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Cardiovascular responses of embryonic alligator (*Alligator mississippiensis*) exposed to 10% O_2 and sodium cyanide (NaCN), a chemoreflex-inducing compound

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ABSTRACT

The possibly interactive effects of changes in atmospheric respiratory gases (hypoxia or hypercapnia) *and* pharmacological chemoreceptor stimulation have not been assessed previously. We present a series of experimental protocols investigating embryonic alligators' capacity to modulate a cardiovascular neural chemoreflex response to a known chemoreceptor stimulant, sodium cyanide (NaCN). We incubated alligator embryos in 21 % (normoxia) and 10 % O_2 (hypoxia) beginning at 20 % of embryonic incubation, and at 70 % and 90 % of incubation we measured heart rate and blood pressure responses to NaCN. These NaCN responses also included examining the effects of NaCN after 1-h exposure to 10 % O_2., ganglionic blockade with hexamethonium chloride and α-adrenergic blockade with phentolamine. Injections of NaCN into the chorioallantoic artery caused a rapid bradycardia followed by a secondary hypertension, which can be attributed to an autonomic nervous system mediated reflex loop. We compared the heart rate response to injections of 1 mg kg^{-1} NaCN before and after a 1-h 10 % O_2 exposure, and it was clear that embryonic alligators lacked capacity to change the intensity of cardiovascular responses to this compound. Hexamethonium greatly lessened the rapid bradycardia, and at 90 % of incubation, the secondary hypertensive response to NaCN appeared due to α-adrenergic stimulation, as phentolamine lessened the response. Collectively, data indicate that while a cardiovascular chemoreflex can be induced by NaCN, the heart rate response lacks plasticity and is not modulated by hypoxic incubation in embryonic alligators.

1. Introduction

The development and plasticity of cardiovascular physiology of embryonic American alligators (*Alligator mississippiensis*) has been well-studied, particularly in response to low levels of oxygen (e.g., Crossley II and Altimiras, 2005; Eme et al., 2011a, 2011b; Eme et al., 2011b; Eme et al., 2013). Naturally occurring hypercapnic (high carbon dioxide) and hypoxic (low oxygen) conditions have been measured in reptilian and megapode birds species that lay eggs in underground or mound nests. Nest saturation due to rainfall, increased microorganisms metabolism, and increased embryonic metabolism in the nest can also cause hypercapnia or hypoxia (Lutz and Dunbar-Cooper, 1984, Booth, 1998). Crocodilians typically lay eggs in large mound nests, and in nests of the American crocodile (*Crocodylus acutus*) and American alligator (*Alligator mississippiensis*) CO_2 levels of ~2–8.5 % (18–65 Torr) and O_2 levels of 11–15 % have been recorded during incubation, likely due to high microorganism respiration (Ackerman, 1980; Lutz and Dunbar-Cooper, 1984; Miller, 2008; Grigg et al., 2010). Modulation of reflexive cardiovascular control has been previously documented for multiple adult reptile species during various environmental or physiological changes, such as responses to feeding in common boa (*Boa constrictor*) and Green iguana (*Iguana iguana*) (Guagnoni et al., 2024; Wang et al., 2021), responses to temperature change in South American rattlesnake (*Crotalus durissus*) and Caiman (*Caiman latirostris*) (Filogonio et al., 2021; Hagensen et al., 2010), and responses to exercise in South American rattlesnake and mammals (Filogonio et al., 2021; Raven et al., 2006). In

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these cases, the focus was on assessing baroreflex control of f_H in each animal model. In response to changes from a control or resting state, either the baroreflex was desensitized or the experimental manipulation did not alter this reflexive control loop.

Studies investigating the modulation of a cardiovascular chemoreflex have focused on mammals (Spicuzza et al., 2005), with data for fish as well. In response to exercise, humans exhibit an increase in chemoreflex sensitivity when studied using hypoxia as a stimulation of the chemoreceptors (Wan et al., 2020). The chemoreflex has also been studied extensively in fish species, please refer to this review (Gilmour and Perry, 2006). In response to chemoreceptor stimulation, the cardiovascular response in one species of fish, the Pacu (*Piaractus mesopotamicu*), involves a rapid bradycardia in response to 1.0 mg kg^{-1} NaCN (Leite et al., 2007). A similar response has been reported in fish, the gulf toadfish (*Opsanus beta*) (McDonald et al., 2010) and the neotenous axolotl (*Ambystoma mexicanum*) (McKenzie and Taylor, 1996). However, the combined effects of hypoxia or hypercapnia with sodium cyanide (NaCN) pharmacology has not been assessed previously.

Pharmacological stimulation of chemoreceptors can be conducted to assess physiological mechanisms that support responses to environmental respiratory gases (oxygen and carbon dioxide) and blood gases. A NaCN-stimulated cardiovascular chemoreflex in embryonic reptiles has been previously investigated in one species, the common snapping turtle (*Chelydra serpentina*), with NaCN injection producing a rapid dose dependent bradycardia coupled to hypotension at higher doses (Eme et al., 2021). In both American alligator and common snapping turtle, acute hypoxia causes a rapid bradycardia not mediated by increases in parasympathetic motor output (Crossley II and Altimiras, 2005; Eme et al., 2011a; Eme et al., 2011b; Eme et al., 2021). Surprisingly, while embryonic snapping turtle possess parasympathetic tone on f_H during the final 30 % of incubation, the hypoxic bradycardia does not appear to be mediated by changes in autonomic tone on the heart suggesting oxygen's direct action on cardiomyocyte contractility. Embryonic alligators lack both parasympathetic and sympathetic tone on f_H through 90 % of incubation, and the hypoxic bradycardia also seems to be driven by the direct action of oxygen level on cardiomyocyte contractility (Crossley II and Altimiras, 2005; Eme et al., 2011a; Eme et al., 2013). Interestingly, embryonic alligators do possess a cardiovascular chemoreflex activated by the 5 HT_3 receptor agonist, phenylbiguanide (PBG), suggesting the capacity for (but no resting tone) autonomic nervous system motor output (Eme et al., 2011b). In fetal sheep (*Ovis aries*), NaCN has been used to elicit a cardiovascular chemoreflex that consists of a pronounced bradycardia (Dawes et al., 1969). This response was attributed to aortic and carotid chemoreceptors, as cutting the innervation of these structures abolished the response (Dawes et al., 1969). In dogs (*Canis lupus familiaris*), intracarotid injections of NaCN also produces a marked bradycardia (Gupta and Singh, 1981). In the perfused carotid labyrinth of the African clawed frog (*Xenopus laevis*) outflow of the internal carotid artery decreases in response to NaCN (Kusakabe and Ishii, 1987). Therefore, NaCN is a compound well established to stimulate a cardiovascular neural chemoreflex.

The plasticity of a PBG-induced cardiovascular chemoreflex has been studied by chronically incubating embryonic alligator in hypoxic (10 % O_2) conditions and measuring mean blood pressure (P_m) and heart rate (f_H) responses to PBG (Eme et al., 2011b). Chronic 10 % O_2 incubation reduced the bradycardic response to PBG injection at 95 % of incubation, suggesting the chemoreflex of embryonic alligators is (plastic) blunted following chronic hypoxia with neural tone becoming attuned to lower blood oxygenation (Eme et al., 2011b). It is important to note in Eme et al. (2011b), embryonic alligators were removed from the hypoxic incubation conditions and studied at 21 % O_2. Therefore, questions remain regarding the capacity of embryonic alligators to modulate the chemoreflex response when exposed to low O_2 levels, and cardiovascular effects of additional chemoreflex inducing agents are unknown.

Both PBG and sodium cyanide (NaCN) have been used to study cardiovascular chemoreflex function in embryonic common snapping turtles (*Chelydra serpentina*) (Eme et al., 2021), and NaCN has been used as well in dogs and chickens (*Gallus gallus domesticus*) (Comroe and Mortimer, 1964; Dawes et al., 1969; Magno, 1973). In response to NaCN, embryonic snapping turtles exhibited a dose-dependent hypotensive bradycardia (Eme et al., 2021). Similar to the responses to PBG in embryonic alligator, the embryonic snapping turtles' cardiovascular responses to NaCN appeared mediated by motor output from the parasympathetic nervous system (Eme et al., 2021). Further, unlike the cardiovascular chemoreflex to PBG, the response to NaCN appears to largely lack plasticity as incubation of embryonic snapping turtles in 10 % O_2 did change the f_H response to NaCN (Eme et al., 2021). The capacity of embryonic snapping turtles or alligators to modulate or change the cardiovascular chemoreflex in response to exposure to prolonged hypoxic conditions are unknown.

We designed a series of protocols to determine the capacity of embryonic American alligators to modulate the cardiovascular response to NaCN. First, we determined the cardiovascular response to NaCN alone (*Protocol 1*), then we examined the mechanism of the NaCN response using the ganglionic blocker hexamethonium chloride (*Protocol 2*). We determined the ability of embryonic alligators to modulate NaCN responses by comparing NaCN-injection before and at the end of a 1-h long exposure to 10 % O_2 (*Protocol 3*). Lastly, we examined the NaCN response after hexamethonium chloride and α-adrenergic blockade with phentolamine (*Protocol 4*). We predicted that NaCN would produce a dose dependent change in f_H and P_m that would strengthen with embryonic alligator age, from 70 % to 90 % of incubation. We predicted that incubation in hypoxic conditions (10 % O_2, beginning at 20 % of incubation) would have no effect on cardiovascular responses to NaCN, similar to embryonic turtles (Eme et al., 2021). We predicted that the response to injection of NaCN before and at the end of a 1-h exposure to 10 % O_2 would be greater in at 90 % of total incubation owing to maturation of the cardiovascular system. We predicted that ganglionic blockade with hexamethonium would blunt or eliminate the heart rate response to NaCN, and that α-adrenergic blockade with phentolamine would blunt or eliminate the blood pressure response.

2. Materials and methods

Ten clutches totaling ~400 fertile American alligator (*Alligator mississippiensis*) eggs were collected from wild nests on the Rockefeller Wildlife Refuge in Grand Chenier, LA and transported to the Department of Biological Sciences at the University of North Texas. Eggs were handled as previously described (Crossley II et al., 2017; Eme et al., 2021; Smith et al., 2019), and initial embryonic age was established according to Ferguson (Ferguson, 1985). Representative eggs ($N = 1$ or 2) from each clutch were dissected and the developmental progression of the embryo compared to Ferguson (1985) and to previous images the researchers' possess of properly developing alligator eggs. All eggs were weighed, numbered, and randomly assigned to 1 L Ziplock© boxes containing a vermiculite:water mixture at a ratio of mass of 1:1. The lidless box mass was recorded for each egg box, and water maintained by adding water three times weekly to bring the box to its original mass. Embryos were incubated in a walk-in environmental control room at 30 °C (Percival Scientific, Perry, IA), ensuring that all embryos developed as females to match previous studies (e.g., Crossley II et al., 2017; Eme et al., 2021). At approximately 20 % of incubation (total incubation is 72 days at 30 °C), 50 % of all eggs were randomly assigned to either a 21 % oxygen group (N21) or a 10 % oxygen group (H10). These oxygen (O_2) levels were based on previous studies on the embryonic development of alligators (Crossley II et al., 2003; Galli et al., 2016; Marks et al., 2013; Tate et al., 2016), and the similarity to previous measures from a crocodilian nest (Lutz and Dunbar-Cooper, 1984). To maintain the O_2 level for each treatment, the boxes were placed in 76 L Ziploc© bags connected to a normoxic (21 % O_2) or hypoxic (10 % O_2) air supply. Normoxic air was supplied using an air pump (LT 11 Whitewater, Pentair Aquatic Eco-Systems, NC, USA) passed through a rotameter flow

controller. Hypoxic air was generated by connecting the air flow between two lines using rotameters (Sho Rate, Brooks instruments division, PA, USA) supplied with compressed N_2 or air supplied by an air pump (Whisper AP 300, Tetra products, VA, USA). Normoxic and hypoxic air was humidified using bubbling chambers and delivered to the bags at a rate of 2–4 L min^{-1}. Gas composition was monitored continuously using an O_2 analyzer (S-3AI, AEI Technologies, Bistrop, TX, USA) and recorded on a computer at 1 Hz. All experiments were approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC no. 11–007).

2.1. Surgical instrumentation

At 70 % ($N = 62$) and 90 % ($N = 86$) of incubation, a subset of the total eggs collected were selected from both conditions and successfully instrumented surgically. Prior to instrumentation, eggs were cleaned with water and candled using a fiber optic light source to locate a tertiary artery of the chorioallantoic membrane (CAM). The location of the vessel was marked, and the egg moved to a custom-made temperature control chamber maintained at 30 °C. A 1 cm^2 section of the shell above the vessel was removed, exposing the eggshell membrane. The membrane was carefully removed, exposing the CAM. The CAM artery was isolated under a dissection microscope (Leica MZ6 Leica Microsystems, Waukegan, Il, USA), and the artery was catheterized with a heat-pulled polyethylene catheter (PE 50; Clay-Adams, Parsippany, NJ, USA) filled with 0.9 % NaCl heparinized solution (100 IU) (Sagent Pharmaceuticals, Schaumburg, 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 cannulation, eggs were moved to a water-jacketed stainless-steel six-chamber experimental apparatus. Individual chambers were ~ 700 cm^3 and contained one egg during each experiment. Each chamber was fitted with a lid with 3 holes providing entry for air tubing and the catheter. Chamber temperature was maintained using a circulating water bath (VWR 1165; VWR International, LLC, PA, USA), and incoming air was warmed before being pumped into chambers (400 $mL \bullet min^{-1}$) by passing air through a 2-m copper coil in contact with the apparatus. The catheter was then fitted with a three-way stainless steel 22-gauge port that was connected to disposable pressure transducer (MLT0699 ADInstruments, CO, USA) connected to an amplifier (Octal Bridge, ADInstruments, CO, USA). The pressure signals were acquired at 40 Hz using PowerLab® (ADInstruments, CO, USA) and ChartPro® software (v 8 ADInstruments, CO, USA). Mean f_H and mean blood pressure (P_m) was derived from the arterial pressure (P_a) signal. Prior to experimentation, the pressure transducers were calibrated against a static vertical column of 0.9 % NaCl. The zero point was set at the top of the experimental chamber. P_m values were corrected for the position of the transducer above the embryo using a vertical column of saline (Crossley II et al., 2003). Embryos were then allowed at least 1 h for f_H and P_m to reach stable values before experimentation began. An additional $N = 17$ embryo preparations were unsuccessful, as indicated by a continuous decline in f_H, and were not used.

2.2. Study protocols

All protocols were conducted in normoxia (except *2.5 Protocol 3*) and began by injecting the embryo with a control saline bolus, 0.9 % NaCl, that was equivalent to the volume injected with each drug. All drugs were administered in a 50 μl bolus followed by a 100 μl saline flush. All embryos were allowed to stabilize for 45–60 min before any initial dose of NaCN was administered. For each protocol, $N = 7 \rightarrow 11$ embryos were used at each developmental timepoint (70 % or 90 % of incubation) and incubation condition (H10 or N21).

2.3. Protocol 1: NaCN dose response

In a set of normoxic and hypoxic incubated embryos at 70 and 90 % of incubation, NaCN was injected at increasing doses of 0.1 mg kg^{-1}, 1.0 mg kg^{-1} and 10 mg kg^{-1}. Between each dose, P_m and f_H were allowed to return to pre-injection values before the next dose was administered.

2.4. Protocol 2: NaCN (1.0 mg kg^{-1}) before and after ganglionic blockade with hexamethonium

In a separate set of normoxic and hypoxic incubated embryonic alligator at 70 % and 90 % of incubation, a single dose of 1.0 mg kg^{-1} NaCN was injected. This dose has previously been shown to stimulate O_2 chemoreceptors without disrupting normal mitochondrial function (Sundin et al., 2000). P_m and f_H were allowed to return to pre-injection values before injection of the ganglionic blocker, hexamethonium chloride (25 mg kg^{-1}). Values were allowed to stabilize for 45–60 min before a second dose of 1.0 mg kg^{-1} NaCN was administered.

2.5. Protocol 3: NaCN (1.0 mg kg^{-1}) before and during continuous 10 % O_2

In a separate set of normoxic and hypoxic incubated embryonic alligator at 70 % and 90 % of incubation, 1.0 mg kg^{-1} NaCN was injected before and following a 1-h exposure to 10 % O_2. The protocol consisted of a 1-h stabilization period, then embryos were injected with 1 mg kg^{-1} NaCN, and after cardiovascular parameters had returned to pre injection values, the O_2 was reduced to 10 % in all experimental chambers. The 10 % O_2 mixture was produced using a gas mixer (model 3500 HL, Sechrist Industries Inc., Anaheim, CA) connected to a house airline and a house compressed nitrogen line. O_2 percentage in each chamber was measured with an O_2 analyzer (S-3AI, AEI Technologies, Bistrop, TX, USA) to ensure the chamber reached to target O_2 percentage. Both P_m and f_H stabilized approximately 1 h after the onset of 10 % O_2. The embryos were then injected with a second dose of 1.0 mg kg^{-1} NaCN. This protocol determined the capacity for the embryo to adjust cardiovascular responses to NaCN during acute exposure to 10 % O_2.

2.6. Protocol 4: NaCN (1.0 mg kg^{-1}) before and after ganglionic blockade with hexamethonium and α-adrenergic receptor blockade with phentolamine

In a separate set of normoxic and hypoxic incubated embryonic alligator at 90 % of incubation only, a dose of 1.0 mg kg^{-1} NaCN was injected before and after injection of hexamethonium chloride (25 mg kg^{-1}) and the α-adrenergic receptor antagonist phentolamine (3 mg kg^{-1}). P_m and f_H were allowed to return to pre-injection values before injection of the ganglionic blocker hexamethonium chloride (25 mg kg^{-1}). Values were allowed to stabilize for 45–60 min before injection of phentolamine (3 mg kg^{-1}) was given. Again, values were allowed to stabilize for 45–60 min before a second dose of 1.0 mg kg^{-1} NaCN was administered.

2.7. Measurements and calculations

Control P_m and f_H were determined as the mean values of a 5 min period prior to all treatments. Responses to NaCN were taken as the mean value from between 15 and 30 s of the maximal P_m and f_H response; the maximal bradycardic f_H response occurred within the first 1-min post injection, and the maximal hypertensive response occurred within the first 5 min. The response to each of the blocking agents, hexamethonium and phentolamine, was taken as the mean of a 5 min period just prior to subsequent injections. For the purposes of the continuous 10 % O_2, the response was taken as the mean value of a 5 min period prior to the second NaCN dose.

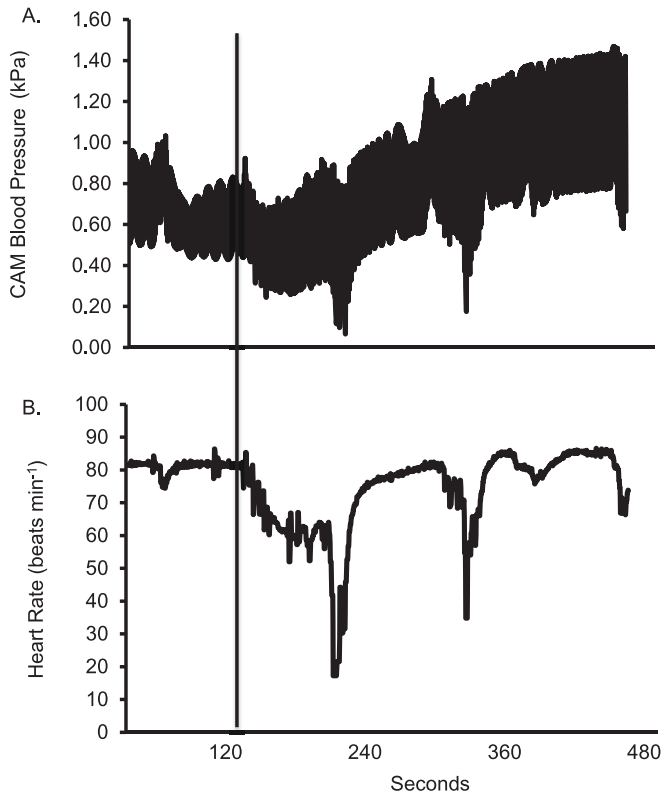

Fig. 1. A representative trace of the CAM arterial blood pressure (A) and heart rate (B) response to an injection of 1.0 mg kg^{-1} sodium cyanide (NaCN) into a CAM vein of an embryonic alligator at 90 % of incubation. The vertical line represents the point of injection. The representative trace is 470 s in length. The response is characterized by the rapid onset of a bradycardia followed by a secondary hypertension followed by a return to pre injection values.

2.8. Statistics

We utilized ANOVA on the relative (percentage) change on arcsine square root transformed values for P_m and f_H. Paired *t*-tests were used to examine whether the drug or hypoxia caused a change in the absolute mean values of P_m and f_H during protocols. The use of ANOVA on relative change, and paired *t*-tests on absolute values is reasonable for our experimental design that includes the final ~1/3 of embryonic incubation. During this time period in all vertebrates, blood pressure rises substantially and heart rate falls, and we wish to compare normoxic embryos (N21) to those for which chronic hypoxia (H10) may cause a consistent hypotensive bradycardia (Eme et al., 2011a, 2011b). Therefore, comparing relative change on P_m and f_H values across incubation condition (N21 vs H10) and development (70 % and 90 %) is prudent as absolute value are so different in magnitude as to wash out any difference between mean values (e.g., Eme et al., 2011a, 2011b; Eme et al., 2021).

The relative percentage change in mean arterial blood pressure (P_m) and heart rate (f_H) responses were compared for NaCN dose response injections (*Protocol 1*; Fig. 2), NaCN injection before and after ganglionic blockade with hexamethonium (*Protocol 2*; Fig. 3), NaCN injection before and after ~1-h exposure to 10 % O_2 (*Protocol 3*; Fig. 5), and NaCN injection before and after hexamethonium *and* α-adrenergic blockade with phentolamine (*Protocol 4*; Fig. 6). Relative changes were arcsine square root transformed prior to a 2-way Repeated Measures (RM)-ANOVA; incubation condition and percentage were the independent variables, and pre- and post-injection for each chemical or pre- and post-hypoxic exposure were the repeated measures. For Fig. 5, only 90 % of incubation embryos were studied, and a 1-way RM ANOVA was used. Significant main effects of the independent variables from the ANOVA

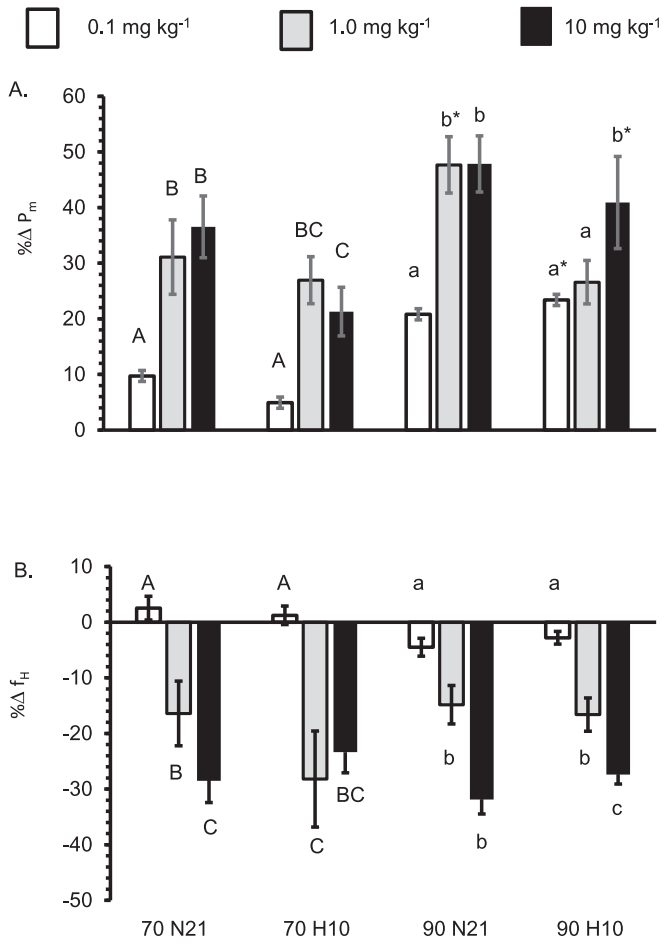

Fig. 2. *Protocol 1*: The percentage change in mean arterial pressure (A; $\%\Delta P_m$) and heart rate (B; $\%\Delta f_H$) in response to sodium cyanide (NaCN) doses of 0.1 mg kg^{-1} (open), 1.0 mg kg^{-1} (grey) and 10.0 mg kg^{-1} (black). Like lettering *within* an incubation percentage indicates similar responses between the different doses of NaCN. An asterisk indicates a significant difference in the response to a given dose of NaCN comparing across N21 and H10 between incubation percentages. Significant outputs from 2-way ANOVA are noted in Table 1. Data are presented as mean values ± S E. Sample size was 9 for the embryos in the N21 group at 70 % of incubation, 8 for the H10 group at 70 % of incubation, and 9 in the N21 and H10 groups at 90 % of incubation.

Table 1
ANOVA table representing significant results for with oxygen conditions (O), incubation percentage (I), and NaCN, Hexamethonium or Phentolamine dose (NaCN, Hex or Phent) as the independent factors and heart rate (f_H) and mean arterial pressure (P_m) as the dependent factors. Data are presented in Figs. 2, 3 and 6.

Variable	Factor	df	Fstat	p	Figure or Table Details and Notes
P_m	O	1	16.6	<0.001	Fig. 2; Asterisks across condition in Fig. 2
P_m	NaCN	2	24.3	<0.001	Fig. 2; Like lettering in Fig. 2 within condition
f_H	NaCN	2	79.4	<0.001	Fig. 2; Like lettering in Fig. 2 within condition
f_H	Hex	1	25.8	<0.001	Fig. 3; no significant effect of condition or incubation percentage, asterisks represent only effect of Hex
f_H	Phent	1	15.2	<0.001	Fig. 6; no significant effect of condition, asterisk represent only effect of Phent

Table 2
Protocol 1: Mean arterial pressure (P_m) and heart rate (f_H) before (Control) and after injections of 0.1, 1.0 and 10.0 mg kg^{-1} NaCN into embryonic alligators that were incubated in 21 % O_2 (N21) or 10 % O_2 (H10). Injections were given at 70 % and 90 % of incubation. An asterisk indicated significant differences within each incubation condition and percentage comparing the control values (Control) to the values after NaCN injection of different doses. Data are presented as mean ± SEM. $P \leq 0.05$. Data were analyzed with paired Students *t*-tests.

% Incubation & Condition	Measure	Control	0.1 mg kg^{-1}	Control	1 mg kg^{-1}	Control	10 mg kg^{-1}
70 N21	P_m(kPa)	0.91 ± 0.06	1.00 ± 0.08*	0.83 ± 0.05	1.10 ± 0.12*	0.80 ± 0.06	1.08 ± 0.09*
70 H10	P_m(kPa)	0.81 ± 0.05	0.85 ± 0.05	0.75 ± 0.03	0.95 ± 0.05*	0.77 ± 0.05	0.93 ± 0.05*
90 N21	P_m(kPa)	1.50 ± 0.11	1.76 ± 0.10*	1.34 ± 0.08	1.96 ± 0.08*	1.48 ± 0.08	2.17 ± 0.09*
90 H10	P_m(kPa)	0.97 ± 0.10	1.20 ± 0.14*	1.02 ± 0.10	1.28 ± 0.12*	1.01 ± 0.11	1.40 ± 0.13*
70 N21	f_H(bt min^{-1})	73 ± 3	74 ± 2	77 ± 2	64 ± 5*	79 ± 2	57 ± 3*
70 H10	f_H(bt min^{-1})	73 ± 3	74 ± 2	74 ± 2	53 ± 7*	75 ± 3	58 ± 4*
90 N21	f_H(bt min^{-1})	82 ± 2	78 ± 3*	83 ± 2	71 ± 4*	81 ± 3	55 ± 3*
90 H10	f_H(bt min^{-1})	74 ± 2	72 ± 2*	81 ± 3	67 ± 3*	78 ± 2	57 ± 2*

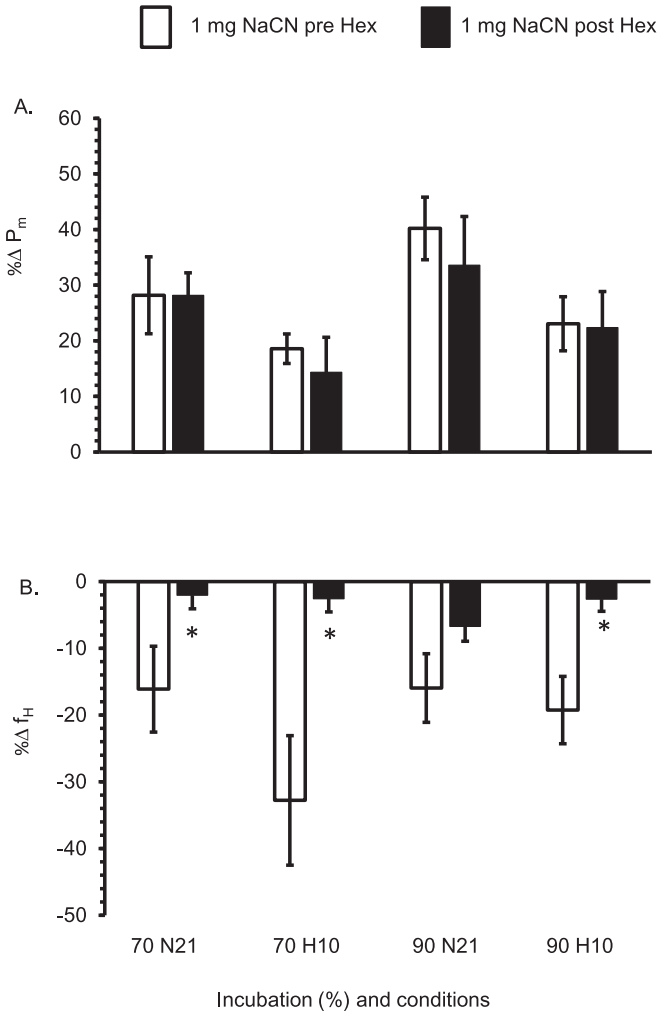

Fig. 3. *Protocol 2*: The percentage change in mean arterial pressure (A; %ΔP_m) and heart rate (B; %Δf_H) in response to an injection of sodium cyanide (NaCN) at 1.0 mg kg^{-1} before (open) and after (black) an injection of hexamethonium chloride (25 mg kg^{-1}). As there was no significant effect of hypoxia or percentage incubation, asterisks indicate a significant difference in the response to NaCN before and after hexamethonium injection within each percentage of development and condition (N21 or H10). Significant outputs from 2-way ANOVA are noted in Table 1. Data are presented as mean values ± SE. Sample size was 8 for the embryos in the N21 group at 70 % of incubation, 7 for the H10 group at 70 % of incubation, and 11 in the N21 and H10 groups at 90 % of incubation.

were followed with a Fisher LSD post hoc test to separate values into distinct subsets (Eme et al., 2021).

Paired student's *t*-tests were used to analyze the individual effects of NaCN doses, hexamethonium or phentolamine on P_m and f_H within incubation condition and percentage of incubation (*Protocol 1, 2,4*; Tables 2,3,4). Paired t-tests were used to compare mean P_m and f_H before and during 1 h of 10 % O_2 (*Protocol 4*; Fig. 4). Data are presented at the mean values ± standard error of the mean (S.E.M.). Statistical significance was determined based on $\alpha = 0.05$ for paired t-tests or a Bonferroni corrected $\alpha = 0.025$ for transformed data (Statistica, ver. 13.0; StatSoft, Tulsa, OK).

3. Results

The response to NaCN injection was a rapid decrease in f_H, followed by a secondary increase in P_a (Fig. 1), and in general, NaCN decreased f_H and increased P_m in a dose-dependent manner, with clear differences between 0.1 mg and the 1 mg and 10 mg doses (Fig. 2; Tables 1, 2). The 0.1 mg kg^{-1} dose had no effect on f_H on either the N21 or H10 embryos at 70 % of incubation (Table 2). The rapid bradycardia and secondary hypertension effect were generally greater at 90 % compared to 70 % incubation, but incubation treatment (N21 vs H10) had little influence (Fig. 2). The 1.0 mg kg^{-1} NaCN dose caused a significant increase in P_m in both conditions at both points of incubation studied (Table 2). In addition, 1.0 mg kg^{-1} NaCN caused a significant rapid decrease in f_H immediately after the injection in both groups and at both incubation points studied (Table 2). The 10.0 mg kg^{-1} dose of NaCN significantly increased P_m and decreased f_H in all embryos studied (Table 2). There was a significant effect of development on the percentage change P_m response to NaCN (Fig. 2; Table 1; 2-way RM ANOVA, $F = 16.6$, $df = 1$, $P \leq 0.001$), and the percentage change in P_m in response NaCN was dose dependent (Fig. 2; Table 1; 2-way RM ANOVA, $F = 24.3$, $df = 2$, $P < 0.0001$; Fig. 2A). At 90 %, the change in P_m in response to 1.0 mg kg^{-1}

Table 3
Protocol 2: The effects of an injection of hexamethonium chloride (25 mg kg^{-1}) on mean arterial pressure (P_m) and heart rate (f_H) given to embryonic alligators that were incubated in 21 % O_2 (N21) or 10% O_2 (H10). Injections were given at 70 % and 90 % of incubation. An asterisk shows that hexamethonium significantly decreased blood pressure at 70 % of incubation in the N21 group. Control values (Control) are before hexamethonium injection and 'Hex" values are after injection. Data are presented as mean ± SEM. $P \leq 0.05$. Data were analyzed with a paired Students t-test.

% Incubation & Condition	Measure	Control	Hex
70 N21	P_m (kPa)	0.70 ± 0.09	0.58 ± 0.07*
70 H10	P_m (kPa)	0.61 ± 0.04	0.57 ± 0.03
90 N21	P_m (kPa)	1.33 ± 0.39	1.28 ± 0.52
90 H10	P_m (kPa)	1.19 ± 0.13	1.14 ± 0.13
70 N21	f_H (bt min^{-1})	79 ± 1	78 ± 2
70 H10	f_H (bt min^{-1})	73 ± 2	72 ± 2
90 N21	f_H (bt min^{-1})	73 ± 4	77 ± 3
90 H10	f_H (bt min^{-1})	72 ± 2	71 ± 2

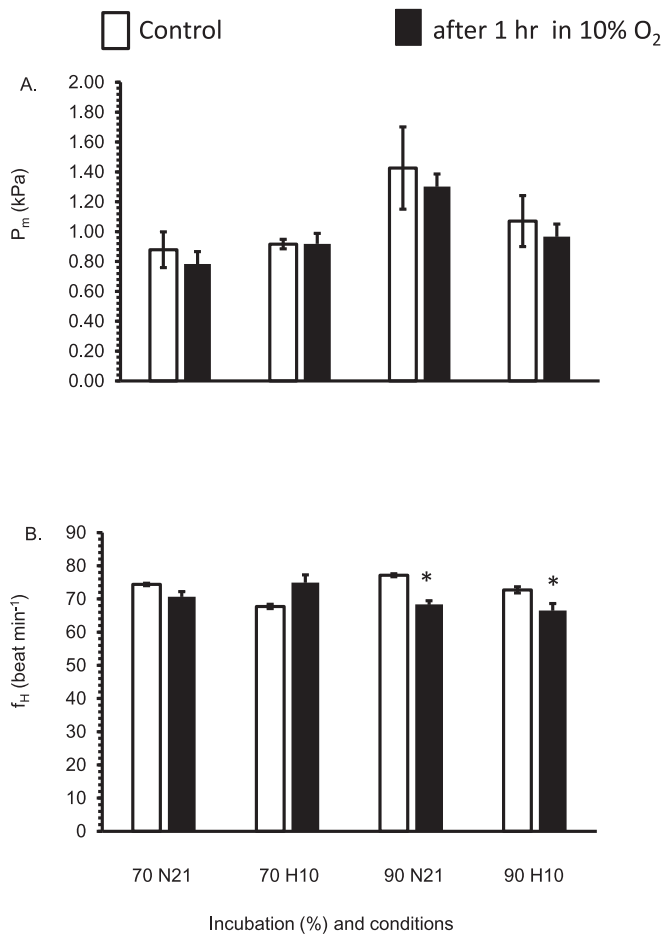

Fig. 4. *Protocol 4*: Mean arterial pressure (A; P_m) and heart rate (B; f_H) before (open column) and after both parameters had stabilized for at least 1 h (black column) after the onset of 10 % O_2 exposure. An asterisk indicated a significant difference in f_H between the values before and during 10 % O_2 exposure within an incubation condition and percentage of incubation. Data are presented as mean values ± SE. Sample size was 7 for the embryos in the N21 group at 70 % of incubation,8 for the H10 group at 70 % of incubation, and 8 in the N21 group and 10 in H10 group at 90 % of incubation.

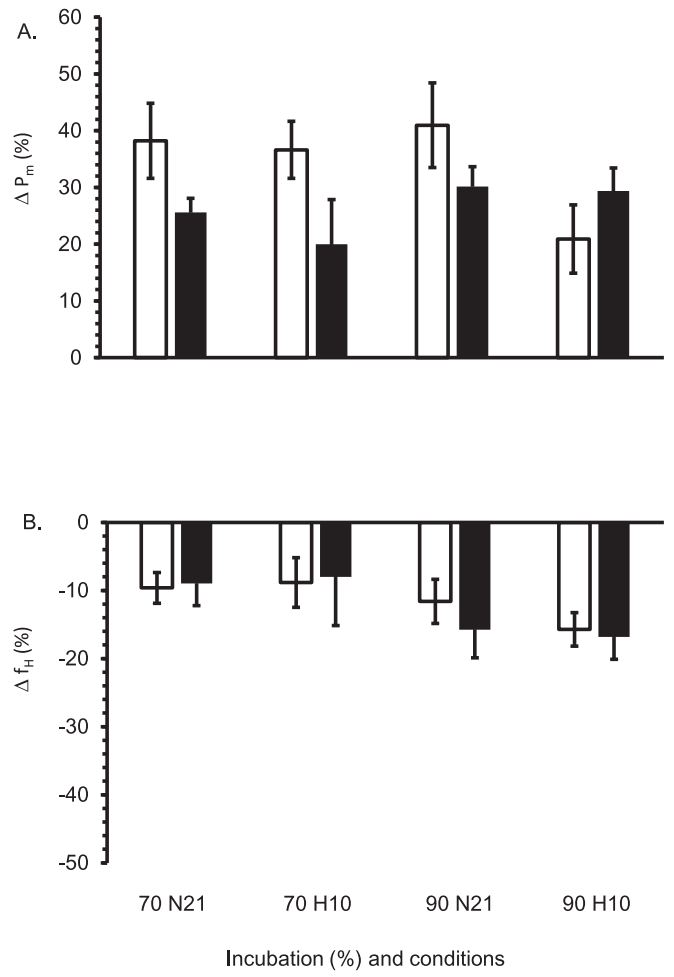

Fig. 5. *Protocol 3*: The percentage change in mean arterial pressure (A; $\%\Delta P_m$) and heart rate (B; $\%\Delta f_H$) in response to an injection of 1.0 mg kg^{-1} sodium cyanide (NaCN) before (open column) and at the end of (black column) at least 1 h exposure to 10 % O_2. Data are presented as mean values ± SE. Sample size was 7 for the embryos in the N21 group at 70 % of incubation, 8 for the H10 group at 70 % of incubation, and 8 in the N21 group and 10 in H10 group at 90 % of incubation.

NaCN was significantly greater in N21 compared to H10. The percentage change in the response to 1.0 mg kg^{-1} NaCN within each incubation condition, N21 and H10, was significantly greater at 90 % compared to 70 % in the N21 group of embryos only (Fig. 2A). There was a significant effect of the dose of NaCN on the relative change in f_H (Table 1; 2-way RM ANOVA, $F = 79.4$, $df = 2$, $P < 0.01$; Fig. 2B). At 90 % of incubation, the decrease in f_H was significant at all doses (Table 2), and the change was significantly dose dependent in both the N21 and H10 groups (Fig. 2B).

Ganglionic blockade with hexamethonium, α-adrenergic blockade with phentolamine, and exposure to 1-h of hypoxia had selected effects on the responses to NaCN. Pre-treatment with hexamethonium chloride blunted the bradycardic response to NaCN (except for 90 % N21 embryos; Table 1; 2-way RM ANOVA, $F = 25.8$, $df = 1$, $P < 0.001$) but did not alter the hypertensive response (Fig. 3). Hexamethonium chloride (25 mg kg^{-1}) injections significantly decreased P_m in the N21 embryos as 70 % of incubation only (Table 3). Hexamethonium had no effect on f_H in all of the groups of embryonic alligators studied (Table 3). Prolonged 10 % O_2 exposure of approximately 1 h had no effect on P_m before NaCN injection (Fig. 4A), but this significantly decreased f_H slightly in the N21 and H10 embryos at 90 % of incubation (Fig. 4B). In addition, the percentage change in P_m and f_H following injection of 1.0 mg kg^{-1} NaCN was similar before and during the prolonged exposure to 10 % O_2 in all groups (Fig. 5A and B). The injection of phentolamine (3 mg kg^{-1}) significantly decreased P_m in both the N21 and H10 embryos at 90 % of incubation (Table 4). The P_m response to 1.0 mg kg^{-1} NaCN was significantly altered by the treatment with phentolamine (Table 1; 1-way RM ANOVA, $F = 15.2$, $df = 1$, $P < 0.0001$) resulting in a significantly dampened response in the H10 embryos (Fig. 6).

Table 4
Protocol 4: The effects of an injection of phentolamine (3 mg kg^{-1}) after injections of Hexamethonium (25 mg kg) in embryos on mean arterial pressure (P_m) and heart rate (f_H) given to embryonic alligators that were incubated in 21 % O_2 (N21) or 10% O_2 (H10). Injections were given at 90 % of incubation. An asterisk indicated significant differences within each incubation condition and percentage comparing the control values to the values after phentolamine injection. Data are presented as mean ± SEM. $P \leq 0.05$. Data were analyzed with a paired Students t-test.

Measure	Condition	Control	Phentolamine
P_m (kPa)	N21	0.97 ± 0.10	0.68 ± 0.06*
P_m (kPa)	H10	0.92 ± 0.12	0.62 ± 0.06*
f_H (bt min^{-1})	N21	74 ± 3	73 ± 3*
f_H (bt min^{-1})	H10	69 ± 3	65 ± 3

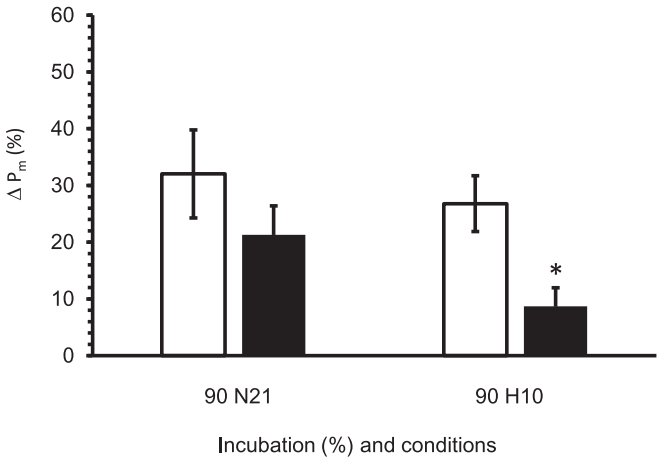

Fig. 6. *Protocol 4*: The percentage change in mean arterial pressure (%ΔP_m) in response to an injection of 1.0 mg kg^{-1} sodium cyanide (NaCN) before (open column) and after (black column) the combined injections of hexamethonium chloride (25 mg kg^{-1}) and phentolamine (3 mg kg^{-1}) for 90 % of development embryos, only. An asterisk indicates a significant difference in the response to NaCN before and after the blocking drug treatments. Significant outputs from 1-way ANOVA are noted in Table 1. Data are presented as mean values ± SE. Sample size was 7 for both the N21 and H10 groups of embryos.

4. Discussion

The cardiovascular response of embryonic American alligators to increasing doses of NaCN differed from embryonic snapping turtles (Eme et al., 2021). Embryonic snapping turtles responded to increasing doses of NaCN with a progressively growing bradycardia when measured at 70 % and 90 % of incubation (Eme et al., 2021). The bradycardia was accompanied by a hypotension in embryonic snapping turtles (Eme et al., 2021). Embryonic alligators exhibit a different pattern of response with development, with the maximal bradycardic response occurring at the 10 mg kg^{-1} dose in embryos at 70 % of incubation in the embryos that were incubated in normoxia (N21) while in the hypoxic group (H10) it occurred at 1 mg kg^{-1} (Fig. 2B). Both embryonic alligator groups in this study responded with the maximal bradycardia to the 10 mg kg^{-1} dose at 90 % of incubation (Fig. 2B). Further, at both incubation timepoints studied the primary arterial pressure response of embryonic alligators was a secondary *hypertension* (Fig. 2A), as opposed to the *hypotension* reported for embryonic snapping turtles (Eme et al., 2021). A basis for the difference in the responses between these two embryonic species is unclear, and no current data exist for other reptiles or extant birds. Future research in adult/juvenile alligators and turtles may reveal if this difference persists or is common in other species.

The ganglionic blocker hexamethonium chloride allowed us to determine if the f_H response of embryonic alligators to NaCN was mediated by an active neural reflex loop. In response to injections of hexamethonium, f_H and P_m were largely unaffected (Table 3). This supports prior findings that embryonic alligators lack autonomic tone on the cardiovascular system at 70 % and 90 % of incubation (Eme et al., 2011a). The f_H response to 1 mg kg^{-1} NaCN was absent after the ganglionic blocker was given (in three of four groups of alligators), indicating that NaCN induced a cardiovascular chemoreflex (Fig. 3B). Interestingly, while the f_H response to NaCN was dampened in normoxic-incubated embryonic alligators at 90 % of incubation, it was not significantly different from the response to 1 mg kg^{-1} dose of NaCN given prior to the blockade (Fig. 3B). NaCN may cause a larger secondary humoral catecholamine release in alligators, and that could cause hypertension (as seen in alligator embryos) as opposed to hypotension (as seen in turtle embryos). For embryonic snapping turtles incubated in normoxia at 90 % of incubation, the NaCN cardiovascular response was eliminated by pretreatment with the cholinergic receptor blocking agent atropine (Eme et al., 2021). This could represent the release of non-adrenergic noncholinergic (NANC) factors that become functional as embryonic alligators mature in normoxia, or NaCN may act on parasympathetic terminals directly or locally on the myocardium causing the release of acetylcholine. A prior study of ischemic myocardium in cats had shown that local production of acetylcholine in vagotomized animals does occur (Kawada et al., 2000a). Further studies could identify mechanisms under which NaCN continues to produce a bradycardic response in normoxic incubated reptiles during the final stages of embryonic incubation.

While the f_H response to NaCN was affected by pretreatment with hexamethonium, the secondary increase in P_m in response to this compound was unaffected (Fig. 3A). In embryonic snapping turtle, NaCN produces a marked hypotension that is coupled to the bradycardia unlike the secondary hypertension presented here for embryonic alligator (Eme et al., 2021). Further, treatment with hexamethonium either eliminated or dampened arterial pressure response in embryonic snapping turtles, suggesting a NaCN induced withdrawal of sympathetic tone on the vasculature of the embryo (Eme et al., 2021). Embryonic alligator responded to NaCN with a persistent hypertension that is unaffected by ganglionic blockade, suggesting the autonomic nervous system is not involved the response (Fig. 3A). NaCN has been previously shown to induce the release of epinephrine from the myocardium of rabbits (Kawada et al., 2000b). If a similar response occurred in embryonic alligators, this could increase the contractility of the heart and increase vascular resistance, resulting in the response found in the current study.

To determine the capacity for embryonic alligators to change the sensitivity to chemoreceptor stimulation, embryonic alligators were studied at the end of a 1 h period in 10 % O_2. In response to 1-h 10 % O_2, f_H was slightly depressed in embryonic alligators at 90 % of incubation with no effects on P_m (Fig. 4A and B). The P_m response was similar to that previously reported for embryonic alligators exposed to only 5 min of 10 % O_2 (Crossley II and Altimiras, 2005). f_H recovered in embryonic alligators at 70 % of incubation after 1 h of 10 % O_2, while embryos at 90 % remained relatively bradycardic (Fig. 4B). Further, when the response to NaCN was compared in the same embryos before the 1 h of 10 % O_2 exposure and at the end of the exposure, there was no difference in change in P_m or f_H caused by NaCN doses (Figs. 5A and B). This finding indicates that embryonic alligators lack the capacity to modulate the cardiovascular chemoreflex.

Finally, we investigated the mechanism that underlies the secondary hypertension in response to NaCN in embryos at 90 % of incubation. Our prediction was that the secondary hypertensive response to NaCN was due to release of catecholamines. Interestingly, blocking α-adrenergic receptors with phentolamine significantly decreased the P_m response in H10 embryos incubated chronically in 10 % O_2, while it showed no effect on N21 embryos incubated chronically in normoxia (Fig. 6). It is important to note that while embryos were ganglionically blocked with hexamethonium, we did not block β-adrenergic receptors; the increase in P_m in response to NaCN may reflect an increase in cardiac contractility in response to β-adrenergic stimulation.

4.1. Conclusion

Our goal was to determine the plasticity of a chemically induced cardiovascular chemoreflex and the capacity for embryonic alligators to change, or modulate, the intensity of the response when subjected to prolonged periods of hypoxia. Unlike the response to the $5HT_3$ receptor agonist PBG, the response to NaCN at 90 % of incubation was similar in embryonic alligators incubated in normoxia or 10 % O_2. We interpret this as indicating the f_H chemoreflex in response to NaCN lacks plasticity as has been previously demonstrated in embryonic common snapping turtles (Eme et al., 2021). Further embryonic alligators do not adjust the response to NaCN when exposed to 1 h of 10 %O_2, suggesting they lack the capacity to adjust the response to NaCN. Therefore, the

cardiovascular chemoreflex is clearly a reflex loop in embryonic alligators, however, the sensitivity to NaCN is not plastic and the animals cannot adjust the response intensity.

CRediT authorship contribution statement

John Eme: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Gil Martinez Bautista:** Methodology, Investigation. **Audrey Keneda:** Methodology, Investigation. **Kevin Tate:** Methodology, Investigation. **George Melancon:** Resources, Methodology. **Dane A. Crossley:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dane A Crossley II reports financial support was provided by National Science Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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