryonic sympathetic regulatory capacity matures prior to hatching, and that if sympathetic regulatory capacity was present, tyramine would elicit a response. We further hypothesized that if sympathetic regulatory capacity matures prior to hatching, tyramine would elicit a stronger response at 90% than at 70% of embryonic development.

## 2. Materials and methods

### 2.1. Alligator embryo acquisition, incubation and hatching

American alligator eggs (*A. mississippiensis*) were obtained from the Rockefeller Wildlife Refuge in Grand Chenier, LA, USA and transported by automobile to the lab. Eggs from each clutch were staged to determine each clutches' post-laying time, as previously described (72 day total incubation period at 30 °C; Ferguson, 1985; Crossley and Altimiras, 2005; Eme et al., 2011a). Embryos were incubated at 30 °C in a walk-in, constant temperature room ensuring that all embryos developed as females. All embryos were incubated in plastic containers, placed in a bed of moist vermiculite mixed in a 1:1 ratio of vermiculite:water. Water content of the vermiculite was determined by mass at the beginning of incubation and maintained by weighing the box twice weekly, with water added as needed.

Eggs (n = 60) in plastic incubation containers were placed inside large Ziploc® bags and sealed, and two holes in each bag allowed parallel inflow and outflow of gas. Air was passed through a $H_2O$-bubbler to ensure adequate water saturation within the bag. Gas composition was monitored with an oxygen analyzer (S-3AII, Ametek Applied Electrochemistry, IL, USA) connected to a PowerLab® data recording system connected to a computer running LabChart Pro® software (v 7 ADInstruments, CO, USA), and data recorded at 10 Hz. In late August-early September of 2011 and 2012, a subset of eggs was allowed to hatch, and these young alligators were subsequently used in experiments in 2012 (n = 6 hatch 2011 'yearlings'; n = 5 hatch 2012 'hatchlings'). Measurements of young female alligators were conducted at 1.5–2 months of age ('hatchlings'), and at 9–12 months of age ('yearlings'). Measurements of embryonic alligators were conducted at 70% and 90% of embryonic development/incubation, which reflected *in ovo* developmental stages 24/25 and 27/28, respectively (stage 26 absent in *A. mississippiensis* embryos; Ferguson, 1985; Eme et al., 2011a,b). All studies were approved by UNT IACUC #11-007.

### 2.2. Young alligator maintenance, surgery and tyramine injection via femoral artery

All alligators were hatched in the lab following incubation at 30 °C (as above), ensuring that all animals were female and 'age-matched'. Alligators were group housed in tanks ranging in size from 0.3 × 1 × 0.3 m plastic tanks to 0.7 × 2 × 0.7 m fiberglass tanks, depending on animal size. Animals were maintained at 26–30 °C with free access to water and fed an *ad libitum* diet of commercial alligator food (56% protein LoneStar Feeds®) and/or fish 1–4 times per week. 'Hatchling' alligators were 1.5–2 months old at the time of surgery and experimentation (n = 5, all animals had absorbed embryonic yolk), and 'Yearling' alligators were 9–12 months old (n = 6). Animals were fasted for 6 to 8 days prior to experimentation. Pressure transducers were calibrated prior to each measurement period against a vertical column of saline, and heart rate was determined with a software tachograph calculated 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 (cm) to recorded pressure (kPa).

Alligators were lightly anaesthetized by placing them in a sealed container with gauze soaked in isoflurane (Isoflo®; Abbott Laboratories, North Chicago, IL, USA). The animal's trachea was intubated and the lungs artificially ventilated using a small rodent ventilator (Model 683; Harvard Apparatus®; Holliston, MA, USA) downstream of a vaporizer providing 0.5–2% isoflurane at 3–6 breaths $min^{-1}$ and 1–4 mL $breath^{-1}$ (Ohmed Fluotec 4 Anesthetic Vaporizer; GE Healthcare; Buckinghamshire, UK). The animal's left rear leg was scrubbed with 70% ethanol, and a 1–2 cm incision was made through the skin of the outer thigh. The skin was blunt dissected away from the underlying musculature, and the femoral artery exposed by removal of the femoral sheath. Under a dissection microscope (Leica WILD M3Z; Leica Microsystems, Waukegan, IL, USA), the femoral artery was blunt dissected away from the femoral vein, and an occlusive catheter was inserted into the artery using heat-pulled, heparinized and saline-filled PE 10 tubing connected to PE 50 tubing. The catheter was secured at the exit point from the thigh using half hitch knots and was secured to the animal's dorsal surface using a series of square knots. The duration of anesthesia was 30–90 min.

In a constant-temperature room at 30 °C, the recovered animal was placed inside a darkened chamber (0.25 × 0.15 × 0.1 m or 0.45 × 0.25 × 0.2 m). An open midline (0.4 cm wide, 10–15 cm long) in the chamber's lid allowed for movement of the catheter with the animal and for fresh air to enter the chamber. The catheter was attached to a pressure transducer (ADInstruments model MLT0699, Colorado Springs, CO, USA) *via* saline-filled PE 50 tubing,

connected to an Octabridge amplifier (ADInstruments), and the pressure signal acquired (40 Hz).

Following at least 24 h post-operative recovery, *all* alligators received a control injection of heparinized saline to document any responses from injection alone, and the volume was equivalent to total volume of each drug injection plus saline flush. Subsequent to the control injection, hatchling and yearling alligators were injected with tyramine (10 mg $kg^{-1}$). Tyramine was administered through a T connector in the arterial catheter line, and flushed into the line with double the injection volume. Tyramine injection concentration was based on previously published values in chicken embryos (Crossley and Altimiras, 2000).

Following completion of each trial, animals were sacrificed with an overdose of isoflurane (Isoflo; Abbott Laboratories), and wet body mass determined ±0.001 g using an analytical balance (Mettler Toledo XS204; Columbus, OH, USA).

### 2.3. Embryonic surgery and tyramine injection before and after ganglionic, cholinergic, dopaminergic and adrenergic pharmacological blockade

At 70% (n = 22) and 90% (n = 27) of development, eggs were removed from their incubators and candled to determine orientation of the embryo and location of an accessible third order chorioallantoic membrane (CAM) artery. Eggs were placed in a temperature-controlled surgical chamber (30 °C), and a portion of the eggshell removed under a dissection microscope (Leica MZ6; Leica Microsystems). A third order CAM artery was isolated for arterial pressure monitoring and drug injection, and an occlusive catheter was inserted into the artery under a dissection microscope using heat-pulled, heparinized and saline-filled PE 50 tubing, as previously described (Crossley and Altimiras, 2005). The catheter was attached to a pressure transducer 1–3 cm above the egg *via* saline-filled PE 50 tubing, connected to an amplifier, and the pressure signal was acquired (40 Hz) as above. Pressure transducers were calibrated, heart rate was determined and blood pressure was corrected, as above.

Following catheterization, embryos were transferred to a six-chamber (730 mL chamber volume, with one embryo per chamber placed on cotton), water-jacketed, stainless steel experimental apparatus and allowed to recover for at least 60 min. Temperature (30 °C) was maintained throughout the apparatus' chambers by recirculating water (30 °C) from a constant temperature circulator (VWR 1165; VWR International, LLC, West Chester, PA, USA). Each chamber in the apparatus had a stainless steel lid, with three small holes that allowed for the catheter line (1 × 5 $mm^3$) and airlines (2 × 3 $mm^3$) to enter the chamber. Air was continuously pumped into each chamber at ca. 0.2 L $min^{-1}$ after passing through a $H_2O$ bubbler connected to a 2-m heating coil lining the chambers. Following a 60 min recovery, *all* embryos received an initial control injection of heparinized saline equivalent to total volume of each drug injection plus saline flush to document any responses from injection alone. Individual total injection volumes did not surpass 5% of total blood volume (ca. 3–5 mL $kg^{-1}$; Tate et al., 2012). All injections were administered through a T connector in the arterial catheter line, and all drug injection concentrations were based on previously published values in chicken and/or alligator embryos (Crossley and Altimiras, 2000; Crossley et al., 2003a,b; Eme et al., 2011a,b). Each drug injection was chased/flushed with 50 μL of saline to insure the drug had entered the CAM artery, 35 μL of drug for 70% embryos and 50 μL of drug for 90% embryos.

A first set of embryos was subjected to tyramine/6-hydroxydopamine (6-OHDA) response protocol at 70% (n = 7) and 90% of development (n = 6). Each embryo was first injected with tyramine (10 mg $kg^{-1}$; stimulates catecholamine release from neuronal storage vesicles) and allowed to recover for at least 90 min. Second, embryos were injected with 6-OHDA (20 mg $kg^{-1}$; neurotoxin that selectively depletes neurotransmitter from dopaminergic and noradrenergic neurons) and allowed to recover for at least 90 min. Third, embryos were injected again with tyramine (10 mg $kg^{-1}$) to determine if 6-OHDA had altered the response to tyramine, and embryos allowed to recover for at least 60 min.

A second set of embryos was subjected to tyramine/hexamethonium response protocol at 70% (n = 11) and 90% of development (n = 6). Each embryo was first injected with tyramine, second with hexamethonium (25 mg $kg^{-1}$; preganglionic nicotinic acetylcholine receptor antagonist) and allowed to recover for at least 30 min, and third with tyramine (10 mg $kg^{-1}$) to determine if hexamethonium had altered the response to tyramine.

A third set of embryos was subjected to tyramine/atropine/phentolamine response protocol at 70% (n = 4) and 90% of development (n = 4). Each embryo was first injected with tyramine (10 mg $kg^{-1}$), second with atropine (3 mg $kg^{-1}$; Sigma-Aldrich, cholinergic receptor antagonist) and allowed to recover for at least 30 min, and third with tyramine (10 mg $kg^{-1}$) to determine if atropine had altered the response to tyramine. Fourth, embryos were injected with phentolamine (3 mg $kg^{-1}$; Sigma-Aldrich nonspecific α-adrenoreceptor antagonist), and fifth embryos were injected with tyramine (10 mg $kg^{-1}$) to determine if phentolamine had altered the response to tyramine.

A fourth set of embryos was subjected to tyramine/atropine response protocol at only 90% of development (n = 6). Each embryo was first injected with tyramine (10 mg $kg^{-1}$), second with atropine (3 mg $kg^{-1}$), and third with tyramine (10 mg $kg^{-1}$) to verify the tyramine response after atropine.

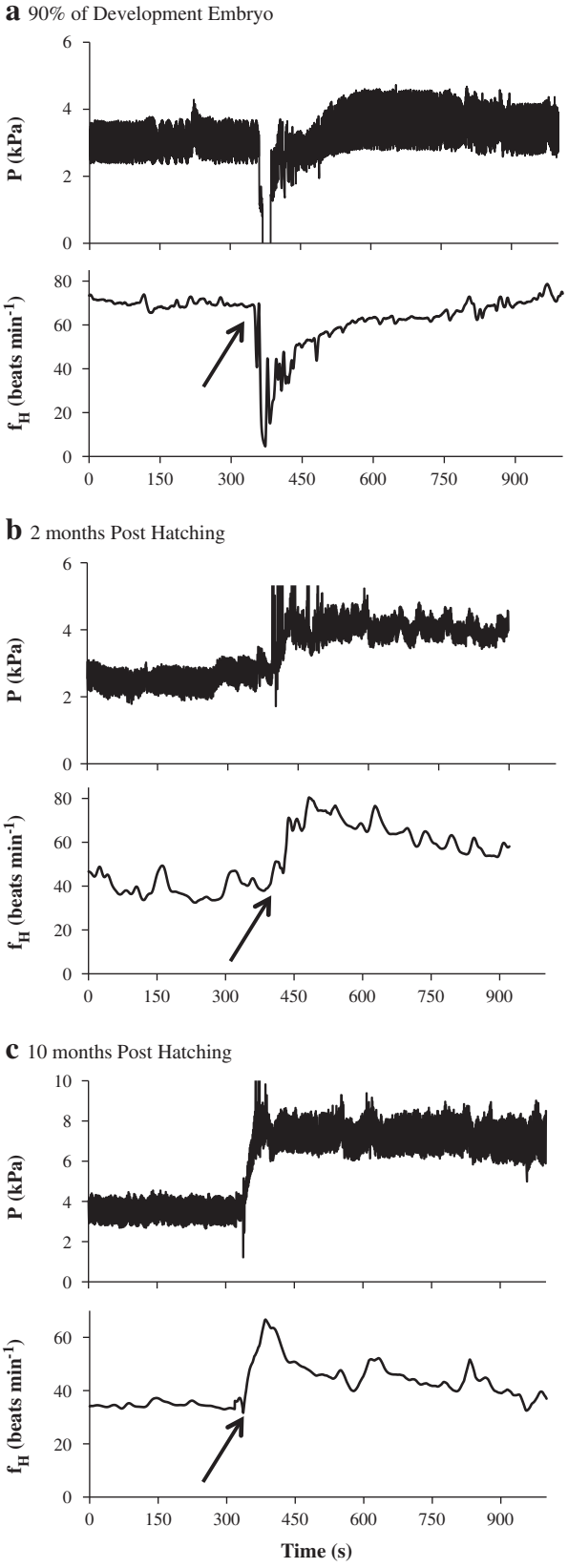
A fifth set of embryos was subjected to tyramine/phentolamine response protocol at only 90% of development (n = 5). Each embryo was first injected with tyramine (10 mg $kg^{-1}$), second with phentolamine (3 mg $kg^{-1}$), and third with tyramine (10 mg $kg^{-1}$) to determine if phentolamine had altered the response to tyramine, independent of previous treatment with atropine.

Following completion of each trial, embryos were sacrificed with an overdose of isoflurane, and embryonic wet mass determined ± 0.001 g using an analytical balance, as above.

### 2.4. Data analyses

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 or animals within each experimental protocol. Within each experimental protocol and age (70% of embryonic development, 90% of embryonic development, hatchling/2 months old, and yearling/9–12 month old), separate 1-way repeated-measures Analyses of Variances (RM-ANOVA) were used to asses differences in absolute or fractional/magnitudinal mean $P_m$ or $f_H$ responses to tyramine before and after other pharmacological blockade. Changes in $P_m$ and $f_H$ for injection responses to tyramine were based on mean values taken over control 5–10 min periods preceding injection compared to mean values taken from two 'phases' 1) immediate response, 10–20 s period within 30 s after tyramine injection, and 2) peak hypertension for 30 s period within 5 min following tyramine injection. Significant RM-ANOVA results were followed by a Student Newman-Keuls *post-hoc* test. For comparisons of the magnitude of a change in $P_m$ or $f_H$ between developmental ages, arcsine square root transformed fractions were used. The magnitude of the effect of tyramine was expressed in relative terms as the absolute change divided by the absolute control level. Because these values are expressed as percentages, data were transformed (arcsine square root) and then compared using ANOVA (Eme et al., 2011a,b).

Changes in $P_m$ and $f_H$ for injection responses to atropine, phentolamine and hexamethonium were based on control mean values taken over 5–10 min periods preceding injection compared to mean values taken from 15 to 20 min stable periods between 10 and 20 min following injection, a similar time period previously used for responses cholinergic, adrenergic and ganglionic blockers (Crossley and Altimiras, 2005; Eme et al., 2011a,b). A paired *t* test compared control mean $P_m$ and $f_H$ values before and after control injections of




saline, and after injections of atropine, phentolamine, and hexamethonium. Changes in $P_m$ and $f_H$ for injection responses to 6-OHDA, were based on mean values taken over control 5–10 min periods preceding injection compared to mean values taken from three 'phases' 1) immediate response, 10–20 s period within 1 min after injection, 2) peak hypertension for 30 s period within 5 min following 6-OHDA injection and 3) 10–25 min stable periods approximately 90 min following injection. A 1-way RM-ANOVA compared resting mean $P_m$ and $f_H$ for each phase of the response to 6-OHDA within each developmental age, followed by a Student Newman–Keuls *post-hoc* test. Two-tailed *t* tests compared resting $P_m$ or mean $f_H$ between yearling and hatchling alligators. Throughout the text, means are given ±SEM. All statistical significance was determined based on $\alpha = 0.05$.

## 3. Results

### 3.1. Young alligator tyramine responses

As previously shown in alligator embryos, control injections of saline did not alter hatchling or yearling $P_m$ or $f_H$ (paired *t* tests $P > 0.2$; Crossley et al., 2003b; Crossley and Altimiras, 2005; Eme et al., 2011a, b). Body mass was 40.4 ± 2.7 g and 150.8 ± 21.0 g for hatchling and yearling alligators, respectively. Hatchling body mass was typical for alligators that have recently finished metabolizing their yolk (ca. 35–60 g hatching mass), and yearling body mass was similar to the ca. 200 g reported previously for 1-year old lab-raised alligators (Eme et al., 2010). Yearling mean resting $P_m$ was 2.7 ± 0.4 kPa and $f_H$ was 27 ± 3 beats min$^{-1}$. Hatchling mean resting $P_m$ (2.6 ± 0.2 kPa) was statistically similar to yearling resting $P_m$, but mean $f_H$ was significantly higher for hatchlings (41 ± 3 beats min$^{-1}$; *t* test, $t = 3.7$, $df = 9$ $P < 0.01$).

Hatchling and yearling alligators responded to arterial injection of tyramine with an immediate and pronounced rise in $f_H$ (Fig. 2 — separate RM ANOVAs: Hatchling $F_{1,4} = 288.55$, $P < 0.0001$, Yearling $F_{1,5} = 259.84$, $P < 0.0001$) and $P_m$ (Fig. 2 — separate RM ANOVAs: Hatchling $F_{1,4} = 71.94$, $P < 0.01$, Yearling $F_{1,5} = 41.65$, $P < 0.01$). No bradycardia or hypotension was observed following tyramine injection (Figs. 1, 2).

### 3.2. Embryonic tyramine responses before and after ganglionic, cholinergic, dopaminergic and/or adrenergic pharmacological blockades

As previously shown in alligator embryos, control injections of saline did not alter embryonic CAM arterial pressure or heart rate (paired *t* tests $P > 0.1$; Crossley and Altimiras, 2005; Eme et al., 2011a,b). Body mass was 14.0 ± 0.7 g and 32.1 ± 1.2 g for embryos at 70% and 90% of embryonic development, respectively, similar to previous data (Crossley and Altimiras, 2005; Eme et al., 2011a,b). Mean resting $P_m$ and $f_H$ were similar to previously published values for alligator embryos at both 70% and 90% of embryonic development (Crossley et al., 2003b; Crossley and Altimiras, 2005; Eme et al., 2011a,b).

Across all embryonic experiments at 70% and 90% of embryonic development, the response to CAM arterial tyramine injection was an immediate and pronounced bradycardia (Fig. 1). This bradycardia was followed by a return to baseline for heart rate in less than 5 min and was accompanied by a significant hypotension, most prominently at 90% of development. At 70% of development, tyramine injection had little effect on pressure for embryos. However, at 90% of development tyramine injection caused a bimodal response, with an initial drop in pressure followed by a hypertensive rebound within 5 min post-injection (Fig. 1).

**Fig. 1.** Representative traces of arterial pressure and heart rate before and after tyramine injection (10 mg kg$^{-1}$) for a developing female embryonic alligator at 90% of embryonic development (a), a 2-month old female alligator (b), and a 10-month old female alligator (c). Tyramine injections were made through a T-connector in a CAM arterial (a) or femoral arterial line (b, c). Tyramine causes an immediate bradycardia and hypotension only in embryonic alligators and not in hatchling or juvenile alligators. For hatchling and yearling alligators, tyramine injection causes a pronounced and immediate increase in pressure and heart rate, but in embryonic alligators the tachycardia was absent and the hypertensive response was more gradual. *Arrows* indicate the time of tyramine injection.

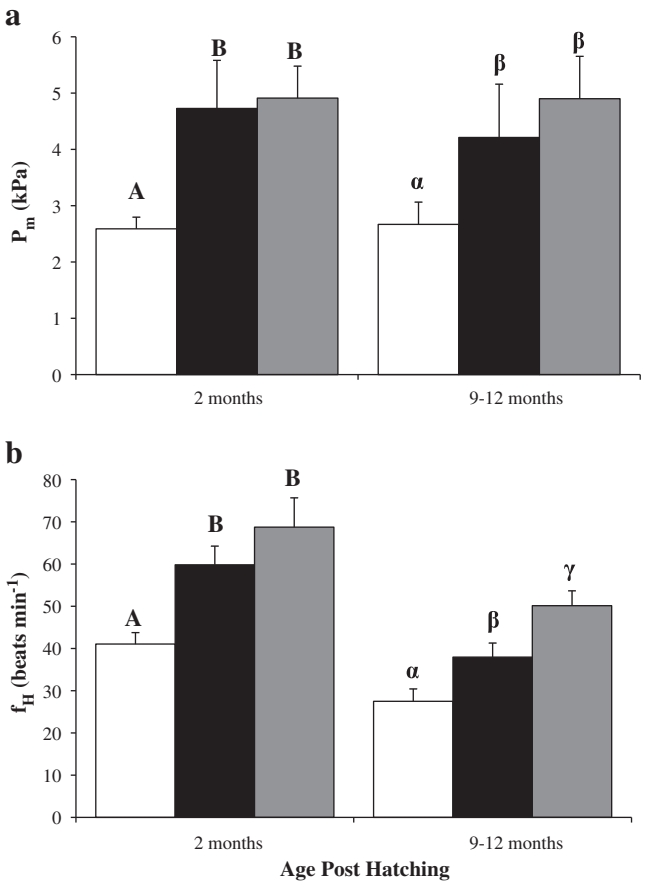



**Fig. 2.** Mean arterial pressure (a) and mean heart rate (b) in hatchling (ca. 2-months; n = 5) and yearling (ca. 9-12 months of age; n = 6) alligators prior to tyramine injection (white bars) and following tyramine injection (grey bars: within 20-30s post injection; black bars: within 5 min post injection). Letters denote separate SNK *post-hoc* comparisons following RM-ANOVA of $P_m$ or mean $f_H$. *Similar letters* indicate levels are not significantly different within each developmental age, and *dissimilar letters* indicate levels are significantly different. *Error bars* are SEM.

6-OHDA injection caused a similar, multifaceted response at both 70% and 90% of development. Injection of 6-OHDA resulted in an immediate (~30 s) and significant bradycardia without changes in $P_m$ (Table 1 — 70% $f_H$ $F_{1,6} = 796.12$, $P < 0.0001$, 90% $f_H$ $F_{1,5} = 1586.3$, $P < 0.0001$). This bradycardia was followed by a significant hypertension that occurred within 5 min of the initial injection (Table 1 — 70% $f_H$ $F_{1,6} = 72.1$, $P < 0.0001$, 90% $f_H$ $F_{1,5} = 54.4$, $P < 0.0001$). $P_m$ and $f_H$ returned to pre-injection levels in both developmental groups within 90 min.

Embryos in the tyramine/6-OHDA response protocol at 70% and 90% of development showed a significant bradycardia after tyramine injection at both 70% and 90% of development before and after 6-OHDA injection, followed by a return to baseline within 5 min post tyramine injection (Fig. 3 — 70% $f_H$ $F_{1,5} = 3183.3$, $P < 0.0001$, 90% $f_H$ $F_{1,5} = 759.4$, $P < 0.0001$). Embryos at 70% of development showed a significant, but slight hypotension immediately after tyramine injection, before 6-OHDA injection, along with a significant, but slight hypertensive rebound (Fig. 3 — 70% $P_m$ $F_{1,6} = 66.8$, $P < 0.0001$, 90% $P_m$ $F_{1,6} = 149.1$, $P < 0.0001$). After 6-OHDA injection, the hypertensive response was not present at 70% or 90% of development (Fig. 3). The magnitude of the hypertensive response to tyramine injection prior to treatment with 6-OHDA was greater for 90% of development embryos compared with 70% of development embryos, while the bradycardia remained constant.

Embryos in the tyramine/hexamethonium response protocol at 70% and 90% of development showed a significant bradycardia after tyramine injection at both 70% and 90% of development before hexamethonium injection, followed by a return to baseline within 5 min post tyramine injection (Fig. 4 — 70% $f_H$ $F_{1,10} = 4216.7$, $P < 0.0001$, 90% $f_H$ $F_{1,10} = 810.9$, $P < 0.0001$). This bradycardia was significantly blunted following hexamethonium injection (Fig. 4). Embryos at 70% and 90% of development showed a significant hypotension immediately after tyramine injection, before hexamethonium injection, followed by a significant hypertensive response (Fig. 4 — 70% $f_H$ $F_{1,10} = 4216.7$, $P < 0.0001$, 90% $f_H$ $F_{1,10} = 810.9$, $P < 0.0001$). After hexamethonium injection, the bradycardia and hypertensive response to tyramine was eliminated in 70% of development embryos, and in 90% embryos the bradycardia was eliminated and the hypertensive response was blunted (Fig. 4). Hexamethonium injection did not significantly alter embryonic $f_H$ and produced a slight hypotension at 70% of development, only (Table 2); this response was similar to previous results (Eme et al., 2011a,b).

Embryos in the tyramine/atropine/phentolamine response protocol at 70% and 90% of development showed a significant bradycardia after tyramine injection at both 70% and 90% of development, before sequential injection with atropine and then phentolamine, followed by a return to baseline within 5 min post tyramine injection (Fig. 5 — 70% $f_H$ $F_{1,3} = 28698.9$, $P < 0.0001$, 90% $f_H$ $F_{1,3} = 554.1$, $P < 0.0001$). Embryos at 70% of development did not show a significant hypotension or hypertensive response after tyramine injection, before atropine and phentolamine injection, however, 90% of development embryos did show a significant hypotension followed by a significant hypertensive response (Fig. 5 — 70% $P_m$ $F_{1,3} = 232.9$, $P < 0.01$, 70% $P_m$ $F_{1,3} = 29.0$, $P = 0.01$). At both 70% and 90% of development, after atropine injection, before phentolamine injection, tyramine injection no longer caused a bradycardia, and after phentolamine injection this lack of bradycardia persisted (Fig. 5). However, after atropine injection, before phentolamine injection, tyramine injection continued to cause a significant

**Table 1**
For embryos in the tyramine/6-OHDA response protocol, mean CAM arterial pressure ($P_m$; kPa) and heart rate ($f_H$; beats min$^{-1}$) before 6-OHDA injection (Pre 6-OHDA; 5–10 min), for the immediate bradycardia response, 10–20 s period within 1 min after injection (Heart Rate Minimum Post 6-OHDA), peak hypertension for 30 s period within 5 min injection (Hypertensive Maximum Post 6-OHDA), and 10–25 min stable periods approximately 90 min following injection (Post 6-OHDA). Data are mean ± SEM.

| Developmental age (%) | Variable | Pre 6-OHDA (n) | Heart rate Minimum Post 6-OHDA (n) | Hypertensive Maximum Post 6-OHDA (n) | Post 6-OHDA (n) |
|---|---|---|---|---|---|
| 70 | $P_m$ | 0.61 ± 0.08$^{A}$ (7) | 0.54 ± 0.05 $^{A}$ (7) | 0.75 ± 0.08$^{B}$ (7) | 0.66 ± 0.09 $^{A}$ (7) |
| 90 | $P_m$ | 1.93 ± 0.20 $^{A}$ (6) | 2.03 ± 0.47 $^{A}$ (6) | 2.74 ± 0.40 $^{B}$ (6) | 2.02 ± 0.23 $^{A}$ (6) |
| 70 | $f_H$ | 83 ± 2 $^{A}$ (7) | 48 ± 6 $^{B}$ (7) | 79 ± 4 $^{A}$ (7) | 85 ± 2 $^{A}$ (7) |
| 90 | $f_H$ | 86 ± 2 $^{A}$ (6) | 45 ± 10 $^{B}$ (6) | 73 ± 4 $^{A}$ (6) | 94 ± 3 $^{A}$ (6) |

$^{A,B}$6-OHDA injection caused a significant, immediate bradycardia followed by a significant hypertension (1-way RM ANOVA, $P < 0.05$; multiple comparisons within each row).

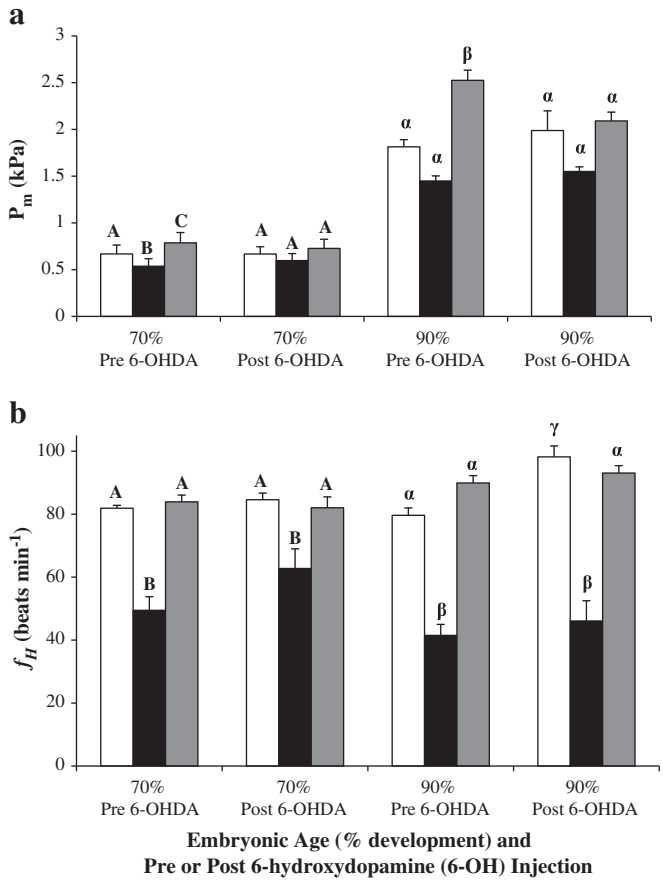



**Fig. 3.** Mean CAM arterial pressure (a) and mean heart rate (b) in 70% (n = 7) and 90% of development (n = 6) alligator embryos prior to tyramine injection (white bars) and following tyramine injection (grey bars: within 20–30 s post injection; black bars: within 5 min post injection) for embryos before (Pre 6-OHDA) and after 6-OHDA injection (Post 6-OHDA). Letters denote separate SNK *post-hoc* comparisons following RM-ANOVA of $P_m$ or mean $f_H$. *Similar letters* indicate levels are not significantly different within each developmental age, and *dissimilar letters* indicate levels are significantly different. *Error bars* are SEM.

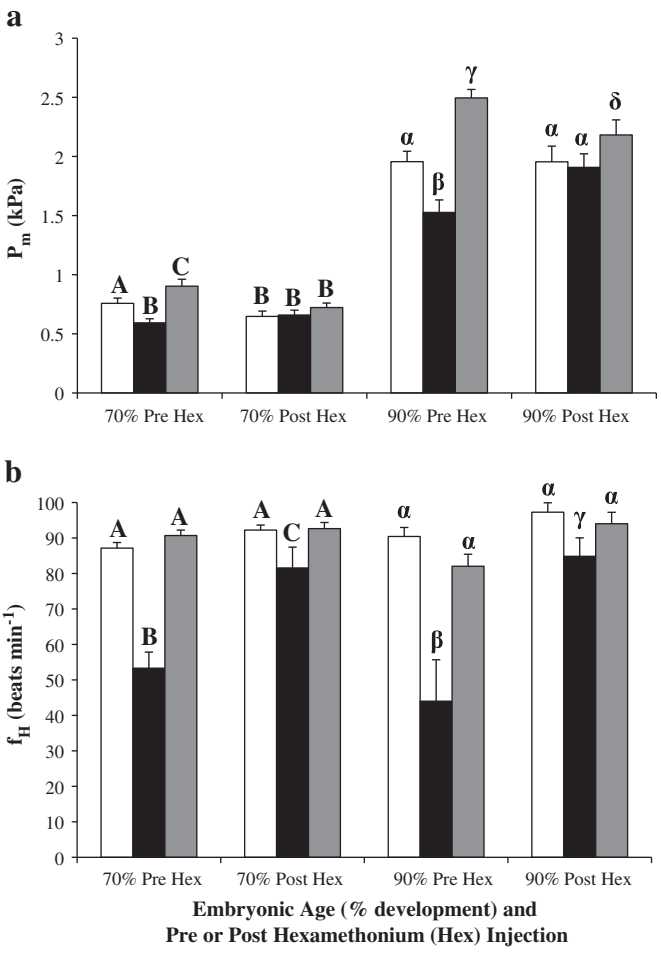



**Fig. 4.** Mean CAM arterial pressure (a) and mean heart rate (b) in 70% (n = 11) and 90% of development (n = 6) alligator embryos prior to tyramine injection (white bars) and following tyramine injection (grey bars: within 20–30 s post injection; black bars: within 5 min post injection) for embryos before (Pre-Hex) and after hexamethonium injection (Post-Hex). Letters denote separate SNK *post-hoc* comparisons following RM-ANOVA of $P_m$ or mean $f_H$. *Similar letters* indicate levels are not significantly different within each developmental age, and *dissimilar letters* indicate levels are significantly different. *Error bars* are SEM.

hypertension. At 90% of development, after sequential injection with atropine and phentolamine, tyramine injection had no effect on $P_m$ (Fig. 5). Atropine injection did not significantly alter embryonic $f_H$, and produced a slight hypotension at 90% of development, only (Table 3); this response was similar to previous results (Eme et al., 2011a,b). Phentolamine injection caused a large and sustained decrease in $P_m$, but no change in $f_H$ (Table 4; paired *t* tests, $t > 3.9$, df = 3 or 8, $P < 0.05$, and $P > 0.1$, respectively); this response was similar to previous results (Eme et al., 2011a).

Embryos in the tyramine/atropine response protocol at 90% of development only showed a significant bradycardia after tyramine injection, before atropine injection (Fig. 6a — 90% $f_H$ $F_{1,5} = 1543.0$, $P < 0.0001$). After atropine injection, this bradycardia was not present following the second tyramine injection. The separate group of embryos in the tyramine/phentolamine response protocol at 90% of development only showed a significant bradycardia after tyramine injection, before phentolamine injection (Fig. 6b — 90% $f_H$ $F_{1,4} = 454.1$, $P < 0.0001$). After phentolamine injection, this bradycardia persisted following the second tyramine injection. Embryos in the tyramine/atropine response protocol at only 90% of development did show a significant hypertensive rebound after tyramine injection (Fig. 7b — 90% $P_m$ $F_{1,5} = 303.1$ $P < 0.0001$). After atropine injection, this hypertensive response persisted following the second tyramine injection. Embryos in the tyramine/phentolamine response protocol at only 90% of development showed a significant immediate hypotension and hypertensive rebound after tyramine injection, before phentolamine injection (Fig. 7b — 90% $P_m$ $F_{1,4} = 211.6$, $P < 0.0001$). After phentolamine injection, the hypotension and hypertensive rebound were not present following the second tyramine injection.

## 4. Discussion

Arterial tyramine injection caused the typical vertebrate response in hatchling and yearling alligators — rise in $P_m$ and $f_H$. The tachycardia and hypertension in hatchling and yearling alligators were immediate responses and characteristic of functional sympathetic nerve terminals. However, in embryonic alligators, tyramine injection caused a substantial bradycardia at both 70% and 90% of embryonic development. This embryonic bradycardia was accompanied by a hypotension, likely attributed to the negative chronotropic effect that resulted from tyramine injection on the embryonic heart. Subsequent to the embryonic hypotension, a gradual but substantial hypertension occurred ('hypertensive rebound'). Closer examination of the embryonic tyramine response using injection of pharmacological blockers suggested tyramine caused an increase in preganglionic and peripheral sympathetic neurotransmitter release.

Yearling mean resting $P_m$ (2.7 ± 0.4 kPa) and $f_H$ (27 ± 3 beats min$^{-1}$) were lower and higher, respectively, than values

**Table 2**
For embryos in the tyramine/hexamethonium response protocol, mean CAM arterial pressure ($P_m$; kPa) and heart rate ($f_H$; beats $min^{-1}$) before (Pre Hex; 5–10 min) and for a 15 min period at least 15 min after injection of hexamethonium (Post Hex). Data are mean ± SEM.

| Developmental age (%) | Variable | Pre Hex (n) | Post Hex (n) |
|---|---|---|---|
| 70 | $P_m$ | 0.75 ± 0.05 (11) | 0.66 ± 0.04* (11) |
| 90 | $P_m$ | 2.16 ± 0.05 (6) | 2.01 ± 0.11 (6) |
| 70 | $f_H$ | 92 ± 1 (11) | 93 ± 1 (11) |
| 90 | $f_H$ | 97 ± 3 (6) | 97 ± 2 (6) |

* Hexamethonium injection caused a significant hypotension at 70% of embryonic development (paired *t* tests, $t > 2.7$, df = 5 or 10, $P < 0.05$).

**Table 3**
For embryos in the tyramine/atropine/phentolamine *and* tyramine/atropine response protocol, combined mean CAM arterial pressure ($P_m$; kPa) and heart rate ($f_H$; beats $min^{-1}$) before (Pre Atropine; 5-10 min) and for a 15 min period at least 15 min after injection of atropine (Post Atropine). Data are mean ± SEM.

| Developmental Age (%) | Variable | Pre Atropine (n) | Post Atropine (n) |
|---|---|---|---|
| 70 | $P_m$ | 0.73 ± 0.11 (4) | 0.66 ± 0.06 (4) |
| 90 | $P_m$ | 1.95 ± 0.21 (10) | 1.55 ± 0.16* (10) |
| 70 | $f_H$ | 93 ± 2 (4) | 93 ± 2 (4) |
| 90 | $f_H$ | 83 ± 3 (10) | 82 ± 2 (10) |

* Atropine injection caused a significant hypotension at 90% of embryonic development (paired *t* tests, $t > 3.2$, df = 3 or 9, $P < 0.05$).

recently published for yearling alligator measured at 28 °C (Bagatto et al., 2012). However, those animals were raised in large enclosures in Louisiana, fed daily, and were five to eight times as large as the lab raised animals in our study. The relationship between body mass, $P_m$ and $f_H$ has not been studied systematically in crocodilians. In addition, reported resting $f_H$ of a free-ranging large alligator (45 kg) of 31 beats $min^{-1}$ was very similar to our value (Smith et al., 1974), as were other published values for alligators (Huggins et al., 1969). This study is the first to report *in vivo* $P_m$ and $f_H$ measured in very young crocodilians, ca. 2 months of age.

The embryonic hypertension following tyramine injection was secondary to an initial bradycardia (Fig. 7). The embryonic hypertension was similar to that previously reported for embryonic chickens (Pappano, 1976; Crossley et al., 2003a), with the embryonic alligator hypertension also mediated exclusively through α-adrenergic receptor stimulation (Figs. 5a and 7). Embryonic chickens showed a mean hypertensive response to tyramine of up to 0.75 kPa at day 19 of incubation (ca. 90% of chicken embryo development), and 90% of development alligator embryos showed a mean $P_m$ increase of ca. 1.0 kPa in response to tyramine injection (Fig. 3). A similar response to tyramine has been reported in anesthetized rats, where phentolamine pretreatment abolished the hypertensive response to tyramine (Khwanchuea et al., 2008) as it did to the embryonic alligator response. Importantly, it appears that the 'pressor response' was not due to positive chronotropic or inotropic stimulation of the heart given that tyramine did not increase heart rate in embryonic alligators. This pressor response was due to a tyramine-induced release of catecholamines, as in mammals, with tyramine acting as both a competitive inhibitor for active catecholamine reuptake and displacing stored catecholamines (Burgen and Iversen, 1965; Scriven et al., 1983). Sympathectomy with 6-OHDA, a neurotoxin that selectively destroys dopaminergic and noradrenergic neurons, eliminated the tyramine hypertension in embryonic alligators, indicating that the source of the α-adrenergic stimulation was release from sympathetic nerve terminals given the specificity of 6-OHDA (Fig. 2a; Nilsson, 1983). In addition, embryonic animals pretreated with the ganglionic blocking agent hexamethonium exhibited a blunted hypertensive response to tyramine (Fig. 3a). While reduced, this

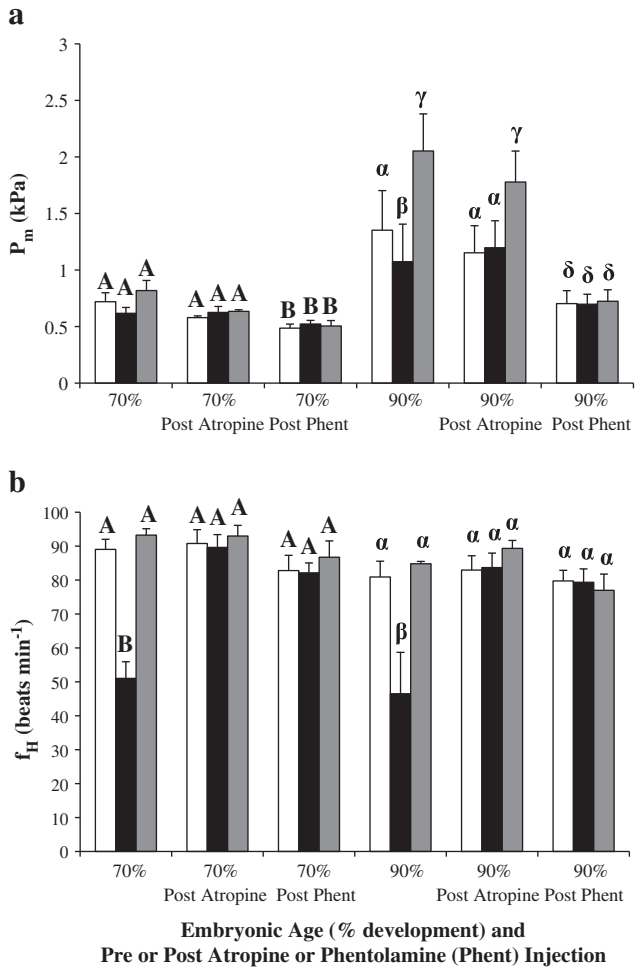



**Fig. 5.** Mean CAM arterial pressure (a) and mean heart rate (b) in 70% (n = 4) and 90% of development (n = 4) alligator embryos prior to tyramine injection (white bars) and following tyramine injection (grey bars: within 20–30 s post injection; black bars: within 5 min post injection) for embryos before sequential injection with atropine followed by phentolamine, after injection with atropine (Post-Atropine), and after injection with phentolamine (Post-Phent). Letters denote separate SNK *post-hoc* comparisons following RM-ANOVA of $P_m$ or mean $f_H$. *Similar letters* indicate levels are not significantly different within each developmental age, and *dissimilar letters* indicate levels are significantly different. *Error bars* are SEM.

**Table 4**
For embryos in the tyramine/atropine/phentolamine *and* tyramine/atropine response protocol, combined mean CAM arterial pressure ($P_m$; kPa) and heart rate ($f_H$; beats $min^{-1}$) before (Pre Phent; 5–10 min) and for a 15 min period at least 15 min after injection of phentolamine (Post Phent). Data are mean ± SEM.

| Developmental age (%) | Variable | Pre Phent (n) | Post Phent (n) |
|---|---|---|---|
| 70 | $P_m$ | 0.65 ± 0.06 (4) | 0.49 ± 0.04* (4) |
| 90 | $P_m$ | 1.60 ± 0.21 (9) | 0.94 ± 0.09* (9) |
| 70 | $f_H$ | 92 ± 2 (4) | 81 ± 2 (4) |
| 90 | $f_H$ | 81 ± 2 (9) | 78 ± 2 (9) |

* Phentolamine injection caused a significant hypotension at 70% and 90% of embryonic development (paired *t* tests, $t > 3.9$, df = 3 or 8, $P < 0.05$).

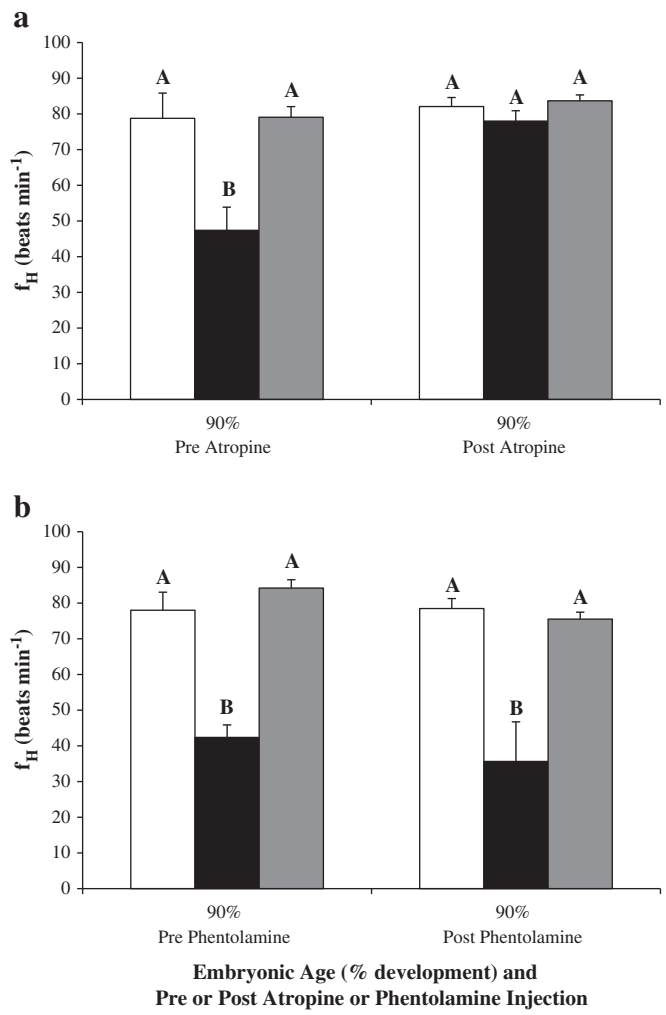



**Fig. 6.** Mean CAM arterial pressure (a) and mean heart rate (b) in 90% of development (n = 5 or 6) alligator embryos prior to tyramine injection (white bars) and following tyramine injection (grey bars: within 20–30 s post injection; black bars: within 5 min post injection). Embryos' responses were measured for separate groups before or after atropine injection only (a, n = 6) and before or after phentolamine injection only (b, n = 5). Letters denote separate SNK *post-hoc* comparisons following RM-ANOVA of mean $f_H$. *Similar letters* indicate levels are not significantly different, and *dissimilar letters* indicate levels are significantly different. *Error bars* are SEM.

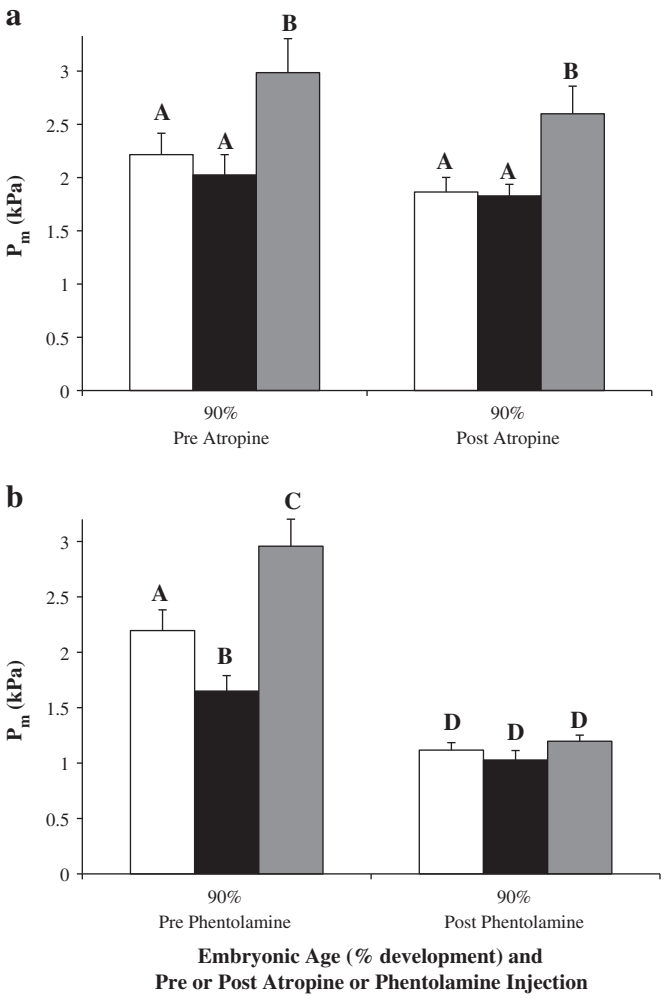



**Fig. 7.** Mean CAM arterial pressure (a) and mean heart rate (b) in 90% of development (n = 5 or 6) alligator embryos prior to tyramine injection (white bars) and following tyramine injection (grey bars: within 20–30 s post injection; black bars: within 5 min post injection). Embryos' responses were measured for separate groups before or after atropine injection only (a, n = 6) and before or after phentolamine injection only (b, n = 5). Letters denote separate SNK *post-hoc* comparisons following RM-ANOVA of $P_m$. *Similar letters* indicate levels are not significantly different, and *dissimilar letters* indicate levels are significantly different. *Error bars* are SEM.

persistent tyramine induced hypertension further suggests that tyramine stimulated preganglionic neurotransmitter release, a unique response compared to other developing vertebrate species (Crossley et al., 2003a).

The pronounced embryonic bradycardia following tyramine injection, mediated *via* cholinergic muscarinic receptor stimulation, represents a unique vertebrate response to tyramine. 6-OHDA injection caused a large bradycardia followed by a hypertension, but cardiovascular values returned to baseline within 45 min. This bradycardic response to 6-OHDA may also be a response unique to embryos. Pretreatment with 6-OHDA did not eliminate the embryonic bradycardia caused by tyramine, which makes the possible release of acetylcholine due to a hypertensive baroreflex unlikely (Fig. 2). This indicated that similar to embryonic chickens, embryonic alligators possessed functional sympathetic neurons that terminate on the heart and vasculature at 70% of incubation, as alligator and chicken embryos both respond to tyramine injection (Crossley and Altimiras, 2000). Further, the capacity for the sympathetic terminals to alter cardiovascular function likely increased between 70% and 90% of embryonic development (Fig. 2a). While the tyramine response did mature *in ovo*, the functional significance of this increased capacity may be critical for post hatching life but of limited importance *in ovo* given that sympathetic tone on the cardiovascular system was limited in embryonic alligators (Eme et al., 2011a,b).

The initial bradycardia following tyramine was only evident in embryonic animals, and this was eliminated by pretreatment with atropine, strongly suggesting that tyramine induces vagal synaptic release of acetylcholine (Figs. 4b; 5). Following ganglionic blockade with hexamethonium, a dramatic reduction in the degree of the tyramine-induced bradycardia was observed, and this observation indicated that acetylcholine release from preganglionic nerve terminals was a source of the embryonic bradycardia (Fig. 3b). While this work verified that sympathetic and parasympathetic tone were not present *in ovo* for alligator embryos (Eme et al., 2011a,b), it demonstrated the capacity for sympathetic regulation was present at 70% of incubation. Therefore, we demonstrated that humoral catecholamine control over embryonic cardiovascular function was the dominant regulator *in ovo*, similar to our previous findings (Eme et al., 2011a,b). A bradycardic response to tyramine has not been previously reported in numerous studies conducted using this drug as a tool to assess autonomic cardiovascular regulation (e.g., Fange and Ostlund, 1954; Pappano, 1976; Schafers et al., 1997; Crossley et al., 2003a; Broadley, 2010). Previous studies included both fetal and embryonic animals, suggesting that the embryonic alligator may be a unique species during development (Tabsh et al., 1982;

Crossley et al., 2003a). The mechanisms underlying the embryonic bradycardic response to tyramine could have been neurotransmitter release from preganglionic parasympathetic/sympathetic synapses either directly due to a cellular mechanism, through peripheral tyramine receptor mediated efferent stimulation, or through central nervous system stimulation by crossing the blood-brain barrier.

## 5. Summary

Our previous work has shown that humoral control of cardiovascular function was dominant over parasympathetic control in alligator embryos, which lacked vagal tone at 30 °C (Eme et al., 2011a,b). Here, we confirmed that response and further demonstrated capacity for parasympathetic and sympathetic regulation was developed but not tonically active *in ovo*. Tyramine produced the stereotypical hypertensive tachycardia in young alligators studied, however, embryonic alligators exhibited a unique initial bradycardic hypotension followed by a hypertension. This suggested that in addition to the classical model for tyramine action, which was exclusively adrenergic neuron terminal release of catecholamines, in embryonic alligators tyramine also induced preganglionic neurotransmitter release. The hypertensive response was due to direct action of tyramine on the sympathetic nerve terminals causing hypertension as previously documented in embryonic chickens (Crossley et al., 2003a). Uniquely, tyramine also altered embryonic alligator cardiovascular function *via* stimulation of preganglionic neurotransmitter release, which resulted in increased vagal output reducing heart rate and increased sympathetic output that aided the hypertensive response. Possible routes by which tyramine induced embryonic preganglionic neurotransmitter release include direct action on the preganglionic neuron, a reflex due to peripheral reception of tyramine or through central nervous system stimulation by crossing the blood-brain barrier.

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