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Cardiovascular physiology of embryonic neotropic cormorants (*Phalacrocorax brasilianus*)

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ABSTRACT

Cardiovascular maturation in avian species has primarily been studied in precocial species of birds, with few studies conducted on altricial species, which make up the majority of avian species. In the precocial species of birds studied to date, cardiovascular regulation is derived primarily from an adrenergic receptor stimulation that is present from approximately 50% to 60% of incubation until hatching. Conversely, the cholinergic modulation of heart rate differs in its timing of activation, as it is reported to be present in some studies at 60% of incubation to as late as after hatching in others. This has led to the speculation that, although adrenergic stimulation is critical to cardiovascular homeostasis, cholinergic stimulation prior to hatching in birds is species-specific and therefore is not critical for cardiovascular homeostasis in embryonic birds. In this work, we conducted a series of studies on an altricial species, the neotropic cormorant (*Phalacrocorax brasilianus*), to gain novel data regarding cardiovascular development in a largely unstudied group of birds. We investigated cholinergic and adrenergic receptor mediated control of both arterial blood pressure and heart rate. We predicted that, given the state of this altricial species at hatching, both cholinergic and adrenergic tone on the cardiovascular system would be functional in the embryo. Our findings indicate that cholinergic tone was present at 90% of incubation. However, there was a pronounced adrenergic tone on the cardiovascular system that was relatively greater than that reported in the other studies of avian embryos. Therefore, our findings support our prediction regarding the function of cholinergic tone and adrenergic tone prior to hatching.

1. Introduction

The study of heart rate and arterial blood pressure in embryonic birds has been the focus of multiple investigations during the past several decades (Altimiras and Crossley II, 2000; Andrewartha et al., 2011; Chiba et al., 2004; Crossley II and Altimiras, 2000, 2012; Crossley II et al., 2003a; Crossley II et al., 2003b; Girard, 1973a, 1973b; Höchel et al., 1998; Swart et al., 2014; Taylor et al., 2014; Tazawa et al., 1992; Van Mierop and Bertuch, 1967). Recent studies have reported that adrenergic tone, or continuous stimulation, on heart rate and blood pressure during development is a common characteristic during embryonic life in bird species, suggesting that adrenergic tone may be vital to embryonic cardiovascular function (Andrewartha et al., 2011; Crossley II and Altimiras, 2000, 2012; Crossley II et al., 2003a; Girard, 1973a; Swart et al., 2014; Taylor et al., 2014; Tazawa et al., 1992; Van Mierop and Bertuch, 1967). However, the function of cholinergic tone, a continuous suppression of heart rate, during embryonic development differs across avian species, which has led to speculation that cholinergic tone is not critical to embryonic cardiovascular homeostasis in developing birds (Andrewartha et al., 2011; Chiba et al., 2004; Crossley II and Altimiras, 2000, 2012; Crossley II et al., 2003a; Crossley II et al., 2003b; Höchel et al., 1998; Swart et al., 2014; Taylor et al., 2014; Tazawa et al., 1992). Although prior works have been essential in furthering our understanding of cardiovascular maturation in avian species, questions regarding cardiovascular maturation in altricial species of birds largely remain.

Avian species can be separated into two categories: precocial species, which are readily mobile, with open eyes, able to thermoregulate, and having the ability to self-feed at hatching; or altricial species, which are immobile, with eyes closed, poor thermoregulators, and fed by their parents at hatching (Ar and Yom-Tov, 1978). Although the majority of avian species are altricial, studies of both embryonic arterial blood pressure and heart rate have primarily been conducted in precocial birds, with relatively few studies conducted on altricial birds (Altimiras and Crossley II, 2000; Andrewartha et al., 2011; Ar and Yom-Tov, 1978; Chiba et al., 2004; Crossley II and Altimiras, 2000, 2012; Crossley II

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et al., 2003b; Girard, 1973b; Höchel et al., 1998; Swart et al., 2014; Taylor et al., 2014; Tazawa et al., 1992; Tazawa et al., 1994; Van Mierop and Bertuch, 1967). Expanding our understanding of cardiovascular development in altricial species will provide evidence of the critical components of cardiovascular function that are shared by developing birds.

In domestic chicken (*Gallus gallus domesticus*) embryos, cholinergic and adrenergic receptors are present in the cardiovascular system early in development, along with the anabolic and catabolic enzymes for acetylcholine and catecholamine production (Berry, 1950; Ignarro and Shideman, 1968a, 1968b, 1968c, 1968d). However, studies have also shown that embryonic chickens develop parasympathetic cholinergic efferent nerves that can release acetylcholine on day 12 of the 21-day incubation period, whereas sympathetic efferent nerves are capable of releasing catecholamines on day 21 (Crossley II and Altimiras, 2000; Higgins and Pappano, 1981; Ignarro and Shideman, 1968d; Pappano, 1977). In regards to functional control in embryonic chickens, adrenergic tone is present on day 12 of incubation, derived from circulating catecholamines, while cholinergic tone varies from being functional at 60% of incubation to being absent during embryonic development (Chiba et al., 2004; Crossley II and Altimiras, 2000, 2012; Tazawa et al., 1992).

Comparatively, Canada geese (*Branta canadensis*), domestic geese (*Anser anser domesticus)*, chicken strains (*Gallus*), emus (*Dromaius novaehollandiae*), and Pekin ducks (*Anas platyrhynchos domestica*) possess both beta-and-alpha adrenergic receptor tone on the cardiovascular system minimally by 70% of incubation (Crossley II and Altimiras, 2000, 2012; Crossley II et al., 2003a; Sirsat et al., 2018; Swart et al., 2014; Taylor et al., 2014). Thus, adrenergic tone on the cardiovascular system is prominent in all embryonic bird species studied to date. For example, Canada geese (*Branta canadensis*) and domestic geese (*Anser anser domesticus*) possess a beta-adrenergic receptor stimulatory tone on heart rate and alpha-adrenergic tone on arterial pressure at 70% and 90% incubation (Swart et al., 2014) a common characteristic in the bird species studied to date. However, the presence of cholinergic receptor tone varies across species from being absent, to becoming functional prior to hatching (Andrewartha et al., 2011; Crossley II and Altimiras, 2000; Higgins and Pappano, 1981; Swart et al., 2014; Taylor et al., 2014). For example, an inhibitory cholinergic tone on heart rate has been reported in two embryonic geese species at 70% and 90% of incubation (Swart et al., 2014). Cholinergic tone on heart rate has also been reported in embryonic emus (*Dromaius novaehollandiae*) (Crossley II et al., 2003a), red jungle fowl chickens (*Gallus gallus*) (Crossley II and Altimiras, 2012), and Pekin ducks (*Anas platyrhynchos domestica*) (Sirsat et al., 2018) prior to hatching. However, the reports of cholinergic tone in the white leghorn chicken vary, with studies reporting cholinergic tone on heart rate becoming functional as early as at 60% of incubation to as late as post-hatching (Chiba et al., 2004; Crossley II and Altimiras, 2000, 2012; Taylor et al., 2014; Tazawa et al., 1992). While questions remain regarding the role of cholinergic tone in precocial species of birds, the function of cholinergic and adrenergic tones on the cardiovascular system have only been investigated in precocial birds and are largely unknown in altricial species. Due to common traits for adrenergic tone and differences in cholinergic tone in embryonic avian species, an investigation conducted on altricial species may provide insight regarding common traits in the ontogeny of cardiovascular regulation in birds.

The goal of this study was to characterize the maturation of cardiovascular function and to determine if cholinergic and adrenergic tones are present in embryos of the neotropic cormorant (*Phalacrocorax brasilianus*), an altricial species. To provide data for future studies we measured body mass, organ masses in this species. Altricial species, including the neotropic cormorant, hatch at an earlier stage of development compared to precocial species, potentially making them more vulnerable to fluctuations in the environment (Ar and Yom-Tov, 1978). Therefore, cardiovascular regulatory mechanisms may be more pronounced in altricial species when compared to precocial species. Based on this assumption, we predicted that baseline f_H would be maintained by a tonic cholinergic and adrenergic stimulation during incubation.

2. Methods and materials

2.1. Species of study

We collected eggs of neotropic cormorants (*Phalacrocorax brasilianus*) from Henderson County, Texas, and Freestone County, Texas, during 2015 and 2016 in the months of May through July, with permission using a Texas Fish and Wildlife Scientific Collection permit (SPR-1114-257) and a U.S. Federal Fish and Wildlife Migratory Bird Scientific Collecting permit (MB57014B-1). Egg collection in Henderson County took place in the Bird Island Wildlife Management Area (WMA) within the Cedar Creek Reservoir, and in Freestone County within a wetland in Richland Creek WMA, North Sector, along the banks of Alligator Creek. A total of 100 eggs from 50 nests were collected during the 2015 breeding season and a total of 150 eggs from 56 nests were collected during the 2016 breeding season from the Henderson County Bird Island WMA. A total of 50 eggs from 16 nests were collected during the 2015 breeding season from Freestone County Richland Creek WMA. Upon collection, the eggs were immediately floated (Westerskov, 1950) to estimate the age of incubation. Once the eggs were collected from the nest, they were placed in egg cartons and immediately placed within a Coleman Cooler (Coleman Company, Inc., Wichita, KS, USA). Eggs were transported directly to the University of North Texas by car. Total time between egg collection from the nest to placement in an incubator at the University of North Texas was <3 h. Upon arrival at the University of North Texas, eggs were candled for a more accurate estimation of the incubation period (Hanson, 1954; Reiter and Andersen, 2008; Westerskov, 1950). A total of 300 eggs were collected from both sites during the study period.

2.2. Egg incubation

Eggs were placed in a Grumbach incubator (Grumbach model BSS 160 Digital, Grumbach Brugeraete GmbH., Asslar, Germany) programmed to maintain a constant temperature (38 °C) and humidity (60%), and eggs were rotated 45° every two hours, similar to the incubation conditions described for the double-crested cormorant, *Nannopterum auritum* (Powell et al., 1996). Eggs collected from Bird Island WMA during the 2015 breeding season were set in the incubator at a vertical position with the air cell facing upwards and were 49% ($n = 100$ eggs) viable, while eggs collected during the 2016 breeding season were set in the incubator horizontally the air cell on the side and were 65% ($n = 150$ eggs) viable. The viability of eggs collected from Richland Creek WMA was 80% ($n = 50$ eggs).

2.3. Estimates of embryonic mass and blood volume

For the purpose of this study, blood volume and embryonic body mass of the neotropic cormorant were estimated to determine drug injection volumes and concentrations to be administered to the study animals. Body mass of the embryonic neotropic cormorant was estimated based on the known masses on each incubation day of altricial embryonic pigeons (*Columba livia*) and hooded crows (*Corvus cornix*) (Romanoff, 1967) from similar-sized eggs. The average wet weight for both species for each incubation percentage relative to the post-hatching mass was used to estimate the embryonic mass at each incubation day in the neotropic cormorant. A sigmoidal equation was used to predict the average wet weight at each incubation percent of the neotropic cormorant (Table 1). The blood volume of neotropic cormorants was estimated based on previously reported blood volumes of embryonic chickens (*Gallus gallus*) at similar masses (Crossley II and Altimiras,

Table 1
Embryonic neotropic cormorant actual mass (Emb) and estimated (Est) wet mass on the incubation day (Day) and incubation percentage (Inc). Sample size for actual embryonic mass is given in parentheses. Actual embryonic masses are presented as mean values ±SEM. Total sample size of viable embryos was 167.

Day	Incubation (%)	Embryo mass (g)	Estimated Mass (g)
16	61.5 (4)	2.67 ± 0.15	3.65
17	65.3 (10)	3.77 ± 0.17	4.69
18	69.9 (21)	5.61 ± 0.32	5.95
19	73 (24)	7.10 ± 0.25	7.41
20	76.9 (16)	8.70 ± 0.21	9.05
21	80.7 (11)	10.31 ± 0.22	10.83
22	84.6 (9)	11.30 ± 0.24	12.68
23	88.4 (38)	12.75 ± 0.26	14.56
24	92.3 (19)	16.09 ± 0.55	16.41
25	96.1 (10)	19.73 ± 0.49	18.18
26	100 (5)	21.66 ± 0.82	19.83

2000).

2.4. Surgical procedures

Starting at day 16 a subset of viable eggs were used for to determine embryonic mass measurements while P_M and $f_{H,}$ measurements were taken in embryos at in 70% an 90% of incubation interval. To complete the physiological measurements, eggs were taken from the incubator and candled to locate a tertiary artery of the chorioallantoic membrane. Once the artery was located, the eggs were placed in a custom-built temperature-controlled (38 °C) surgical chamber. Under a dissection microscope (Leica MZ6 Leica Microsystems, Waukegan, Il, USA) within the chamber, a 10 mm × 10 mm opening was made in the shell using a 20 g needle. Once the artery was exposed, the artery was catheterized with a heat-pulled polyethylene catheter (PE 50; Clay-Adams, Parsippany, NJ, USA) containing heparinized (100 IU) saline at 0.9% NaCl (Sagent Pharmaceuticals, Schaumbug, IL, USA). Once the catheter was in place, a piece of 6–0 silk suture secured the catheter to the vessel, and the catheter was glued to the eggshell with cyanoacrylate glue.

After catheterization, the eggs were placed in a temperature-controlled experimental apparatus set at 38 °C containing six separate water-jacketed holding cells. Temperature was maintained using a recirculating constant-temperature water bath (VWR International, LLC, West Chester, PA, USA). Each individual chamber was fitted with a steel lid with two 6 mm diameter holes for externalizing the catheter and to supply the chamber with air. Air continuously passed through the chamber after passing through a humidifier and then through a copper coil submerged in a water bath kept at 38 °C. Each chamber in the experimental apparatus was continuously supplied with air at a flow rate of 200 mL min^{-1}. The catheter was attached to a pressure transducer (MLT0670 BP, ADInstruments, Colorado Springs, CO, USA), which was connected to a bridge amplifier (ML224 Quad amp, ADInstruments, Colorado Springs, CO, USA). The amplifier was connected to a data acquisition system (Powerlab 16/30, ADInstruments, Colorado Springs, CO, USA), and the signal was acquired on a computer (Mac Mini, Apple Incorporated, Cupertino, CA, USA) with acquisition software (LabChart 7, ADInstruments, Colorado Springs, CO, USA) at a frequency of 100 Hz. Heart rate was derived from the blood pressure signal. Prior to experimentation, the pressure transducers were calibrated against a static vertical column of saline (0.9% NaCl). The zero point was set at the top of the experimental chamber. Because floating and candling provided only rough estimates of incubation time, for the physiological measurements eggs were grouped into incubation windows: 70% of incubation (days 18–20), and 90% of incubation (days 23–25) for the purposes of the study.

Experiment: We studied embryos at 70% and 90% of incubation to determine the function of cholinergic and adrenergic tone on P_M and f_H to mirror prior studies of embryonic birds for the purposes of future comparison. Prior to each drug injection, the P_M and f_H response to a control injection of heparinized (100 IU) saline (0.9% NaCl) <5% of the estimated total blood volume was measured in embryos in the 70% (n = 34) and in the 90% (n = 30) of incubation interval. This protocol was carried out only on the embryos that stabilized during the 1-h post-surgical period, to measure any possible effects of the injection volume alone. Of these, a subset of embryos were injected with either atropine (3 mg kg^{-1}) to assess the response to cholinergic receptor blockade (sample size at 70% was n = 8 and at 90% was n = 8) or a series of two drugs to assess the response to beta-adrenergic receptor blockade with 3 mg kg^{-1}propranolol (sample size at 70% was n = 19 and at and 90% was n = 20), followed by an injection of the alpha-adrenergic receptor blocker phentolamine (3 mg kg^{-1}) (sample size at 70% was n = 14 and at 90% was n = 13). Sample size decreased between the two adrenergic blockers because some embryos injected with phentolamine failed to reach stable values as f_H rapidly declined. These embryos were eliminated from the phentolamine effect analysis. Drugs were purchased from Sigma-Aldric, St. Louis, MO, USA. Drug concentrations were selected based on prior studies of avian embryos (Crossley II and Altimiras, 2000, 2012; Swart et al., 2014). After each injection, parameters were allowed to stabilize for 45–60 min. Total injection volumes were < 5% of total estimated blood volume.

In all cases, embryos were euthanized with an overdose injection of pentobarbital. Embryonic mass was then measured, and the embryos in the 70% and 90% incubation interval were dissected to determine organ masses.

2.5. Morphological data

Embryonic mass was determined for all incubation days, starting at day 16 (Table 1). Masses of the heart, liver, lungs, kidneys, and brain were measured at 70% and 90% of incubation. Masses were measured using a balance (Mettler Toledo XS204, CH-8606 Greifensee, Switzerland). Due to collection errors for the group at 70% of incubation, yolk masses were determined only for embryos at 90% of incubation. Embryos were aged by comparing their physical features to those previously recorded for embryonic double-crested cormorants (*Nannopterum auritum)* (Powell et al., 1998). The sample size for each tissue is presented in Table 2.

2.6. Statistical analysis

All statistical significance differences were determined based on $p \leq 0.05$. Paired *t*-tests were used to compare P_M and f_H before and after the saline and each drug injection in the groups in the 70% and 90% of incubation intervals. A one-way ANOVA was used to analyze egg and embryonic mass differences, comparing the embryos at 70% and 90% of incubation intervals. An ANCOVA, with embryonic mass used as a covariate, was used to compare the organ masses between the two points of incubation studied. All statistical analyses were conducted using the software program Statistica (Statistica v13; StatSoft, Tulsa, OK, USA).

3. Results

3.1. P_M and f_H response to injections

Saline injections equal to the volume given for each drug injection increased P_M slightly (+0.06 kPa) but significantly in embryos in the 70% of incubation interval (paired t-test $p \leq$ 1.16E-06) and in the embryos in the 90% of incubation (+0.07 kPa) interval (paired t-test $p \leq$ 0.01), without changing f_H at either time points (Fig. 1A and B).

3.2. Cholinergic blockade

Injection of 3 mg kg^{-1} of atropine had no effect on P_M in embryos in either the 70% or 90% of incubation interval (Fig. 2A). Cholinergic blockade in embryos in the 90% interval resulted in a significant (paired

Table 2
Mass of the egg, yolk, embryo, heart, liver, lung, kidney, and brain of neotropic cormorants in the incubation interval (Inc) of 70% (days 18–20) and 90% (days 23–25). An asterisk indicates significant differences between incubation intervals in embryonic mass based on the one-way ANOVA which was used to analyze the differences in egg mass and embryonic mass. A double asterisk indicates a significant difference between incubation intervals based on the ANCOVA with embryonic mass as the covariate which was used to determine if there were differences between organ mass that was not simply due to increasing embryonic mass. Sample size is given in parentheses. Data are presented as mean values ± SEM. In all cases, significant effects are designated as $p \leq 0.05$.

Inc. (%)	Egg (g)	Yolk(g)	Embryo (g)	Heart (g)	Liver (g)	Lung (g)	Kidney (g)	Brain (g)
70	30.09 ± 0.40(30)		5.73 ± 0.27	0.044 ± 0.003	0.093 ± 0.008	0.106 ± 0.006	0.057 ± 0.005	0.308 ± 0.016
90	31.35 ± 0.65(24)	4.31 ± 0.22	15.16 ± 0.73*	0.105 ± 0.004**	0.309 ± 0.019	0.195 ± 0.011(21)	0.229 ± 0.017(21)	0.527 ± 0.016(20)

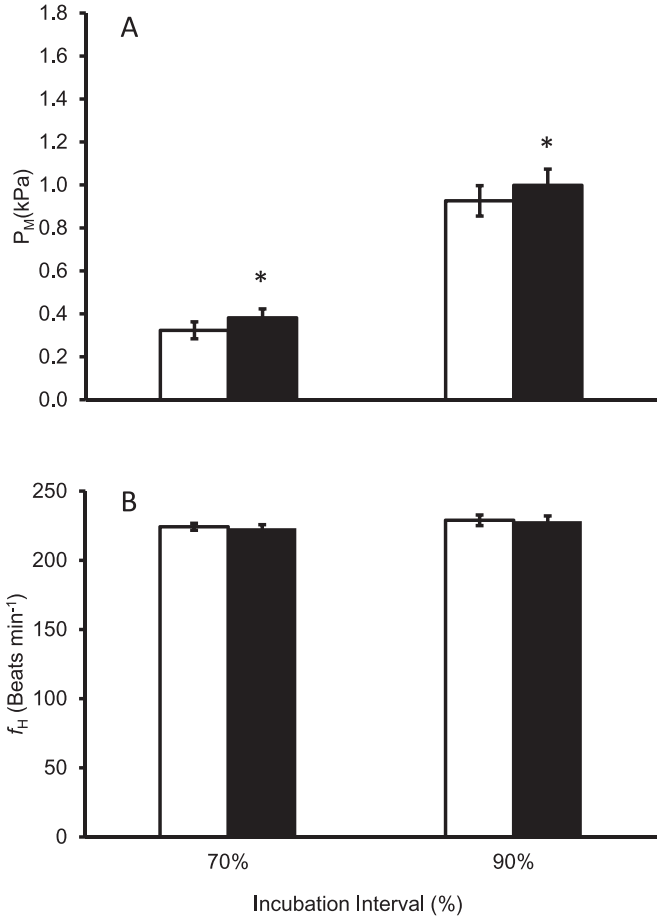

Fig. 1. Embryonic neotropic cormorant mean arterial pressure, P_M (A) and heart rate, f_H (B) before (open column) and after (filled column) an injection of saline. An asterisk indicates a significant response. Sample size was $n = 35$ at 70% of incubation and $n = 31$ at 90% of incubation. Data are presented as mean ± SEM.

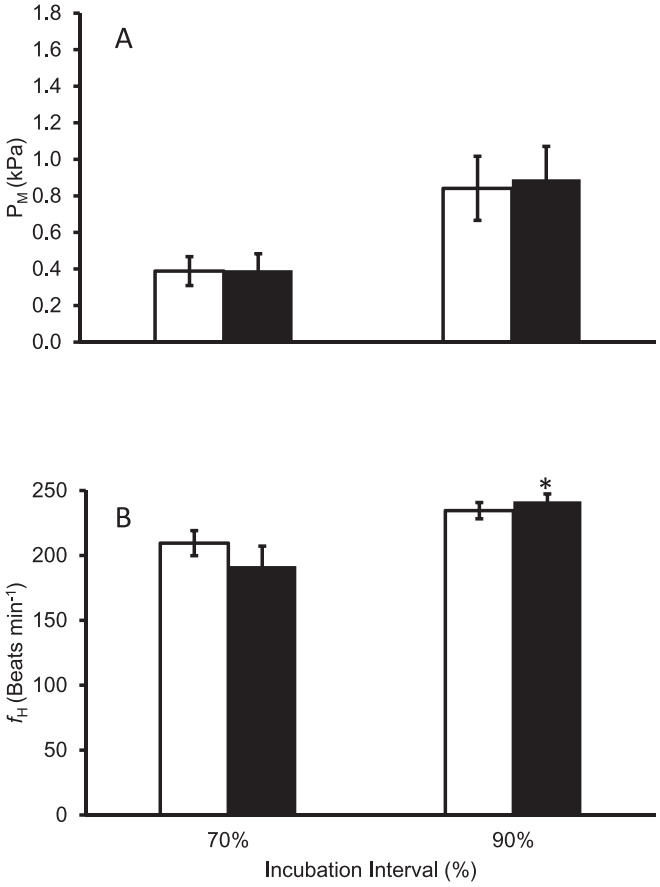

Fig. 2. Embryonic neotropic cormorant mean arterial pressure, P_M (A) and heart rate, f_H (B) before (open column) and after (filled column) an injection of 3 mg kg^{-1} atropine. An asterisk indicates a significant response to the injection. Sample size was n = 8 at 70% of incubation, and $n = 8$ at 90% of incubation. Data are presented as mean ± SEM.

t-test p ≤ 0.01) increase in f_H from 236 ± 6 beats min^{-1} to 242 ± 7 beats min^{-1} (Fig. 2B).

3.3. Adrenergic blockade

A beta-adrenergic blockade with 3 mg kg^{-1} of propranolol increased P_M and decreased f_H in embryos at 70% and 90% of incubation (Fig. 3A and B). At 70%, P_M significantly (paired t-test $p \leq 2.80E-06$) increased from 0.27 ± 0.04 kPa to 0.56 ± 0.07 kPa (Fig. 3A), while f_H decreased significantly (paired t-test $p \leq 4.21E-09$) from 227 ± 3 beats min^{-1} to 178 ± 6 beats min^{-1} (Fig. 3B). At 90%, P_M increased significantly (paired t-test $p \leq 1.01E-06$) from 0.87 ± 0.07 kPa to 1.45 ± 0.11 kPa (Fig. 3A) and f_H decreased significantly (paired t-test $p \leq 5.70E-14$) from 231 ± 3 beats min^{-1} to 165 ± 5 beats min^{-1} (Fig. 3B). These changes in P_M were equivalent to an approximately 158 ± 47% increase in the group at 70% of incubation and an 85.5 ± 19.4% increase in the group at 90% of incubation. The changes in f_H in response to the propranolol injection were equivalent to a 22.3 ± 2.3% and a 29.0 ± 1.5% decrease at 70% and 90% of incubation, respectively.

The alpha-adrenergic blockade with 3 mg kg^{-1} of phentolamine decreased P_M in embryos in both the 70% and 90% of incubation intervals without affecting f_H (Fig. 3C and D). In the 70% of incubation interval embryos, phentolamine significantly (paired t-test p ≤ 0.01) decreased P_M from 0.62 ± 0.07 kPa to 0.31 ± 0.06 kPa (Fig. 3C), and at 90% of incubation, P_M significantly (paired t-test $p \leq 3.15E-08$) decreased from 1.49 ± 0.08 kPa to 0.49 ± 0.06 kPa (Fig. 3C). These reductions in P_M were equivalent to a 48.9 ± 9.0% and a 66.5 ± 5.1% decrease in the 70% and 90% of incubation interval embryos respectively (Fig. 3C).

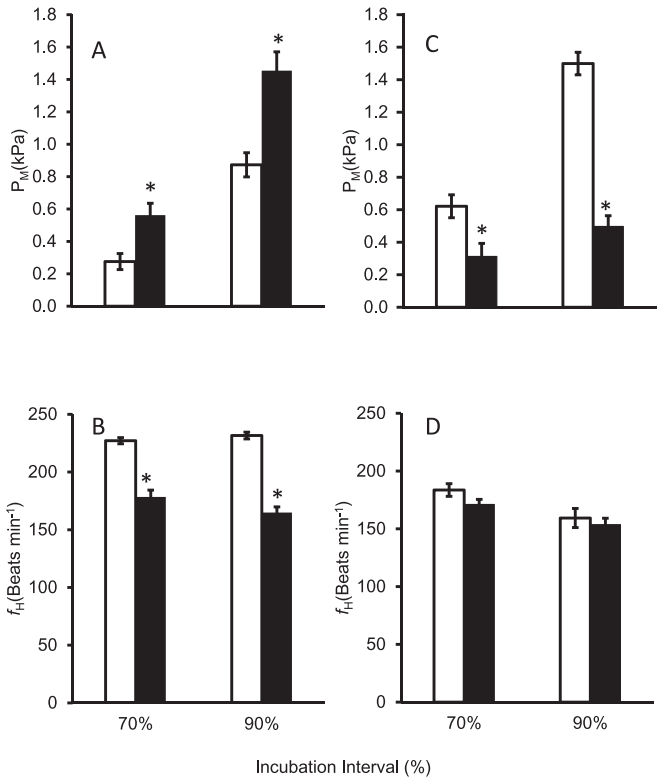

Fig. 3. Embryonic neotropic cormorant mean arterial pressure, P_M and heart rate, f_H before (open column) and after (filled column) an injection of 3 mg kg^{-1} propranolol (A and B respectively) followed by before (open column) and after (filled column) phentolamine (C and D respectively). An asterisk indicates a significant response to each injection. Sample size for propranolol injections was $n = 19$ at 70% of incubation and $n = 20$ at 90% of incubation. Sample size for phentolamine injections was $n = 14$ at 70% of incubation and $n = 13$ at 90% of incubation. Data are presented as mean ± SEM.

3.4. Morphological features

Egg mass was similar at the two points of development studied. Embryonic mass increased significantly (F value = 170) from 5.73 ± 0.27 g in the 70% of incubation interval to 15.16 ± 0.73 g in the 90% interval (Table 2). Masses of the heart, liver, lungs, kidneys, and brain also increased from 70% of incubation to 90% of incubation. However, using embryonic mass as a covariate, there was no significant difference in any of the organs measured, with the exception of the heart. Heart mass was significantly different (F value = 4.79) between the incubation intervals and decreased in percentage of embryonic mass from 0.79 ± 0.05 to 0.70 ± 0.02% between the 70% and 90% incubation intervals (Table 2).

4. Discussion

To date, the majority of cardiovascular studies of avian development have focused on precocial species of birds. Our findings represent novel measurements of both P_M and f_H in an altricial species, the neotropic cormorant. Further, our findings revealed that, although P_M rises from 60% of incubation to 90% of incubation in this species, f_H remains relatively constant. In addition, at the two incubation intervals studied, adrenergic tone was prominent. This finding is similar to that reported in precocial bird species, and it suggests that adrenergic tone is critical for cardiovascular homeostasis in embryonic birds. (Altimiras and Crossley II, 2000; Andrewartha et al., 2011; Crossley II and Altimiras, 2000, 2012; Crossley II et al., 2003a; Girard, 1973a; Swart et al., 2014; Taylor et al., 2014; Tazawa et al., 1992; Van Mierop and Bertuch, 1967). However, our prediction that baseline f_H would be maintained by a prominent cholinergic tone at both points of incubation studied was not supported by the data, as it was only weakly present at 90% of incubation. This suggests that the function of cholinergic tone on f_H prior to hatching is species-specific.

4.1. Response to drug injections

Cholinergic tone on the heart was only present at 90% of incubation and was minimal; injection of atropine only increased f_H by approximately 3% in the neotropic cormorant (Fig. 2A and B). Based on the current study and past works, the presence or absence of cholinergic tone on f_H may be dependent several factors including the studied species, the study temperature, and study methods, as cholinergic tone has been reported to be both functional and nonfunctional prior to hatching even within the same strain of domestic chicken (Andrewartha et al., 2011; Chiba et al., 2004; Crossley II and Altimiras, 2012; Crossley II et al., 2003a; Swart et al., 2014; Taylor et al., 2014).

4.2. beta-adrenergic and alpha-adrenergic tones

Adrenergic antagonist injections caused pronounced changes at 70% and 90% of incubation in the neotropic cormorant. Focusing on the effects of the beta-adrenergic antagonist propranolol, injections caused a substantial hypertension (Fig. 3A) at both points of incubation studied, resulting in an approximately 104% and 67% increase from pre-injection values in P_M at 70% and 90% of incubation, respectively (Table 3). The propranolol injection also resulted in a pronounced decrease in f_H in both 70% and 90% incubated embryos (Fig. 3B). These findings illustrate that there is a clear stimulatory beta-adrenergic tone on f_H and, given the intensity of the P_M response, suggest there is also a vasodilatory tone on P_M as well (Fig. 3A). In prior studies of avian species, beta-adrenergic tone on the cardiovascular system has been consistently reported. Our current finding agrees with multiple studies that have also reported a beta-adrenergic tone maintaining both basal f_H and P_M across multiple embryonic avian species, suggesting that beta-adrenergic tone is critical for embryonic cardiovascular homeostasis (Altimiras and Crossley II, 2000; Andrewartha et al., 2011; Crossley II and Altimiras, 2000, 2012; Girard, 1973a; Swart et al., 2014; Taylor et al., 2014; Tazawa et al., 1992; Van Mierop and Bertuch, 1967).

The origins of the beta-adrenergic tone in the neotropic cormorant embryo were not explored in the current study. However, prior investigations of avian species have determined that, although the sympathetic nervous system is potentially functional, the beta-adrenergic tone is derived from circulating catecholamines (Andrewartha et al., 2011; Crossley II and Altimiras, 2000; Higgins and Pappano, 1981; Swart et al., 2014; Taylor et al., 2014). Further, an increase in beta-adrenergic tone on f_H with development has been reported in species of geese embryos and may be attributed to an increase in circulating levels of catecholamines (Crossley II and Altimiras, 2000; Swart et al., 2014). Interestingly, although the percentage changes in the response to beta -adrenergic blockade were not statistically tested in the current study, the tone on f_H was relatively stable, 21% versus 28% from pre-injection values at 70% and 90% of incubation, respectively, while the tone on P_M appeared to decrease (Table 3). Although the basis for this change in the neotropic cormorant was not tested, it may represent a transition of the beta-tone on P_M as development progresses in this species. However, the source of adrenergic stimulation was not investigated and further study is needed to determine the origin.

Unlike the beta-adrenergic tone on both f_H and P_M in the neotropic cormorant, alpha-adrenergic tone was only present on P_M (Fig. 3C). The strength of this tone appeared to be greater than in other species previously studied (Table 3) at both intervals of development studied. In particular, the alpha-tone on P_M in the neotropic cormorant embryos in the 70% of incubation interval appeared to be markedly greater than in the other species studied to date (Table 3), suggesting a greater

Table 3
Comparison of the P_M (kPa) response to propranolol (Prop) and phentolamine (Phento) in embryonic birds at 70% and 90% of incubation (Inc). Values are taken from the current study and either estimated or stated values from prior studies. The values for the neotropic cormorant (*P. brasilianus*), chicken strains (*G. gallus*) white leghorn chicken (WLH), broiler chicken (B) Red Jungle Fowl (RJF), Pekin duck (*A. platyrhynchos domesticus*), domestic Goose (*A. anser domesticus*), Canada goose (*B. canadensis*), and Emu (*D. novaehollandiae*) (Crossley II and Altimiras, 2000, 2012; Crossley II et al., 2003a; Sirsat et al., 2018; Swart et al., 2014) are included.

Inc. (%)	Species	PreProp P_M	PostProp P_M	PropP_M%Δ	PrePhento P_M	PostPhento P_M	Phento P_M %Δ	Source
70	*P. brasilianus*	0.28	0.56	104.0	0.62	0.31	−49.69	Current study
	WLH *G. gallus*	1.60	1.63	1.9	1.62	1.27	−21.60	Crossley II and Altimiras, 2000
	A. anser domesticus	1.25	1.38	10.4	1.38	1.22	−11.59	Swart et al., 2014
	B. canadensis	1.30	1.46	12.3	1.46	1.25	−14.38	Swart et al., 2014
	D. novaehollandiae	1.90	2.05	7.9	2.05	1.70	−17.07	Crossley II et al., 2003a
90	*P. brasilianus*	0.87	1.45	66.6	1.49	0.49	−67.11	Current study
	WLH *G. gallus*	2.60, 2.00	2.95, 2.60	13.5, 29.0	2.95, 2.60	1.75, 1.90	−40.68, −30.2	Crossley II and Altimiras, 2000, 2012
	B *G. gallus*	3.25	3.99	21.9	3.14	2.36	−24.5	Crossley II and Altimiras, 2012
	RJF	2.90	3.60	24.1	3.30	2.20	−31.9	Crossley II and Altimiras, 2012
	A. anser domesticus	2.40	2.49	3.75	2.49	1.98	−20.48	Swart et al., 2014
	B. canadensis	2.55	2.65	3.92	2.65	2.23	−15.85	Swart et al., 2014
	D. novaehollandiae	2.55	3.45	35.29	3.45	1.95	−43.48	Crossley II et al., 2003a
	A. platyrhynchos domesticus	2.50	2.74	9.6	2.41	1.12	−53.5	Sirsat et al., 2018

dependence on circulating catecholamines to maintaining P_M. Prior studies of embryonic domestic chickens reported an alpha-tone on f_H (Crossley II and Altimiras, 2000). Similar findings were reported in two species of embryonic geese and embryonic emus that responded to alpha-adrenergic blockade with a clear bradycardia (Swart et al., 2014; Crossley II et al., 2003a). Although speculative, the differences between prior studies and the current work may be based on a difference between precocial versus altricial species of embryos.

4.3. Cholinergic and adrenergic tone within precocial and altricial species

Our findings demonstrate that adrenergic tone is a main factor in maintaining baseline f_H and P_m in embryos at the 70% and 90% of incubation intervals in neotropic cormorants. This finding regarding adrenergic tone in the altricial neotropic cormorant is similar to findings of studies conducted on precocial species (Andrewartha et al., 2011; Crossley II and Altimiras, 2000, 2012; Crossley II et al., 2003a; Girard, 1973a; Swart et al., 2014; Taylor et al., 2014; Tazawa et al., 1992; Van Mierop and Bertuch, 1967), even though altricial species hatch at a relatively early stage of development when compared to precocial species. The identification of adrenergic tone on the cardiovascular system at 70% and 90% incubation in multiple precocial embryonic birds, and now in the altricial neotropic cormorant as well, indicates that the maturation of adrenergic tone on f_H and P_m may be critical to maintain cardiovascular function prior to hatching.

5. Summary

Neotropic cormorant embryos at 70% and 90% of incubation possessed a strong beta-adrenergic tone on P_M and f_H. Neotropic cormorant embryos also showed a continuous alpha-adrenergic tone on P_m. Therefore, our findings support the prediction that adrenergic tone maintains baseline P_M and f_H in the embryonic neotropic cormorant. In regard to cholinergic tone, our predication that baseline f_H would be maintained by tonic cholinergic stimulation is partially supported. Although cholinergic tone was not shown in embryos at 70% of incubation, a slight cholinergic tone on f_H was identified in 90% incubated embryos. Our findings provide further evidence that adrenergic regulation is critical for maintaining homeostasis of the cardiovascular system during embryonic avian development.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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