DEVELOPMENT OF CARDIOVASCULAR REGULATION IN EMBRYOS OF THE DOMESTIC FOWL *(Gallus gallus),* WITH PARTIAL COMPARISON TO EMBRYOS OF THE DESERT TORTOISE *(Gopherus agassizii)*

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In adult vertebrates, cardiovascular regulation is accomplished by numerous systems with neural, hormonal and local components responsible for the majority of regulation. These regulatory components work in concert to maintain the essential function of blood perfusion to adult tissues. Given the essential nature of this function it is therefore surprising that the development of cardiovascular regulation during gestation is poorly understood. The majority of what is known is based on a single vertebrate model, the fetal lamb.

The fetal lamb has been used in multiple studies due to the clear clinical applications and has been pivotal in understanding the onset of regulation in developing vertebrates. However, study on the fetal lamb is limited to the latter 40% of gestation and has the added complication of an *in-utero* developmental strategy. Therefore the primary focus of this dissertation was to characterize basic cardiovascular regulation in the chicken embryo to provided the needed information for it's use an alternative to the fetal lamb.

Developing chicken embryos rely on both alpha and beta adrenergic tones to maintain normal heart rate and arterial blood pressure during incubation. However, on day 21, just prior to hatch, these animals lose both tones on arterial

pressure suggesting the onset of adult regulation. Cholinergic tone, however, was absent throughout chicken development indicating that it must mature during the neonatal life.

Adult cardiovascular reflexes become apparent late in chicken development with a clear baroreflex specifically operating initially on day. However, an adult response to changes in ambient gas tension was absent during incubation suggesting embryos possess unique regulatory systems that are absent in adult chickens. This mechanism is comprised entirely of adrenergic systems with no cholinergic action during change in ambient gas tension.

Similar developmental patterns were determined in embryos of the desert tortoise suggesting fundamental differences between in-utero and ex-utero developing vertebrates.

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CHAPTER I

CARDIOVASCULAR REGULATION DURING DEVELOPMENT

Introduction to the Topic

Cardiovascular regulatory systems are separated into two major control units, local control factors and central reflexive regulators (Persson 1996), that can be further divided into systems responding hemodynamic stimuli, resulting in an agonist or antagonistic affect on cardiovascular function (Persson 1996). Each regulatory unit can work simultaneously, with the interaction resulting in an optimization of hemodynamic performance under specific stress conditions. As an example, heart rate in a resting adult mammal is optimized by the summation of parasympathetic and sympathetic input to the heart (Levy and Martin 1995). During bouts of hemodynamic stress, one system will augment its action while the other withdraws its action (Kirchheim 1976). Additionally, each component may centrally alter the activity of the opposing system adding to the complexity of resting cardiovascular function (Levy and Martin 1995). In extreme cases, this regulation must function at close to optimal levels while the heart is cycling at a rate of 25 Hz. At these atypical levels, regulatory systems must ensure proper pressure gradients are maintained allowing gas transfer to all tissues. This

situation, while extreme, emphasizes the importance of cardiovascular regulation in adult mammals as well as the difficulties of developing a regulatory model.

Selective analysis of individual components has been integral in constructing a model of adult cardiovascular regulation (Persson 1996). Selective analysis requires the isolation of individual components via pharmacological blockade, surgical/chemical denervation or transgenic knockout techniques (Persson 1996). Cardiovascular responses to challenge will then reflect the possible role of a given regulatory component, as well as its interaction with other systems. Investigations utilizing numerous experimental techniques have combined to form the current "Guyton" model of cardiovascular regulation in adults mammals (Guyton and Young 1979). While the Guyton model of adult cardiovascular regulation has been developed, the understanding of the development of cardiovascular regulation remains poorly understood.

The majority of the understanding of cardiovascular regulation comes from a single mammalian model, the fetal lamb. Because of the clear clinical applications several studies have been conducted on fetal sheep and have provided important information of cardiovascular regulatory mechanisms during mammalian ontogeny (Assali et al. 1978). In addition, studies on fetal sheep have delineated critical periods when regulatory systems become operative during gestation (Assali et al. 1978, Shinebourne et al. 1972, Segar et al. 1992, Giussani et al. 1994). In summation, numerous investigations have shown that autonomic nervous control becomes operational and is integral for the

maintenance of cardiovascular function in the last third of sheep gestation. Further, several locally released vasoactive substances and humoral components play an important role in fetal cardiovascular regulation (Giussani et al. 1994). While the fetal lamb has been an important model, working with this animal limits the period of analysis to the latter 40% of incubation as well as possessing the added complication of in-utero development. Therefore, an alternative model needs to be explored which allows agreater greater window of analysis as well as eliminate the in-utero complication.

An ideal candidate is the chicken embryo and would allow analysis of regulatory systems before cardiovasuclar innervation occurs as well as eliminating the extensive protocols needed for in-utero studies. In addition, using the chicken embryo would have the added advantage of ease of environmental manipulation. Several studies have been conducted on developing chicken embryos and have focused on cellular and anatomical characteristics of cardiovascular regulation. These classical studies determined a time line for the presence of cellular receptors, as well as physical contact between the autonomic nervous system and the developing cardiovascular system (Romanoff 1967, Pappano 1977). While these investigations established the period during which physical integrity of regulatory systems were complete, the operation of cardiovascular regulation was undetermined. Later pharmacological studies attempted to address to determine the operation of cardiovascular regulation utilizing selective autonomic agonist and antagonists. These works

characterized cardiovascular response to numerous autonomic receptor agonists and provided important essential about the functional integrity of the regulatory mechanisms (Tazawa 1981a, Tazawa et al. 1985, Tazawa et al. 1992, Girard 1973a). However, the resting influence of these receptors on cardiovascular function was unknown.

Pappano first recognized this analytical deficiency and attempted to determine windows of developmental time when neuro-stimulation could elicit changes in cardiovascular function in embryonic chickens (Pappano 1977). Utilizing atrial field stimulation in combination with autonomic drugs, his work established a developmental window when centrally mediated parasympathetic and sympathetic action was possible in embryonic chickens. However, the importance of autonomic tonic input and possible autonomic role in cardiovascular response to hemodynamic challenges during development was undetermined.

Given our general lack of knowledge of cardiovascular regulation during development the primary focus of my research was to characterize basis regulatory mechanism embryonic chickens. The first step was to characterize autonomic tone on the developing cardiovascular system. Autonomic tone in adult chickens is a sum of both sympathetic and parasympathetic action of the cardiovascular system maintaing function within so range. Given the importance of the autonomic system in adult chickens, I determined that a thorough investigation into the onset of tone needed to be completed. Once I had

completed this work, I then choose to focus on the sympathetic division to determine when it became functionally viable. Following completion of the autonomic analysis, I then needed to determine when adult cardiovascular reflexes became operational specifically the baroreflex and the chemoreflex. Once I had determined when these reflexes became operational, I then choose to determine mechanisms that were responsible for any chemoreflex reactions followed by an isolation of any possible autonomic mechanisms. Finally I choose to do a similar analysis on embryos of the desert tortoise (*Gopherus agassizii*) for comparative purposes.

This thesis is divided into chapters based on hypothesises, presented below, that I constructed to address each of the steps described in the previous paragraph.

Hypothesis 1 addressed in Chapter 2.

- During the development of chickens, tonic autonomic control of cardiovascular function will not become operative soon after the initial neural branches reach the heart.

As described in Chapter 2, a clear adrenergic tone was present early in development while cholinergic systems remain inactive. Once adrenergic tone was established, it became necessary to determine if hemodynamic stress could induce cholinergic action as well as the augmentation of existing adrenergic influence.

Hypothesis 2 addressed in Chapter 3.

- Baroreflex control of cardiovascular function will not become operational during the latter half of chicken incubation.

As evident in Chapter 3, baroreflex control of cardiovascular function became sporadically functional in the latter 3 days of chicken development. In addition, the gain in embryos was below that known to occur in adults. Further cholinergic responses were limited during chicken development. Again, adrenergic action seemed to be the predominant mechanism for alteration of cardiovascular function in embryonic chickens. The integrity of sympathetic nervous innervation of the heart then needed to be addressed. This would determine if a lack of reaction to presser challenge was due to an immature mechanism.

Hypothesis 3 addressed in Chapter 4.

- Sympathetic integrity will not be complete by day 19 of a 21-day incubation period.

Hypothesis 3 was rejected as indicated in Chapter 4 however, the anticipated maturation of response intensity to tyramine was absent. Therefore, the results in Chapter 4 further suggested that a significant portion of maturation must occur in post-hatch life. To solidify the findings suggested thus far,

changes in ambient gas levels were applied to assure that reflex regulation remained inactive during the majority of chicken incubation.

Hypothesis 4 addressed in Chapter 5.

- Chemoreflex control of cardiovascular function will not exhibit a similar developmental pattern to that described for the baroreflex. Adult responses will not be active until late in chicken development.

While chemoreflex responses appeared as anticipated in the latter 4 days of chicken incubation, these responses were distinctly embryonic as they were lacking the well-documented adult responses. It was, therefore, necessary to determine if embryonic changes in cardiovascular function were due to any central regulatory component.

Hypothesis 5 addressed in Chapter 6.

- Central regulation has no part in cardiovascular responses to change in ambient gas composition during chicken development.

Given the absence of a classical hypotensive tachycardia, known to occur in adults throughout chicken incubation, it was anticipated that regulatory systems played no role in embryonic responses. Pharmacological manipulation, however, revealed an important adrenergic response to hypoxia was present in

embryos at 18 days of incubation and older. Thus, it was necessary to determine the origin of this hypoxic adrenergic action.

Hypothesis 6 addressed in Chapter 7.

- Chemical sympathectomy and ganglionic blockade will not reveal that direct sympathetic action on the cardiovascular system during hypoxia is possible initially on day 18 of chicken development.

As discussed in Chapter 7, chemical sympathectomy and ganglionic blockade revealed that sympathetic action during hypoxia produced a mild chronotropic response. However, the majority of cardiovascular reactions appeared to be via other mechanisms. These studies have quantified regulation of ex-utero development in a single species of vertebrates, thus similarities in responses from a different vertebrate group needed to be tested.

Hypothesis 7 addressed in Chapter 8.

- Embryonic cardiovascular responses to pharmacological manipulation in a reptilian species, the desert tortoise, will not exhibit distinct characteristic in comparison to chicken embryos given differences in reproductive strategies.

These tests indicated that reptiles share some of the characteristics found in chicken embryos, while exhibiting others that were distinctly reptilian.

Techniques and experimental protocols have been discussed within each chapter with differences in manipulations noted. In addition, each chapter has been constructed as individual units to contain all components of published manuscripts.

CHAPTER II

ONTOGENY OF AUTONOMIC CONTROL OF CARDIOVASCULAR FUNCTION IN THE DOMESTIC CHICKEN, *Gallus gallus*

Introduction

The chicken embryo has long been a primary model for the study and understanding of cardiorespiratory function during the ontogeny of vertebrates (Keller 1997). Although many studies have concentrated on the initiation and maturation of autonomic cardiovascular regulation, little is known of its functional significance during embryonic development. In addition, autonomic control of peripheral vascular beds, essential for blood pressure regulation in adults, is vaguely understood at best. Early studies established the presence of muscarinic and adrenergic sensitive receptors in the heart of chicken embryos during the first quarter of incubation (Berry 1950, Cullis and Lewis 1936). In addition, previous studies found that neurotransmitter anabolic and catabolic enzymes were also present in the embryonic chicken heart by day 5 (Gifford et al. 1973, Ignarro and Shideman 1968, Zachs 1954). Together with morphological data, these studies provided the foundations for determining the ontogenetic sequence leading to cardiac autonomic regulation. However, they did not establish levels of autonomic influence on the developing cardiovascular system.

A functional assessment of autonomic efferents that modulate heart rate was carried out by Pappano et al. (1977) using field stimulation methods. Changes in embryonic heart rate following atrial field stimulation indicated that parasympathetic release of acetylcholine can be elicited on day 12 of incubation, while sympathetic release of noradrenaline is possible on day 21(Pappano and Loffelholz 1974). While this demonstrates the functional integrity of autonomic efferent pathways, it does not imply the existence of an autonomic tone on the cardiovascular (CV) system. A functional autonomic tone requires the maturation of afferent and central elements which may not occur until later in development.

The objectives of this study were to determine when the tonic autonomic regulation of CV function in developing chicken embryos became operational. Results were compared with existing data from other species in an effort to establish possible trends in CV development of vertebrates.

Materials and Methods

Animals and incubation conditions

Plymouth chicken eggs of the Russ208 strain were purchased from Fællesrugeriet (Randers, Denmark) and transported under constant temperature conditions, 30°C, to the Department of Zoophysiology, University of Aarhus. On arrival, eggs were placed in an incubator at 38 °C and turned manually twice a day until day 18 of incubation.

Measurement of blood pressure and heart rate

At selected developmental ages, eggs were removed from the incubator, candled to trace the major chorioallantoic arteries with a soft pencil and placed in a vermiculite bath (38 °C). A chorioallantoic artery was exposed by removal of a small portion of eggshell. The smallest branching vessel was occlusively cannulated with a polyethylene catheter (PE-90 1.27 mm OD / 0.86 mm ID, Clay-Adams) with the tip heat pulled to an outer diameter of 0.5 mm under a Leitz dissection microscope. Silk suture was used to secure the catheter to the vessel after careful alignment of the two, and the catheter was glued to the eggshell with cyanoacrylic glue (VetBond 3M). Blood pressure traces were obtained via the fluid filled catheter connected to a Statham pressure transducer (P23), which was calibrated against a static water column. The transducer was connected to a Beckman recorder (R511A) for proper amplification of the signal before being sampled at 500 Hz by a computer via a Data Translation card (DT2801A) and LabView custom-made acquisition software. Heart rate (f_H) was obtained from the pressure signal. Details on the frequency response of the catheter-filled system as well as the zero pressure reference are discussed in chapter 3.

Experimental protocol

Blood pressures were allowed to stabilize for 5-10 min prior to each experimental treatment. The autonomic antagonists atropine, propranolol and prazosin were subsequently injected and their effects recorded until arterial pressure and f_H had stabilized. Injection volumes were normalized for each embryonic age group to 5% of the total blood volume based on literature data

(Romanoff 1967). Drug dosages were calculated taking into account the total mass of living tissue in the egg (including total embryonic wet mass as well as the total mass of the egg membranes (Romanoff 1967)). The following doses were used: atropine - 1mg·kg^{-1} (SIGMA); propranolol - 3 mg·kg^{-1} (SIGMA); prazosin - 1 mg·kg^{-1} (SIGMA); (see Table 1). The efficacy of these dosages was tested in preliminary experiments, as the antagonist blocked the effects of a subsequent injection of the agonist (Figure 1). Special care was taken to avoid large volumes of dead space in injection lines with each drug administration. Double bored catheters were utilized in day 8 embryos to insure that flush volumes would be sufficient without exceeding a total injection volume of 5% total volume of blood.

Catecholamine Analysis

A blood sample was obtained from each embryo following the completion of an experimental protocol, for later catecholamine analysis. Blood was allowed to flow freely from the arterial catheter due to suction induced occlusion of the CAM vasculature. Blood samples were mixed with 5 μL of an EGTA/glutathione solution (0.2M/0.2M) to prevent catecholamine oxidation and immediately spun down to separate the plasma. Samples were maintained at -70 °C until analysis was carried out (within 1 month). HPLC analysis of plasma catecholamines was performed as previously described (Fritsche and Nilsson 1990).

Statistics

A paired Student t-test was used to access significant differences between pre and post drug injection for all variables measured on each day of development. A one-way ANOVA was employed to determine significant differences in arcsine transformed percentage change in heart rate (f_H) and mean arterial pressure (Map) between days of incubation. Fisher's LSD post-hoc comparison was used to isolate significant differences between days of incubation. The fiduciary level of significance for all tests was taken at $p<0.05$. All data are presented as mean ± 1 s.e.m.. For all days of study n=5 eggs were used to determine cardiovacular responses.

Results

General pharmacological effects

General effects of each antagonist on cardiovascular function are presented in Figure 2.2, which demonstrates the impact of atropine, propranolol and prazosin in a day 19 chicken embryo. At this stage, propranolol clearly reduces f_H while elevating Map (B), followed by a reduction in Map and f_H after prazosin treatment (C). In addition, atropine produced no change in blood pressure or heart rate (A).

The values of f_H, systolic pressure (Sys), diastolic pressure (Dia) and mean arterial pressure (Map) before and after injection of the antagonist on a given day of incubation are presented in Tables 2.2-2.4. Blood pressure was in general agreement with that of prior research in embryos at similar stages of development (Girard 1973, Hoffman and Van Mierop 1971, Tazawa 1981a, Van

Mierop and Bertuch 1967). However, f_H was lower compared to that of prior work (Tazawa 1981), which could be attributed to differences in the control of temperature as well as the chicken strains. Atropine injection had no significant effect on any tested variable, with the exception of f_H on day 12. Propranolol exhibited a clear negative effect on f_H at all stages tested, with a mean reduction of 33 ± 5 beat·min^{-1}. In addition, Map was significantly elevated by propranolol injection (average of 0.25±0.06 kPa) on all days of incubation, with the exception of day 21. Prazosin induced a significant reduction in Map from day 8 to 20 (pressure decrease of 0.031 ± 0.01 kPa to 1.27 ± 0.29 kPa at day 8 and 20 respectively), while reducing f_H on days 12, 15, and 19.

Developmental Differences

Atropine had little effect on f_H and Map at all stages of development (Table 2.2); thus, there were no significant differences between stages (Fig.2.3). The pattern of sensitivity to propranolol was constant through development, reducing f_H by an average of 16 -18% (Fig.2.4A). Map on day 21 was not affected by propranolol, a result significantly different from the elevation found on days 12, 15 and 20 (Table 2.3 and Fig.2.4B). An ontogenetic change in prazosin sensitivity was also evident in both Map and f_H during incubation. The reduction in both f_H and Map was significantly greater Fig.2.5) on day 19 than all other days, with the exception of day 20. Similar results were exhibited on day 20 of incubation, with Map falling significantly more than on all other days (Fig.2.5),

with the exception of days 12, 15 and 19. In addition, heart rate changes on day 20 were significantly different (Fig.2.5) than those found on days 8,15 and 21.

Catecholamines levels during development

Noradrenaline (NA), adrenaline (A), and dopamine (D) concentrations showed a significant change as embryonic maturation progressed (Table 2.5). Noradrenaline concentration peaked on day 19 at 169.4 ± 52.0 $ng \cdot ml^{-1}$, a level significantly higher than that on earlier stages ($p<0.05$). Adrenaline exhibited a similar increasing to 80.6 ± 23.2 $ng \cdot ml^{-1}$ on day 19, again significantly higher than that of earlier stages ($p<0.05$). Finally, dopamine peaked at 9.1±1.3$ng \cdot ml^{-1}$ on day 20, significantly higher than that of earlier stages ($p<0.05$).

Discussion

Tonic regulation of the cardiovascular system during chicken development exhibits distinct characteristics suggesting that embryonic and adult regulatory mechanisms are unique during the latter half of incubation. The profound importance of adrenoreceptors in maintaining cardiovascular function demonstrated in this study, suggests that humoral control prevails over neural control during embryonic life. This point was further illustrated by the notable absence of muscarinic tone on the cardiovascular system over the same developmental period. The role of each regulatory system during avian development, as well as how these findings compare to other vertebrate groups, will now be discussed.

Cardiac Vagal Tone

Parasympathetic tone was absent throughout the stages studied, as indicated by the lack of a chronotropic reaction following atropine injection (Fig.2.3). This finding is in general agreement with other studies in which atropine injection produced no effect on embryos from 13 to 16 day of incubation (Haque et al. 1995, Tazawa et al. 1992).

The absencesof parasympathetic tone indicates that a considerable delay exists between the presence of components required for functional efferent cholinergic output (Pappano and Loffelholz 1974) and the onset of cholinergic tone. It is well established that muscarinic receptors are present in chicken embryos between days 2-3 of development (Cullis and Lewis 1936), as are the enzymatic components necessary for the synthesis and degradation of acetylcholine (Gifford et al. 1973, Zachs 1954). Cardiac cholinergic innervation via the sinal branch of the vagus nerve also appears during the first week of embryonic development (Kuratani and Tanaka 1990). Finally, studies utilizing atrial field stimulation further illustrate the potential for parasympathetic activity as early as day 12 of development (Pappano and Loffelholz 1974). However, although the functional components of this tone are present from 50% of development, they appear inactive and no vagal tone appears until neonate life. It could be argued that embryonic muscarinic receptors are insensitive to atropine during development, but this is highly unlikely considering that the chronotropic effects of acetylcholine could be successfully eliminated via pre-treatment with atropine (Fig.2.1).

Collectively, these results suggest that normal CV development occurs in the absence of tonic vagal input. The potential presence of a vagal tone under specific circumstances was not addressed in this work and should be considered. Indeed, long-term recordings of instantaneous heart rate in embryos between 14-17 days have documented fast decelerations of heart rate, which could be the result of brief vagal activation (Haque et al. 1995, Hochel et al. 1998). However, these studies showed opposite effects and lacked statistical treatment; thus, conclusions must be drawn with caution. In chapter 3 it was demonstrated that hypertensive stress was insufficient to induce vagal action until external pipping (19-20 d), indicating that the parasympathetic system is not functional during chicken ontogeny.

A non-function parasympathetic system has also been noted during the ontogeny of the African clawed frog (*Xenopus leivis*), that possess no vagal tone from NF stages 33/34 to 54 (Fritsche, personal communication) and the desert tortoise (*Gopherus agassizii*) (Crossley, unpublished results). Thus, vagal tone may be absent in several terrestrial vertebrate groups which exhibit ex-utero embryonic development. The data available for the fetal lamb is less clear, with some studies producing no effects of atropine (Reller et al. 1989, Thornburg and Morton 1986, Thornburg and Morton 1983) while others have reported an increasing cholinergic tone (Wakatsuki et al. 1992, Walker et al. 1978). Given this confusion, statements related to vagal function in vertebrates with uterine

development are limited; however, vagal tone in developing birds and possibly other ex-utero developing species systems is absent.

Cardiac and Vascular β-adrenergic Tone

A clear β-adrenergic chronotropic tone on the heart was demonstrated via propranolol injections during all stages studied, inducing on average an 18% reduction in embryonic f_H (Fig. 2.4). These results agree with those of Tazawa et al. (1992), who found similar responses to β-antagonist injection in chicken embryos from day 13 to 16. This reaction to β-blockade was unchanged throughout development suggesting that cardiac β-adrenergic regulation is constant from day 8 of incubation. This was also the case for Map throughout incubation, with the notable exception of day 21, which demonstrated no treatment effect.

Thus, the resulting cardiovascular response is one typical of a β-adrenergic antagonist as can be seen from the dynamic changes in Figure 2.2B. The blockade of vascular β-adrenoceptors caused vasoconstriction and the blockade of cardiac β-adrenoceptors lowered f_H. Although f_H stayed low, stroke volume must have increased to compensate for the transient drop in cardiac output and maintained pressure. This, in turn, unmasked the vasoconstriction associated with β-blockade.

The existence of β-adrenergic receptors in the vasculature has been previously suggested in chicken embryos at 6 and 13 to 16 days of age (Koide and Taun 1989, Tazawa et al. 1992). Similar effects have been elicited in

embryos during day 6 and 14 of incubation (Koide and Taun 1989, Saint-Petery and Van Mierop 1974). Although the present pharmacological protocol does not allow a proper distinction between cardiac and vascular changes, it is clear that the chicken embryo responds to β-adrenergic blockade in a fashion similar to that in most other vertebrates. While a cardiac β-adrenergic tone seems to be almost universal in vertebrates, the degree of hemodynamic changes due to β-adrenoreceptor vasodilation might be accentuated in embryos, especially if the chorioallantoic vasculature has an active population of β-adrenergic receptors. This has been suggested for the placenta of the fetal lamb during the later third of gestation (Carter 1993). Therefore, β-adrenergic receptors may have important consequences for the regulation of shunting and blood oxygenation during vertebrate development.

Adrenergic Tone: α–receptor mediated

The pattern of α–adrenergic sensitivity varied substantially throughout embryonic development. A strong α–adrenergic tone on Map was demonstrated in chick embryos from 12 to 20 days of development (Table 2.4), with day 19 and 20 embryos exhibiting a heightened sensitivity to injection (Fig 2.5). Similar results have been reported in embryonic chickens from day 6 -16 (Koide and Taun 1989, Saint-Petery and Van Mierop 1974). Changes in CV sensitivity were not addressed in the aforementioned studies, limiting the extent of any comparison. Similar results have been obtained in fetal sheep, which show an

increase in α–adrenergic tone, with a magnitude that peaks at the end of gestation (Assali et al. 1977).

α–Blockade decreased f_H from day 12 to 19, with a marked effect on day 19 (Fig.2.4). Several studies have noted a lack in α–mediated chronotropic effects on the CVS in chicken embryos from day 6 to day 16 (Koide and Taun 1989, Saint-Petery and Van Mierop 1974). The negative chronotropic effects demonstrated in the current study may therefore be the result of vasodilation and reduced venous return followed by a reduced f_H. A peak in Map coinciding with the peak in f_H on day 19 further supports this explanation.

Humoral versus neural Adrenergic control

A proper understanding of the effects of adrenergic blockade (both β- and α–) requires the distinction between humoral and neural effectors. The participation of sympathetic efferents in CV function during the last days of chicken incubation has been controversial. However, it is generally accepted that adrenergic fibers are inactive until, at least, day 18 of chicken incubation (Higgins and Pappano 1981, Pappano and Loffelholz 1974). As such, it appears that humoral catecholamines support cardiovascular function via cardiac and vascular β -adrenoceptors and via vascular α–adrenoceptors. Interestingly, the plasmatic levels of catecholamines change dramatically over embryonic maturation. As indicated in Table 2.5, the plasma levels of noradrenaline and adrenaline changed 10 and 600 fold respectively throughout ontogeny, with the ratio of A to NA rising as well. While the levels determined in this study are

elevated in comparison to previous work (Dragon et al. 1996), the developmental pattern is similar.

The rise in plasma catecholamine levels is not paralleled by an increased cardiac sensitivity to propranolol. This finding has a physiological relevance to prevent the supramaximal stimulation of the heart that would result otherwise. On days 17-19 the pacemaker tissue and the ventricle exhibit a decreased sensitivity to catecholamines (Higgins and Pappano 1981, Loffelholz and Pappano 1974), probably due to the saturation of the receptors, down regulation or desensitization. In this regard, the activity of catabolic enzymes responsible for their inactivation also peak on days 19-20 in cardiac tissue of chickens, with catechol-o-methyl transferase reaching 0.7 $mmol \cdot h^{-1} \cdot mg\ protein^{-1}$ and monoamine oxidase reaching 50 $mmol \cdot h^{-1} \cdot mg\ protein^{-1}$ (Ignarro and Shideman 1968).

If not related to cardiac stimulation, the progressive rise of catecholamine levels raises the question of whether there is an alternative physiological purpose? The established increase in catecholamine concentration may be instrumental in the improvement of blood oxygenation via an increase in synthesis of carbonic anhydrase and 2,3-DPG (Dragon et al. 1996). If this was a primary function of elevated catecholamines, it could be pivotal in maintaining oxygen transport during the period when diffusive gas exchange is becoming progressively limited. Catecholamines are also important for changes in blood

oxygen affinity as well as the transition to ATP as the main allosteric modulator of the hemoglobin in embryonic chickens (Dragon et al. 1996).

In addition, it is interesting that plasma catecholamine levels also rise during the latter third of development in fetal sheep (Cheung 1990, Jones 1980, Lewis et al. 1982), which suggests that this is a general trend in vertebrate development. Moreover, the ratio of A to NA rises during late ontogeny in the chicks, a finding also demonstrated in fetal sheep (Cohen et al. 1984, Lewis et al. 1982, Lewis and Sischo 1985, Widmark et al. 1989).

Thus, catecholamines may be involved in the timing of internal pipping by regulating the blood flow to the extra–embryonic circulation and insuring adequate perfusion pressures to maintain gas exchange. As the CAM begins to regress, prior to the onset of lung ventilation, the tonic contribution of α–adrenoreceptors would be more important than that from β -adrenoreceptors, increasing resistance and reducing extra-embryonic blood volume. This hypothesis agrees with earlier data indicating that catecholamine effects are maximal at day 19 (Girard 1973) and, while definitive proof requires further study, it seems highly plausible.

Conclusion

A progressive maturation of autonomic cardiovascular control coordinated with morphological development has been the classical picture of cardiovascular ontogeny. In this general framework, parasympathetic control is assumed to precede sympathetic regulation, while the role of humoral catecholamines is

vaguely understood. This study, however, has revealed several flaws in the classical understanding of this system in birds, which could be extended to cardiovascular development in other vertebrate species. Data from the present study indicate the following: 1) Vagal tone is absent through the ontogeny of chickens. 2) Circulating catecholamines exhibit profound maturation changes in both concentration and the ratio of adrenaline: noradrenaline. 3) β -Adrenergic tone is present at 8 days of development with no further maturation of function. 4) α–Adrenergic tone is present at day 8, primarily affecting vascular tone, with an increase in magnitude as development progressed. Clearly each component of CV regulation undergoes a unique ontogenetic sequence with the adrenergic system composing the principal functional system during embryonic life.

Basal CV performance and maturation is achieved without tonic vagal input during chicken development. This is in sharp contrast to adults, which exhibit a pronounced vagal tone (Bagshaw and Cox 1986, Butler and Jones 1968, Jones and Johansen 1972). It must be assumed that CV function comes under vagal influence at "birth" with maturation occurring during postnatal development. Evidence for this post-natal onset of regulation is found in Chapter 3 work in which baroreflex activity is present at external egg piping. This trait, or lack of, has also been found in embryos of the desert tortoise *Gopherus agassizii* (D.Crossley, Chapter 8), suggesting that at least one reptilian species exhibit a similar ontogenic pattern. In addition, several studies have shown no or limited vagal tone on the CVS in fetal lambs in the latter third of gestation (Reller et al.

1989, Thornburg and Morton 1986, Thornburg and Morton 1983). Parasympathetic tone may therefore be of little importance to CV development in all vertebrates. Expansion of this experimental approach to other vertebrates will determine if vagal function is equally absent in other groups.

While this study and others have eliminated vagal input from basal CV function, the adrenergic system is operative in at least chickens and desert tortoise. As described here, chicken embryos rely on adrenergic tone to maintain basal CV function throughout development. Since there is a lack of sympathetic neural activity during ontogeny, adrenergic tone must be accomplished via circulating catecholamines (Pappano and Loffelholz 1974). The correlation between catecholamine concentrations and peak in CV sensitivity further supports their role in basal CV function of embryonic chickens. Similar characteristics are apparent in fetal lamb, which exhibit predominantly α–adrenergic activity with catecholamine levels peaking at term (Assali et al. 1977, Giussani et al. 1994, Reller et al. 1989). Collectively, these data suggest that embryonic vertebrates depend on hormonal adrenergic influence to control CV activity and possibly assure successful embryonic life.

TABLE 2.1

Drug amounts and injection volumes utilized at each stage of development.

	8d	12d	15d	19d	20d	21d
V_{blood} [a]	0.52	1.61	2.35	2.9	2.81	2.61
WM_{embryo} [b]	1.1	5	12.5	22.1	25.8	28.4
$WM_{membranes}$ [c]	1.8	3.9	5.2	4.9	4.6	4.5
$V_{inj,total}$ [d]	30	80	120	150	150	135
Atropine [e]	2.9	8.9	17.7	27	30.4	32.9
Propranolol [f]	8.7	26.7	53.2	80.9	91.3	98.7
Prazosin [g]	2.9	8.9	17.7	27	30.4	32.9

a V_{blood} - estimated volume of blood (μL) (Romanoff 1967)

b WM_{embryo} - wet mass of the embryo (g) (Romanoff 1967)

c $WM_{membranes}$ - wet mass of extraembryonic membranes including chorioallantoic and yolk membranes (g) (Romanoff 1967)

d Vinj,total - total volume injected (μL) = 1/3 drug + 2/3 flush volume

e Atropine (mg) - dose = 1 $mg \cdot kg^{-1}$;

f Propranolol (mg) - dose = 3 $mg \cdot kg^{-1}$

g Prazosin (mg) - dose = 1 $mg \cdot kg^{-1}$

TABLE 2.2

Heart rate and blood pressure data before (C) and after injection (T) of atropine.

		f_H	Sys	Dia	Map
8d	C	199±13	0.44±0.06	0.24±0.04	0.30±0.05
	T	196±7	0.41±0.06	0.21±0.04	0.28±0.05
12d	C	214±8	0.87±0.04	0.41±0.05	0.57±0.04
	T	205±10*	0.88±0.04	0.39±0.05	0.56±0.04
15d	C	227±6	2.25±0.13	1.10±0.13	1.49±0.12
	T	225±5	2.30±0.11	1.06±0.13	1.47±0.12
19d	C	206±9	3.21±0.09	1.86±0.07	2.31±0.05
	T	207±9	3.35±0.17	1.78±0.12	2.30±0.13
20d	C	251±9	3.39±0.22	2.19±0.15	2.59±0.15
	T	259±5	3.56±0.38	2.27±0.22	2.70±0.27
21d	C	260±9	4.60±0.58	2.57±0.15	3.25±0.25
	T	270±11	4.72±0.53	2.47±0.22	3.22±0.26

Data as mean ± s.e.m.. f_H – heart rate (beat·min^{-1}); Sys - systolic pressure (kPa); Dia - diastolic pressure (kPa); Map – mean arterial pressure (kPa). Asterisk (*) - significant difference after injection ($p < 0.05$).

TABLE 2.3

Heart rate and blood pressure data before (C) and after injection (T) of propranolol.

		f_H	Sys	Dia	Map
8d	C	191±5	0.38±0.04	0.20±0.04	0.26±0.04
	T	165±6*	0.44±0.05*	0.23±0.04	0.30±0.05*
12d	C	183±11	0.84±0.04	0.37±0.07	0.53±0.06
	T	161±13*	1.02±0.07*	0.48±0.1*	0.66±0.09*
15d	C	223±11	2.14±0.09	1.00±0.09	1.38±0.08
	T	188±14*	2.65±0.04*	1.14±0.08**	1.64±0.06*
19d	C	200±14	3.42±0.18	1.85±0.15	2.38±0.14
	T	169±15*	3.94±0.27*	2.05±0.22	2.68±0.23*
20d	C	256±6	3.41±0.20	2.08±0.14	2.52±0.15
	T	207±4*	3.97±0.24**	2.19±0.22*	2.78±0.21*
21d	C	251±14	4.39±0.50	2.47±0.15	3.11±0.25
	T	227±10*	4.64±0.51	2.40±0.14	3.15±0.23

Data as mean ± s.e.m.. Abbreviations as in Table 2. Asterisk (*) - significant difference after injection ($p < 0.05$).

TABLE 2.4

Heart rate and blood pressure data before (C) and after injection (T) of prazosin

		f_H	Sys	Dia	Map
8d	C	150±14	0.58±0.07	0.30±0.06	0.39±0.06
	T	145±13	0.53±0.07*	0.28±0.06	0.36±0.07*
12d	C	189±11	1.02±0.10	0.53±0.10	0.69±0.10
	T	179±10*	0.84±0.11*	0.43±0.10*	0.57±0.10*
15d	C	212±5	2.60±0.05	1.17±0.08	1.65±0.04
	T	202±7*	2.11±0.03*	0.81±0.06*	1.24±0.03*
19d	C	169±11	3.79±0.23	1.88±0.18	2.52±0.18
	T	114±20*	1.85±0.25*	0.75±0.06*	1.11±0.10*
20d	C	209±4	3.85±0.23	2.07±0.20	2.66±0.19
	T	194±7	2.81±0.22*	1.27±0.21*	1.78±0.20*
21d	C	233±11	4.83±0.36	2.38±0.25	3.20±0.25
	T	229±12	4.09±0.39	1.71±0.18	2.50±0.24

Data as mean ± s.e.m.. Abbreviations as in Table 2. Asterisk (*) - significant difference after injection ($p < 0.05$).

TABLE 2.5.

Plasma catecholamine concentrations ($ng \cdot ml^{-1}$) at each day of incubation tested.

	NA	A	D
8d	17.7±3.9a	0.1±0.1a	0.8±0.2a
12d	65.0±8.5a	2.3±0.3a	3.2±0.4b
15d	55.7±12.6a	2.4±0.8a	1.1±0.3a
19d	169.4±52.0b	80.5±1.0b	5.4±0.8b
20d	137.0±15.9b	71.2±8.8b	9.1±1.3c
21d	97.3±40.2a	51.2±19.9b	3.2±1.0b

Data as mean ± s.e.m.. Common letters indicate similar effects of drug injection between developmental stages.

FIGURE LEGENDS

Figure 2.1

A) Effects of acetylcholine pre- and post-atropine administration on blood pressure in a day 18 embryo. Notice that the immediate bradycardic effect of acetylcholine (the heart stopped and pressure dropped below 1 kPa, and recovered immediately thereafter) disappear after atropine. B) Effects of isoprenaline pre- and post-propranolol administration on blood pressure in a day 15 embryo. Notice the hypotensive effect of isoprenaline disappearing when the embryo is pre-treated with propranolol.

Figure 2.2

An illustration of the experimental protocol used throughout the study displaying an original trace taken from embryo #5 at day19 of incubation. Changes in pulsatile pressure and f_H are shown pre and post injection (at the arrows) of atropine (A), propranolol (B) and prazosin (C).

Figure 2.3

Effects of 1 $mg \cdot kg^{-1}$ atropine injection on embryonic heart rate (A) and mean arterial pressure (B) at each day of development tested. Data plotted as mean percentage change ± 1s.e.m.. Asterisk (*) represent significant differences induced by the antagonist ($p<0.05$).

Figure 2.4

Effects of 3 $mg \cdot kg^{-1}$ propranolol injection on embryonic heart rate (A) and mean arterial pressure (B) at each day of development tested. Data plotted as mean

percentage change ± 1s.e.m.. Asterisk (*) represent significant differences induced by the antagonist ($p<0.05$). Common letters indicate similar effects of drug injection between developmental stages as determined by a one way ANOVA on arcsine transformed percentage changes.

Figure 2.5

Effects of 1 $mg \cdot kg^{-1}$ prazosin injection on embryonic heart rate (A) and mean arterial pressure (B) at each day of development tested. Data plotted as mean percentage change ± 1s.e.m.. Asterisk (*) represents significant differences ($p<0.05$). Common letters indicate similar effects of drug injection between developmental stages as determined by a one way ANOVA on arcsine transformed percentage changes.

Figure 2.1

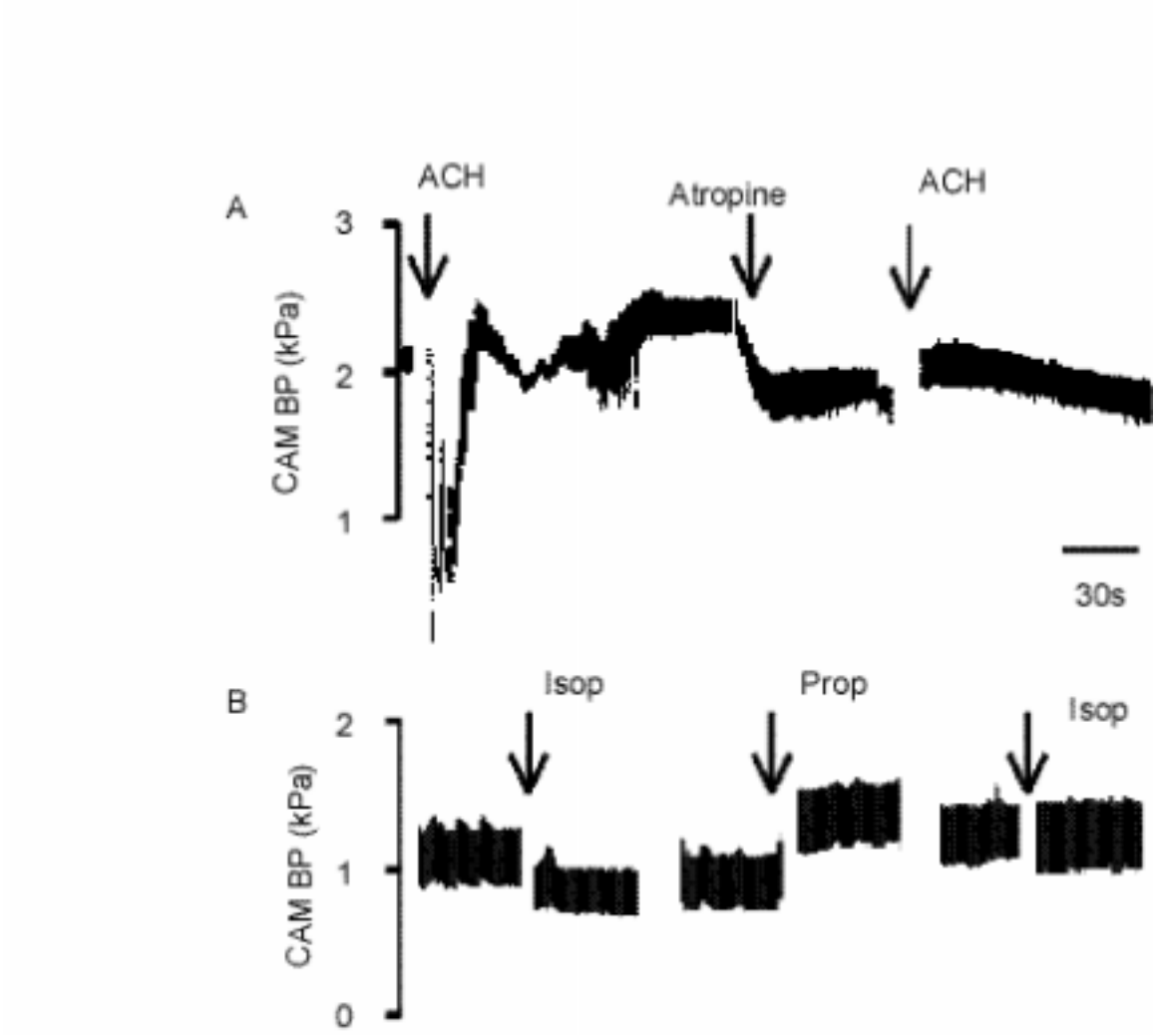

A
ACH
Atropine
ACH
CAM BP (kPa)
3
2
1
30s
B
Isop
Prop
Isop
CAM BP (kPa)
2
1
0

Figure2.2

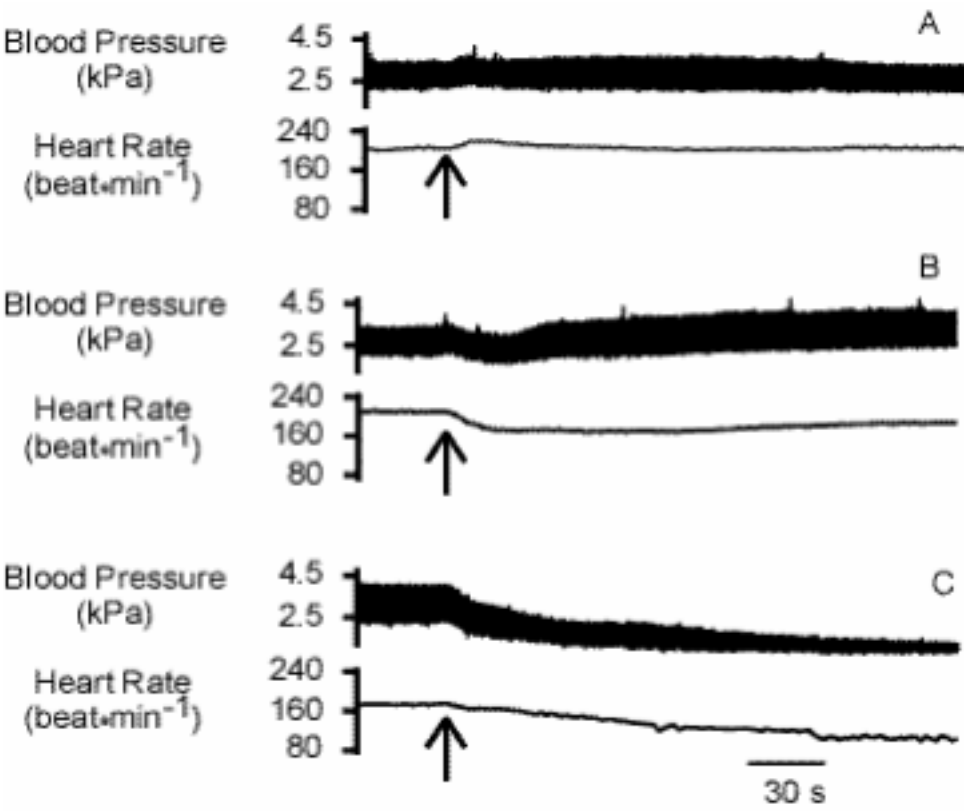

A
Blood Pressure
(kPa)
4.5
2.5
Heart Rate
(beat•min-1)
240
160
80
B
Blood Pressure
(kPa)
4.5
2.5
Heart Rate
(beat•min-1)
240
160
80
Blood Pressure
(kPa)
4.5
2.5
C
Heart Rate
(beat•min-1)
240
160
80
30 s

Figure 2.3

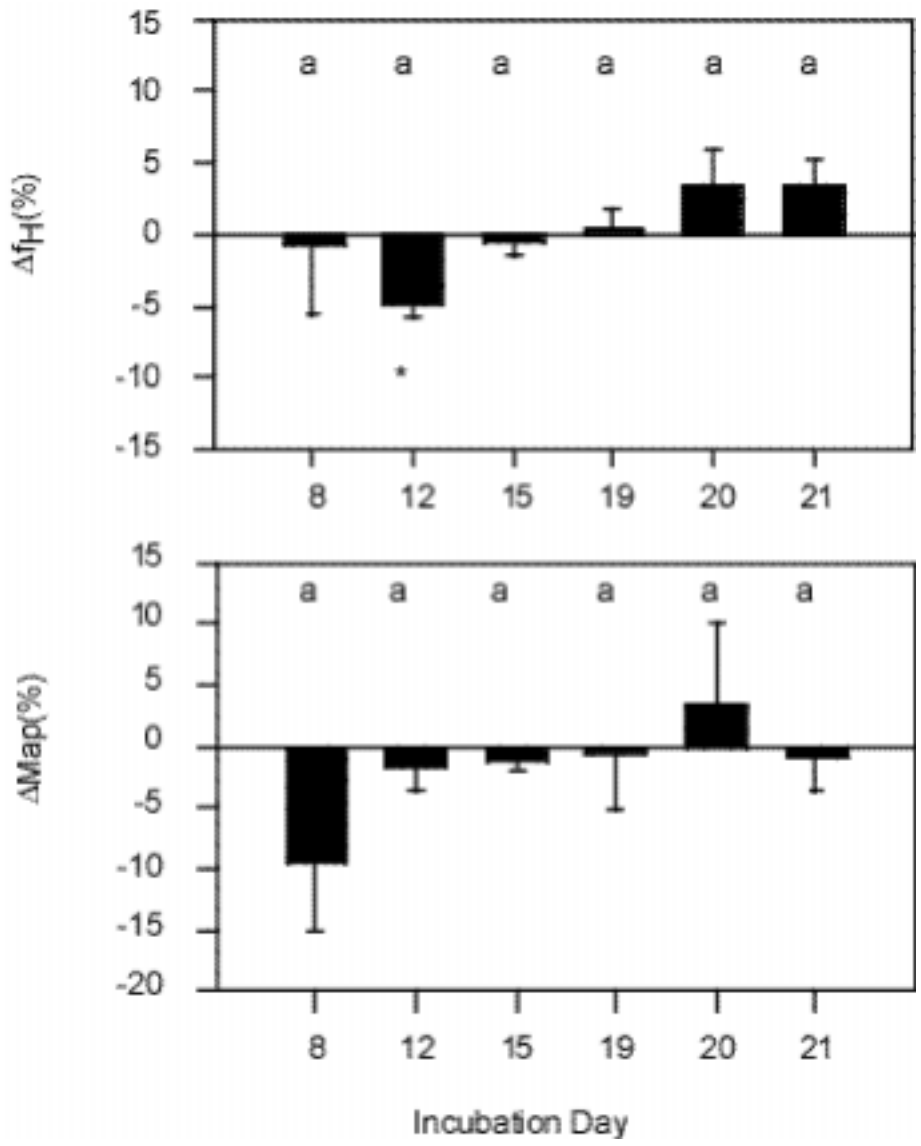

15
10
5
0
-5
-10
-15
a a a a a a
*
8 12 15 19 20 21
ΔfH(%)
15
10
5
0
-5
-10
-15
-20
a a a a a a
8 12 15 19 20 21
ΔMap(%)
Incubation Day

Figure 2.4

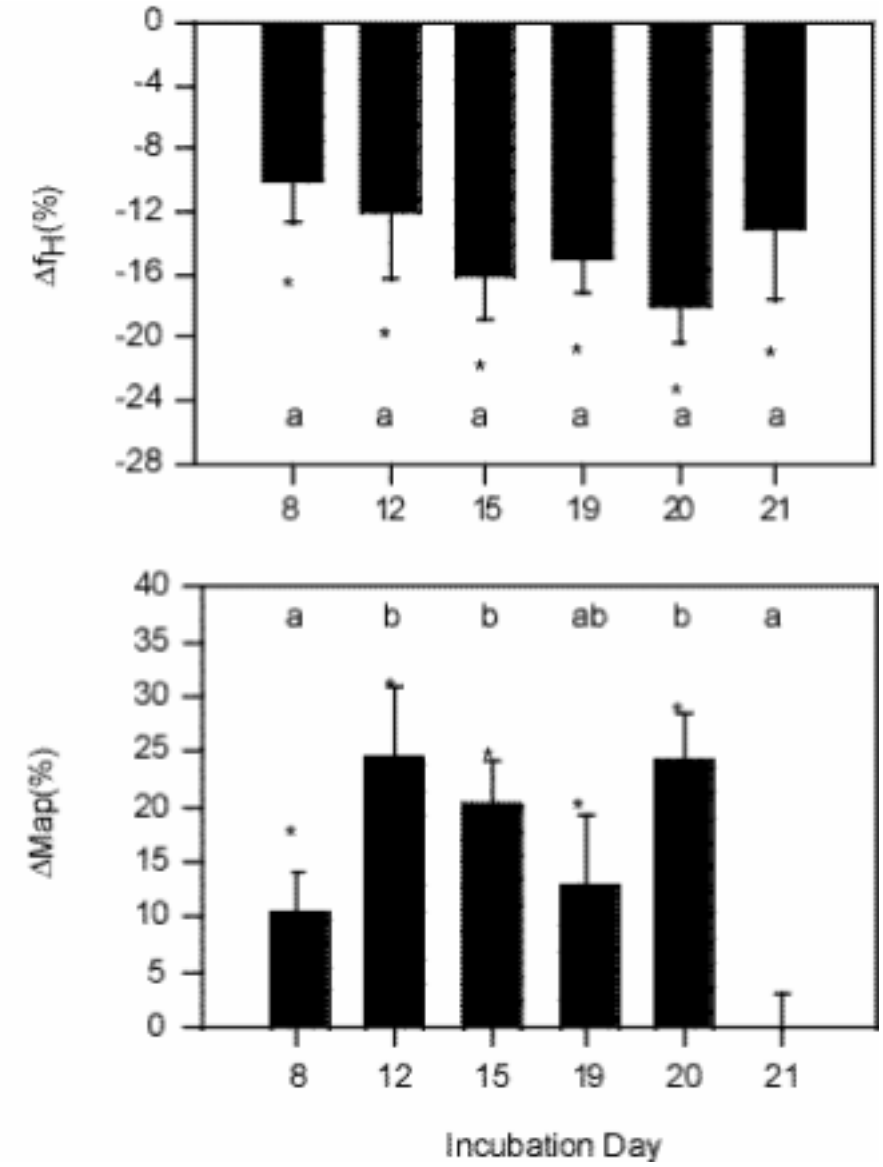
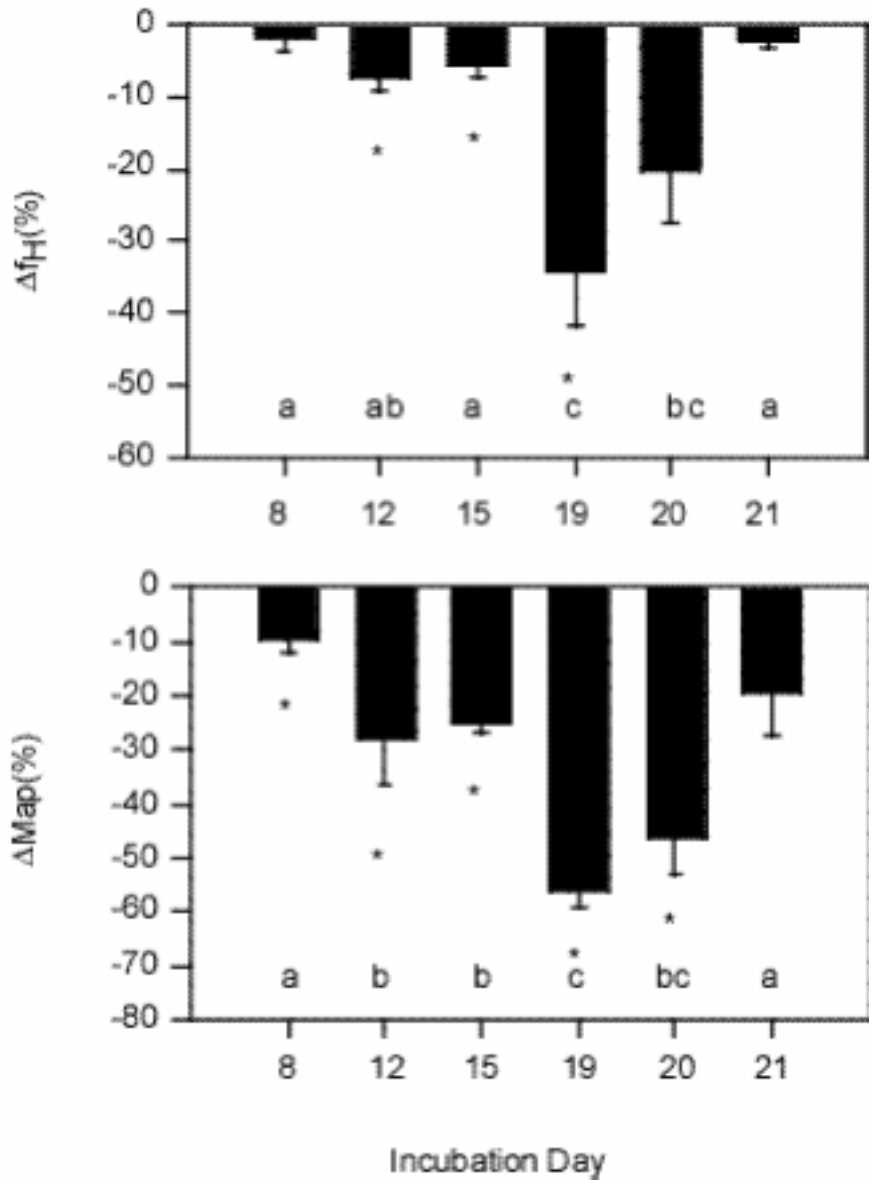

0
-4
-8
-12
-16
-20
-24
-28
ΔfH(%)
a a a a a a
8 12 15 19 20 21
40
35
30
25
20
15
10
5
0
ΔMap(%)
a b b ab b a
8 12 15 19 20 21
Incubation Day

Figure 2.5


0
-10
-20
-30
-40
-50
-60
Δf_H(%)
a
ab
a
c
bc
a
8
12
15
19
20
21
0
-10
-20
-30
-40
-50
-60
-70
-80
ΔMap(%)
a
b
b
c
bc
a
8
12
15
19
20
21
Incubation Day

CHAPTER III

CONTROL OF BLOOD PRESSURE MEDIATED BY BAROREFLEX CHANGES OF HEART RATE IN THE CHICKEN EMBRYO (Gallus gallus)

Introduction

Pressure differences between the arterial and venous sides of the cardiovascular system provide the necessary driving force to ensure adequate gas, as well as other essential components, are transfered to tissues. Alterations in arterial pressure that may compromise gas transfer are counteracted by both central and local homeostatic mechanisms. These mechanisms offset the perturbation, thereby avoiding hypertensive or hypotensive episodes and the myriad of pathologies these conditions could induce (Van Vliet and West 1994).

Among regulatory systems, the baroreflex is the most prominent short-term compensatory mechanism utilized during arterial pressure challenges. This compensatory system results in alterations of cardiac output and peripheral resistance via the cardiac and peripheral limb of the baroreflex respectively. Although baroreflex responses are well characterized in a variety of adult vertebrates (Altimiras et al. 1998), little is known of the maturation of this mechanism.

In the fetal lamb, the standard mammalian model, an unequivocal baroreflex response is present during the final trimester of gestation (Dawes et

al. 1980, Maloney et al. 1977, Segar et al. 1992, Shinebourne et al. 1972). While this model is attractive for its link to clinical pediatrics, an alternative experimental model with short extra-uterine development would be instrumental in exploring the course of baroreflex maturation at different organizational levels. Such a model would also be important to the construction of a generalized picture of the ontogeny of cardiovascular regulation in vertebrates.

The chicken embryo may be such a vertebrate model, with the advantage of a shortened gestational time andease of embryonic manipulation. In addition, mammalian-like circulation of the chicken embryo with an extra-embryonic circuit involved in gas exchange (the chorioallantois) analogous to the placenta make findings applicable to mammalian development (Metcalfe and Stock 1993). Further, its use in the analysis of chemoreflex cardiovascular regulation has been recently shown (Mulder et al. 1998).

Finally, the maturation of many physiological processes is well characterized in chicken embryos, providing essential information for an in-depth study. From previous studies on autonomic cardiovascular regulation, functional vagal innervation appears on day 12 of development (Pappano and Loffelholz 1974), implying that a hypertensive baroreflex could be operational during the latter half of chicken ontogeny. This work sought to test the hypothesis that baroreflex function appears during incubation as it occurs in the fetal lamb.

Materials and Methods

Animals and incubation conditions

Experiments were conducted in two separate locations. Chicken embryos up to 19 days of incubation were studied at the Department of Biological Sciences, University of Nevada, Las Vegas (referred to as LAB1). Chicken embryos from 19-21 days of incubation as well as hatchlings were studied at the Department of Zoophysiology, University of Aarhus (referred to as LAB2). LAB1 studies utilized White Leghorn chicken eggs of the Hyline II strain purchased from Hyline (LakeView, CA) and incubated in a walk-in environmental chamber (Labline Instruments Inc., Melrose Park, IL). LAB2 studies utilized Plymouth chicken eggs of the Russ 208 strain purchased from Fællesrugeriet (Randers, Denmark) and incubated in a custom made incubator. In both cases incubation temperature was maintained at 38±0.5 °C and 60% humidity.

Cannulation procedures

At selected developmental ages, eggs were removed from the incubator and candled to locate a major chorioallantoic artery. For each day of study a minimum of 5 eggs were used for study. Arteries were distinguished from veins based on their course under the shell and the direction of vessel branching. Once the course of the artery had been traced, it was exposed by opening a small window (smaller than 10 x 10 mm) in the eggshell and peeling off the underlying shell membranes. A fluid-filled, polyethylene cannula (PE-90 1.27 mm OD / 0.86 mm ID, Clay-Adams) heat pulled to a tip diameter smaller than 0.5 mm OD was used to occlusively catheterize the smallest branching artery. The operation was carried out under a dissection microscope with the aid of fine microsurgery

instruments under temperature controlled conditions. In all cases surgical procedures were completed within 15 minutes. Special care was taken to avoid bleeding due to the extensive anastomosed chorio-allantoic membrane (CAM) circulation. In all procedures, a ligature was placed downstream to eliminate retrograde flow and heat cauterization was used for smaller vessels. The catheter was then carefully aligned with the vessel and glued to the eggshell with tissue glue (VetBond 3M). Following completion of the procedure, eggs were placed in a temperature controlled vermiculite bath (38 °C) for the duration of each study, commonly less than one hour.

Studies on recently-hatched chicks were conducted on day 2 post-hatch under halothane anesthesia. The animals were anaesthetized with halothane in a closed chamber until righting and corneal reflexes disappeared. Halothane, 0.5-1%, (in air) was delivered thereafter through a plastic gas flow chamber adjusted to the head of the animal. The level of anesthesia was carefully maintained to allow spontaneous ventilation and prevent pedal reflexes. The right femoral artery was occlusively cannulated for blood pressure and heart rate recording.

Blood pressure measurement and calibration

Blood pressure traces were obtained via the fluid filled catheter connected to a P23 Statham pressure transducer, which was calibrated against a static water column. Heart rate (f_H) was obtained from the pressure signal.

Pressure transducers were connected to a recorder (Narco Biotrace or Beckman R21) for proper amplification and sampled at 250 Hz by a computer via an AD card and LabView custom-made acquisition software.

The small diameter of the catheter heat-pulled tip was observed to dampen the frequency-response of the pressure recording system in earlier embryos, when CAM circulation is still poorly developed (9 and 12 days of incubation). Since the goal of the study was the analysis of baroreflex responses induced by alteration of mean blood pressure, no attempt was made to correct for the frequency response of the catheter. In all cases, data are reported as mean arterial pressure (Map).

In previous studies where blood pressure was characterized during development in chicken embryos, there has been no mention of a reference point of circulatory pressures (Girard 1973, Tazawa 1981a). Studies in the fetal lamb have used the pressure in the amniotic sac or the pericardium as reference (Dawes et al. 1980, Reller 1989, Shinebourne et al. 1972). Both approaches would involve a more extensive surgery if employed in chicken embryos. Thus, the problem was solved in two steps. During the experimental protocol, zero pressure was arbitrarily set at the top edge of the eggshell (Figure 1). Once the experiment was completed, the animal was euthanized and quickly frozen at –20 °C. Following this procedure, the egg was cut along the longitudinal axis to determine the relative position of the embryo and the heart. The distance from

the heart to the eggshell was then taken as a pressure head (error) offsetting the measured values.

Experimental protocol and drug infusion

The experimental protocol consisted of the pharmacological manipulation of mean blood pressure via a dose-related alteration of peripheral resistances. The α–adrenoceptor agonist phenylephrine (PHE) was used to increase Map by inducing a generalized vasoconstriction of the peripheral vasculature, while sodium nitroprusside (SNP) induced general vasodilation by the release of nitric oxide. A representative trace of drug effects in early (9d) and late embryos (21d) is shown in Figure 2.

All injection volumes were normalized to 5% of the total blood volume (V_{blood}) at a given embryonic age as determined from literature data (Romanoff 1967, see chapter 2 for details). Preliminary experiments demonstrated that a 5% V_{blood} bolus of saline had no apparent cardiovascular effects.

Drug concentrations were based on preliminary tests to establish the degree of vasoactivity. All dosages were normalized to the total wet mass of the embryos and egg membranes, and ranged between 20-100 $mg \cdot kg^{-1}$ for SNP and PHE. Progressively greater concentrations of one randomly selected drug was injected into each embryo, with cardiovascular effects recorded. The entire protocol lasted less than 60 minutes to avoid secondary alterations in hydration conditions when the eggshell was opened. Embryos were then euthanized with

xylocaine (5 mg) before freezing. Hatchlings were euthanized with Halothane as well as injection of potassium chloride through the femoral catheter.

Calculations and statistics

The gain of the baroreflex was calculated as:

Gain = $(-1)^* \Delta f_H/\Delta Map$

And expressed as $beats \cdot min^{-1} \cdot kPa^{-1}$

Since a baroreflex is characterized by reciprocal responses between heart rate and blood pressure, the slope of this response would be negative, while the gain is positive.

To establish meaningful intra-specific and inter-specific comparisons, gain was normalized with respect to control Map and f_H as:

Normalized Gain = Gain * Map / f_H

And expressed as $\%\Delta f_H \cdot \%Map^{-1}$

Resting blood pressures between stages were compared using a one-way ANOVA and the Newman-Keuls posthoc test. f_H-Map sensitivity was analyzed using common linear regression methods. Tests were carried out using Statistica statistical software.

A zero gain ($\mu=0$) would be predicted if f_H and Map were totally independent of each other. Thus, the experimental gain at each stage was tested against $\mu=0$ with the two-tailed t-distribution. Data are shown as mean ± s.e.m. Significant differences were all taken at the fiduciary level $p<0.05$.

Results

Map shows a consistent increase during incubation, from 1.01 ± 0.02 kPa at day 9 to 3.18 ± 0.28 kPa during external pipping (21d), (Table 3.1). Blood pressure in earlier stages (9d, 12d and 15d) was significantly lower than in later stages ($p<0.05$). No differences in blood pressure were observed between the two strains of chickens used at day19 (2.68 ± 0.29 kPa WL vs. 2.40 ± 0.21 kPa Ply).

In early embryos, the patterns of change in heart rate and blood pressure were similar as vascular tension was manipulated. This trend was altered in late embryos, which exhibited a reciprocal relation between parameters, i.e. increased pressure inducing a bradycardia while decreased pressure resulted in a tachycardia (Fig. 3.2). Changes in f_H-Map sensitivity over development are detailed in Table 3.2. Utilizing 139 pharmacological injections in 54 embryos, f_H-Map sensitivity can be estimated as the average slope (Fig. 3.3). Slope of the f_H-Map curve was positive during early development up to day 19, indicating a lack of baroreflex responses. The largest slope of 204 beats·min^{-1}·kPa^{-1} was observed on day 9, and decreased to 9 beats·min^{-1}·kPa^{-1} at day 19. The coefficient of determination (r^2), i.e., the amount of variance explained by a linear relationship between both variables decreased likewise from 88% at day 9 to 17% at day19, see Table 2). In late embryos, (21d) and 2-day old hatchlings f_H-Map were reciprocal, indicating active baroreflex function. This was best shown by excluding those drug trials where f_H and Map varied concurrently, thus providing a maximal estimate of the f_H-Map slope when only baroreflex responses

occurred. In this case, the maximum slope was negative from day 18 onwards, and peaked on day 20 at –34 beats·min^{-1}·kPa^{-1} ($p<0.05$; Table 2).

These results were mirrored when calculated per animal basis as shown in Figure 3.4A and Table 3.3. The average baroreflex gain (Gain) was negative during early development up to day 19, with a minimum value of -246 ± 75 beats·min-1·kPa^{-1} at day 15 and increased progressively to -37 ± 0 beats·min^{-1}·kPa^{-1} at day 19 (Fig.3.4A). Values from day 9 and 15 were significantly different from all other stages ($p<0.05$). Later in development, gain became positive, oscillating between 25 ± 19 beats·min^{-1}·kPa^{-1} at day 20 to 53 ± 43 beats·min^{-1}·kPa^{-1} at day 21. Only the highest sensitivity on day 15 was shown significantly different from zero ($p<0.05$).

The maximal gain of the baroreflex ($Gain_{max}$), obtained by averaging the gain of the animals that showed the response was positive from day18 to the end of development. Gain values increased progressively from 13 ± 7 beats·min^{-1}·kPa^{-1} at day 18 to 105 ± 83 beats·min^{-1}·kPa^{-1} in two day hatchlings. In Figure 4B the relative proportion of animals with prevalent baroreflex responses are shown. Responses with sensitivities between ± 10 beats·min^{-1}·kPa^{-1} were considered uncertain. No animals had a prevalent baroreflex until day 18. The frequency increased to 33% at day 19, 44% at day 20and 56% at day 21.

Discussion

The present investigation was undertaken in an effort to establish the critical period at which baroreflex regulation becomes operational in chicken

embryos. In addition, change in gain was determined to establish the pattern of of baroreflex development during embryonic life. The data definitely show that this reflex is inactive throughout 90% of incubation, and it demonstrates a increase in gain for late embryonic development to early neonatal life.

Critique of method

The absolute blood pressures reported are based on heart position as determined after freezing. This approach could have resulted in a displacement of the embryo during freezing, altering the overall calibration. This is a legitimate concern considering the heterogeneous nature of an egg, with an aqueous albumen phase and a lipid yolk phase. However, the potential error is relatively unimportant considering the reduced dimensions of the egg (4 cm as an estimate for the short axis) and the embryonic age used in this study. The maximal error if the zero was set at a fixed point along the midline would be ± 0.2 kPa, equivalent to a range of ±20- ±6% difference in pressure from day 9 to 21. A similar method has also been utilized in the fetal lamb by adjusting the zero reference to the midline of the uterus (Shinebourne et al. 1972). Therefore, the reference method proposed here or an alternative should be considered if working with embryos prior to 9 days of age.

An additional criticism could be the use of pharmacological tools to manipulate peripheral resistance in an attempt to invoke the cardiac limb of the reflex. This experimental approach eliminates the ability of the embryo to alter peripheral resistance known to be an important component of the baroreflex and

potentially could bias our conclusions. Indeed, several studies in the fetal lamb have established that the use of phenylephrine to load the baroreceptors render higher gains in comparison to the use of vascular occluders (Dawes et al. 1980, Maloney et al. 1977). It is important to recognize that while sensitivities of the cardiac limb might be overestimated, a reflex was not masked by pharmacological challenge in the fetal lamb, indicating that this approach is valid.

Data Comparisons

Blood pressure values reported in this study are in good agreement with previous work on chicken embryos (Fig. 3.5), and display a progressive rise throughout development. No significant pressure differences were evident between the two strains of chicken used (White Leghorn and Plymouth) at day19 of incubation (Table 3.1). Subsequently, the results were pooled and the two strains considered identical in relation to blood pressure.

Arterial pressures measured in neonates (2 days post hatching) in this study were considerably lower than previously measured in non-anaesthetized animals (Girard 1973). This could be attributed to a depression in cardiac function usually associated with halothane anesthesia (Biscoe and Millar 1964).

Onset of baroreflex function

As shown in Figure 3.2, cardiovascular responses were markedly different between early and late embryos. Late embryos demonstrated a reaction consistent with a functional adult baroreflex response. This response appeared progressively, starting at day 18 of incubation. However, considering the low

gains of the baroreflex at this stage (Table 3.3), the onset of baroreflex regulation should be considered at day 19 of incubation. This is equivalent to 90% of incubation in chicken embryos, which is within the range of baroreflex onset in several mammalian species. It should be noted, however, that little information could be gained from a direct comparison, since the range between species is from 60% of gestation in the fetal lamb (Shinebourne et al. 1972) to neonatal appearance in the rabbit (Gootman et al. 1979).

In chicken embryos, the baroreflex activation at 90% incubation time does not appear as an all or none event i.e. some embryos had a reflex while others lacked it at all times. Activation in chicken embryos was progressive with the percentage demonstrating a baroreflex gradually increasing over the last 10% of development. Mild reflexive responses were first evident in day 18 embryos, while the average slope $\Delta f_H/\Delta Map$ was positive due to the limited number showing a negative slope. If positive slopes were excluded, the average gain in day 18 embryos would be positive at 13± 7 beats·min^{-1}·kPa^{-1}. By day 19, 33% of the embryos showed consistent baroreflex responses and this proportion increased at day 20 and 21. This finding was similar to what has been reported in the fetal lamb (Shinebourne et al. 1972), with the frequency of animals displaying baroreflex responses increased from 60% to 80%. The variability of activation appearance between individuals was unexpectedly wide. This variablity suggests that onset is less reliant on developmental age, possibly indicating that other

factors such as mean arterial pressure, the onset of lung ventilation or the relative contribution of each limb may varying between individuals.

Data from the fetal lamb provide evidence for the importance of other factors determining the onset of baroreflex. Results from the fetal lamb have shown that the baroreflex is triggered when the set point for arterial pressure is reached while the operational set point increases with development, simultaneously to blood pressure increases (Blanco et al. 1988, Segar et al. 1992). Hence, it is possible that individual differences in resting arterial pressure could explain the absence of baroreflex responses. However, there appears to be no correlation between reflex gain and control Map. Thus, this may not explain the variation in onset age demonstrated in embryonic chickens. The possible coupling between the onset of lung ventilation and the onset of baroreflex control is unlikely. In the fetal lamb, baroreflex activity is present prior to respiration. Further, 20% of chicken embryos during days 20 and 21 showed no baroreflex activity, despite the fact that the animals were already ventilating. A change in the relative contribution of each limb of the baroreflex between embryos remains a viable possibility, but without new experimental data, this is only a speculation. It is possible that embryos relying on the peripheral limb of the reflex show a diminished heart rate effect. In fact, studies in the fetal lamb have suggested that the baroreflex is more dependent on peripheral resistance than on heart rate (Blanco et al. 1988). Clearly, this could be the case in chicken

embryos but, as stated, extensive experimentation is necessary to clarify this issue.

Mechanisms of blood pressure control

Although a functional vagal component is present by day 12 of incubation (Pappano and Loffelholz 1974), our results suggest no activation by pressure changes until day 19. In Chapter 2, it was demonstrated that atropine has no chronotropic effect throughout incubation, a finding in direct conflict with the current results. It is proposed that the vagus is functional but inactive during normal beat-to-beat cardiovascular regulation. However, during periods of vascular stress in late stages of development, at least those induced with hypotensive or hypertensive drugs, the vagus could be transiently operational. This hypothesis, while not directly tested in these studies, has support from circumstantial experimental data discussed in Chapter 2 and remains a viable scenario.

A second possible explaination for the existence of baroreflex responses without a tonic efferent vagal activity could involve sympathetic efferents. However, this possibility is less likely considering that in mammals, sympathetic innervation is only involved in hypotensive episodes and that functional chronotropic adrenergic innervation is not operative until 21 days of incubation (Pappano and Loffelholz 1974). Again, further experimentation is required to clarify this issue during the ontogeny in chickens.

Pre- and post-natal maturation of the baroreflex

A clear reflex maturation occurred prior to hatching as illustrated in Figure 3.4. $\Delta f_H/\Delta$Map slope was initially positive, indicating a feed-forward influence of arterial pressure on heart rate. This feed-forward effect can be explained by mechanical properties of the system. Increased Map results in an increased venous return, thus decreasing filling time as well as raising the degree of SA stretch, elevating heart rate.

A similar phenomenon was observed in the fetal lamb (Faber et al. 1974). The inverse scenario explains cardiac events that follow infusion of SNP in early embryos. These effects included a reduction in heart rate, which followed SNP induced reduction in arterial pressure. Overall, these reactions are clearly non-homeostatic given the lack of pressure recovery following the administration of vasoactive drugs in early chicken embryos. In addition, they unveil the limited physiological repertoire early embryos have to compensate for acute stress, as previously shown during anoxia (Mulder et al. 1998) or hypoxia (Tazawa 1981a). Feed-forward effects were less accentuated in day 18 embryos and older, which could be attributed to the appearance of reflex cardiovascular regulation via baroreflex as well as chemoreflex regulation as recently suggested (Mulder et al. 1998). The degree of baroreflex maturation from day 18 to hatching was less definitive in the present study. Based on average gain, there appeared to be no significant difference from zero gain due to the level of data variability (Table 3.3). If, however, an analysis is conducted on only those embryos that displayed a prevalent baroreflex, the gain is significantly different from zero in day 19 and

20 embryos. In addition, a steady increase in gain from day 18 to hatching is demonstrated (Table 3.3); thereafter, there is a progressive maturation of the cardiac limb of the baroreflex in chicken embryos. Utilizing literature values for adult chickens this maturation is incomplete at hatch and must continue to reach a normalized gain of 0.92 $\%\Delta f_H \cdot \%Map^{-1}$ (Bagshaw and Cox 1986) from 0.55 $\%\Delta fH \cdot \%Map^{-1}$ at day 21. Although the recorded gain in 2 day post-hatching chickens were also similar to adult baroreflex gain (Table 3.3), such high values could be simply related with depressed mean arterial pressure due to anaesthetic effects. Clearly, data on non-anaesthetized post-hatched chicks is needed to further verify if baroreflex maturation is complete 2 days after hatching.

Although the maturation of the reflex prior to hatching was expected, previous studies in the fetal lamb provided different conclusions. Several authors have indicated that baroreflex gain increases (Dawes et al. 1980, Shinebourne et al. 1972) while other have shown no change (Maloney et al. 1977) or even a decrease (Faber et al 1974). Thus, it appears unlikely that baroreflex maturation follows a similar pattern in different vertebrate species. This statement is based on a single comparison of the development of arterial pressure and the appearance of baroreflex regulation in the chicken embryo and the fetal lamb. As shown in Figure 3.5, the appearance of a baroreflex in the lamb occurs when arterial pressure is 50% of adult blood pressure, while in chicken embryos blood pressure is only 21% of the adult arterial pressure. This conclusion has been formulated before (Gootman et al. 1979) but it is not demonstrated, which

prompts for more detailed inter-specific studies to understand the driving forces of cardiovascular regulation.

Conclusion

Relevance of Baroreflex Activity *in ovo*

This work has provided the basis for two conclusions. First, there is a late onset of baroreflex regulation and second, the gain of the reflex exhibits maturation over the final 3 days of incubation in the chicken embryo. Although the maturation of baroreflex CVS control is clearly significant for newly hatched precocial chicks, its late appearance questions its importance *in ovo*. Indeed, the pharmacological manipulation of blood pressure shows the potential for embryonic baroreflex regulation, but fails to reveal if baroreceptors are active during normal development.

This shortcoming has been addressed in the fetal lamb with recordings from baroreceptor afferents. There is an increased activity in the carotid nerve in synchrony with the pressure pulse (Blanco et al. 1988), showing that baroreceptors are loaded. In addition, studies in lambs with baroreceptor denervation revealed an increased variability in blood pressure, suggesting that blood pressure homeostasis was impaired without baroreflex feedback (Itskovitz et al. 1983, Yardley et al. 1983). Thus, baroreflex control of blood pressure is relevant for the correct development of peripheral resistance (Itskovitz et al. 1983) and aid in the proper development of organ systems (Dutton et al. 1978).

Finally, baroreflex control has been implicated in cardiovascular homeostasis during fetal movements, specifically fetal breathing movements (Dawes et al. 1972, Fouron et al. 1975). Although movements in the chicken embryo occur as early as day 5 and increase substantially at the end of development (Kou 1932), distinct individualized movements of the thoracic muscles do not occur until 17-18 days of incubation (Kou 1937). Given the space restriction due to the size of the embryo, the compression of vessels and sudden changes in cardiovascular resistance would be more likely at those late stages. In addition, the initiation of increased pulmonary blood flow, which must occur during internal and external pipping, could result in resistance changes requiring fast regulation from the baroreflex. The correlation between baroreflex onset, as well as gain, and embryonic breathing movements or proper organ development, needs to be verified experimentally.

TABLE 3.1

Control mean arterial pressures at different stages of incubation

	Strain	Map	N
9d	WL	1.01± 0.02	(3) a
12d	WL	1.08± 0.08	(4) a
15d	WL	1.15± 0.08	(5) a
18d	WL	2.22± 0.17	(6) b
19d	WL	2.68± 0.29	(4) b
19d	Ply	2.40± 0.21	(8) b
20d	Ply	2.80± 0.13	(9) b
21d	Ply	3.18± 0.28	(9) b
Hatch	Ply	2.40± 0.33	(5) b

Data as mean ± s.e.m. (N). MAP – mean arterial pressure (kPa); WL – White Leghorn strain; Ply – Plymouth strain. Dissimilar letters indicate significant differences in Map.

TABLE 3.2

f_H-Map sensitivity at different stages of development based on the analysis of the f_H-Map slope per trial.

	Slope	r	Slopemax	r
9d	204 (7)	0.94 *	-	-
12d	91 (7)	0.76 *	-	-
15d	160 (8)	0.83 *	-	-
18d	16 (9)	0.73 *	-2 (2)	-
19d	9 (33)	0.41 *	-25 (7)	0.92 *
20d	0 (36)	0	-34 (12)	0.74 *
21d	-5 (31)	0.26	-16 (14)	0.56 *
Hatch	-21 (8)	0.44	-13 (4)	0.28

Slope - f_H-Map slope (beats·min^{-1}·kPa^{-1}). Number of trials in parentheses. r indicates the goodness-of-fit to a linear regression of the data. Slopemax - f_H-Map slope when only baroreflex responses were considered. * - significant differences from zero sensitivity ($p<0.05$). N=5 for all analyses unless noted.

TABLE 3.3

Baroreflex gain in the latest stages of development.

	Gain	% Gain	$Gain_{max}$	$\%Gain_{max}$
18d	-10±8 (6)	-0.09	13±7 (2)	0.06
19d	-37±0 (12)	-0.41	21±7 (4) *	0.22
20d	25±19 (9)	0.25	40±21(7) *	0.44
21d	53±43 (9)	0.48	60±48(8)	0.55
Hatch	40±38 (5)	0.34	105±83(2)	0.93
Adult		0.92		

Data as mean ± s.e.m.. Gain - Average baroreflex gain (beats·min^{-1}·kPa^{-1}); % Gain - Normalized (unitless variable); Gainmax – maximal baroreflex gain (see text for details) (beats·min^{-1}·kPa^{-1}); %Gainmax – normalized maximal baroreflex gain (unitless). (*) – significant differences from zero gain ($p<0.05$). N=5 for all analyses unless noted.

FIGURE LEGENDS

Figure 3.1

Schematic drawing of the experimental setup. In particular, notice the zero pressure reference point is the eggshell, not the level of the embryonic atrium. Subsequently pressures were corrected by adding an offset factor as described in the Material & Methods section.

Figure 3.2

Changes in blood pressure (kPa) and heart rate (beat·min^{-1}) after injection of sodium nitroprusside and phenylephrinein a 9 day embryo (dotted line) and 21 day embryo (continuous line).

Figure 3.3

Plot of the heart rate changes induced by pharmacological alteration of blood pressure at different stages of development. From top-to-bottom and left-to-right: 9d (n=7), 12d (n=7), 15d (n=8), 18d (n=9), 19d (n=33), 20d (n=36), 21d (n=31) and 2d hatchlings (n=8). Each data point corresponds to one pharmacological trial from the number of trials specified above at each stage. Solid lines represent the best linear fit through the entire data set, dotted lines represent the best linear fit through those trials with a positive baroreflex response ($Slope_{max}$).

Figure 3.4

A) The average gain for the relationship between heart rate and on each day of development. Data as mean ± s.e.m.

B) Relative frequency of animals showing a prevalent baroreflex response. Open bars – non-reciprocal heart rate-blood pressure change; closed bars – baroreflex response (reciprocal heart rate-blood pressure change) and shaded bars – uncertain responses (see text for description).

Figure 3.5

Comparison between mean blood pressure and baroreflex gain in chicken and sheep from the start of incubation to adulthood. Pressure data in circles and crosses tracked by a dotted line. Data from this study shown as open circles (mean ± s.e.m.) in the chicken plot; notice that most of the error bars are inscribed within the symbols. Closed triangles tracked by a broken line represent baroreflex gain. Map data obtained from previous studies (Girard 1973, Hochel et al. 1998, Tazawa 1981, Van Mierop and Bertuch 1967). Data on baroreflex gain based on results from this study and previous literature (Bagshaw and Cox 1986, Dawes et al 1980, Maloney et al. 1977, Segar et al. 1992, Shinebourne et al. 1972). Hatching time is indicated by the shadowed line.

Figure 3.1

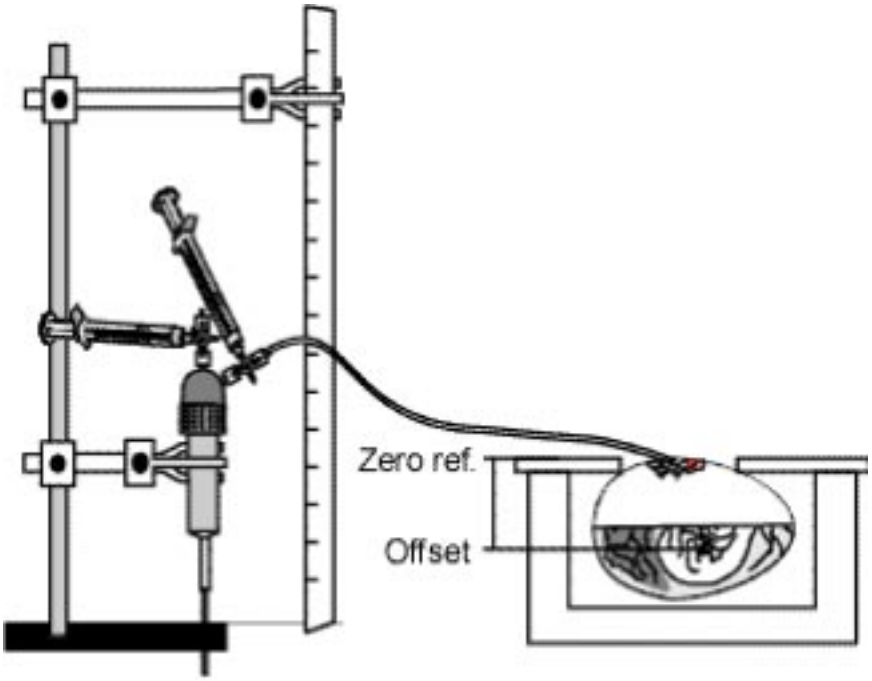


Figure 3.2

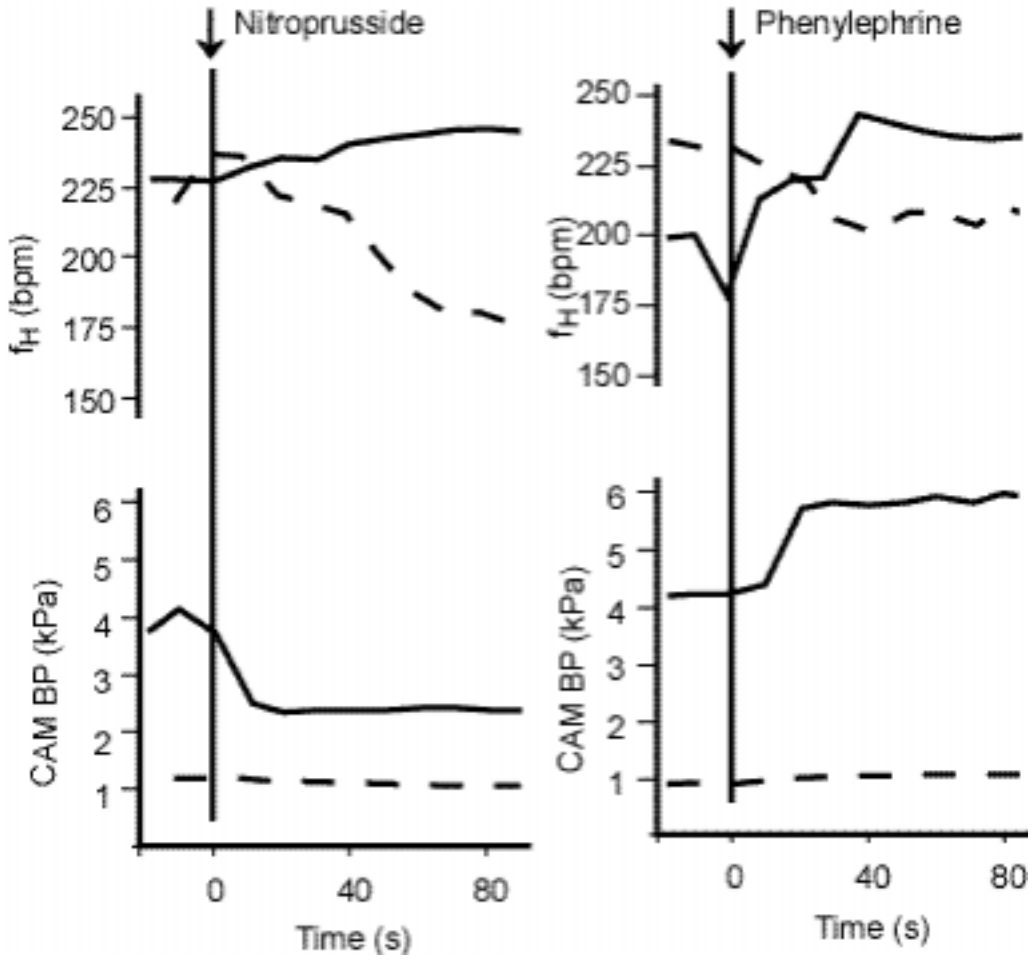

Nitroprusside
Phenylephrine
250
225
200
175
150
f_H (bpm)
6
5
4
3
2
1
CAM BP (kPa)
0
40
80
Time (s)

Figure 3.3

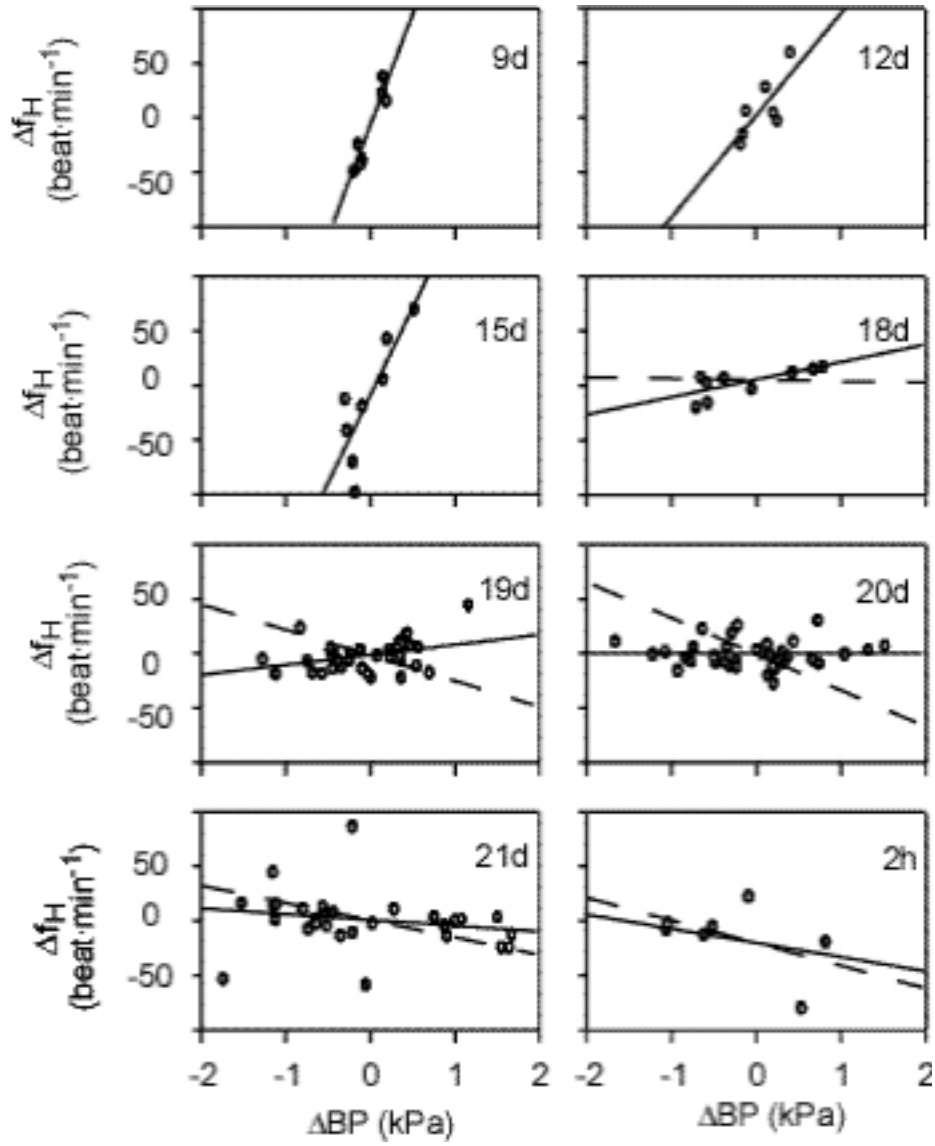

9d
12d
15d
18d
19d
20d
21d
2h
Δf_H (beat·min^{-1})
50
0
-50
-2
-1
0
1
2
ΔBP (kPa)

Figure 3.4

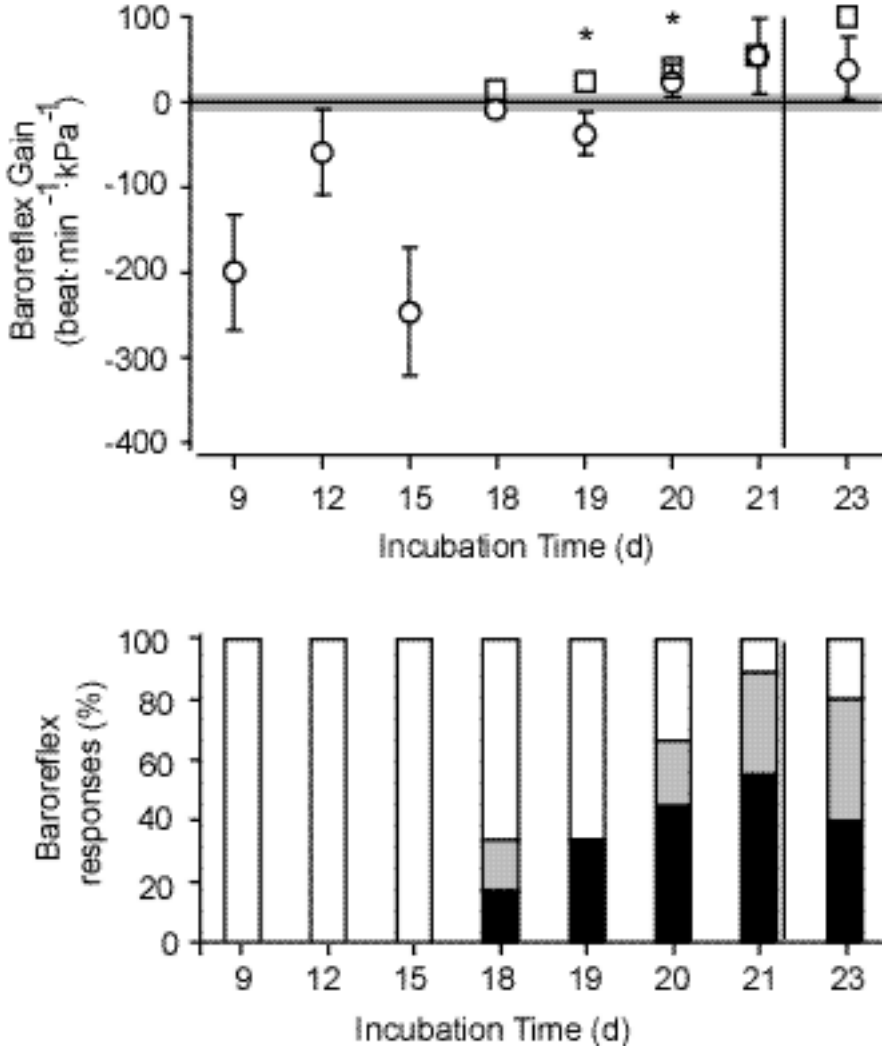
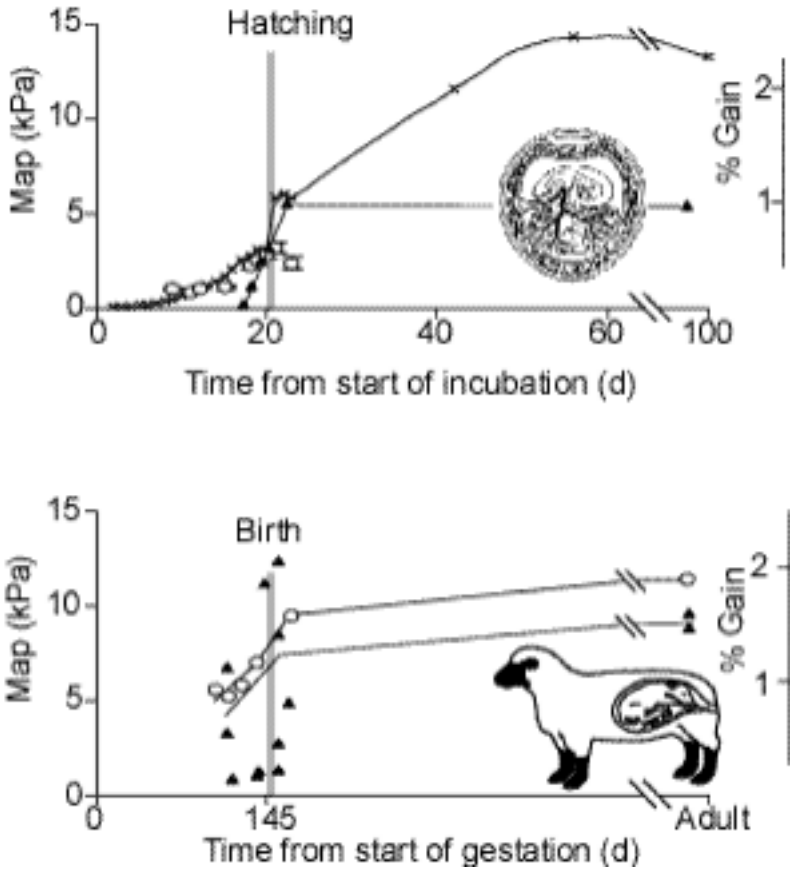

100
0
-100
-200
-300
-400
Baroreflex Gain
(beat·min -1·kPa-1)
*
*
9
12
15
18
19
20
21
23
Incubation Time (d)
100
80
60
40
20
0
Baroreflex
responses (%)
9
12
15
18
19
20
21
23
Incubation Time (d)

Figure 3.5


Hatching
Map (kPa)
% Gain
Time from start of incubation (d)
Birth
Map (kPa)
% Gain
145
Adult
Time from start of gestation (d)

CHAPTER IV

ASSESSMENT OF SYMPATHETIC VIABILITY DURING DEVELOPMENT IN THE DOMESTIC CHICKEN, *Gallus gallus*.

Introduction

In adult chickens, the homeostatic mechanisms that maintain cardiovascular function during periods of environmental stress have been thoroughly characterized (see classic studies of Butler 1967, Ray and Fedde 1969). When exposed to reduced ambient oxygen levels, chickens exhibit a pronounced tachycardia due, in part, to sympathetic augmentation of cardiovascular function (Butler 1967). While the importance of sympathetic influence on cardiovascular function is understood in adult chickens, its role during development is unknown. A recent study has indicated that a strong adrenergic tone is evident during the latter half of chicken incubation (Crossley Ch 2). Thus, during chicken development an adrenergic neurohumoral system is essential for the maintenance of resting cardiovascular function, but the extent of sympathetic contribution to this system is unknown.

Structural components of the sympathetic nervous system that contact the cardiovascular system have been relatively well characterized during the development of chickens. Adrenergic receptors are present on the heart and respond to stimulation during the first quarter of embryonic chicken development

(Cullis and Lucas 1936, Berry 1950). In addition, catabolic enzymes necessary for the production and oxidation of catecholamines are active within a similar time frame (Gifford 1973, Ignarro and Shideman 1968, Zachs, 1954). Morphological analysis of embryonic cardiovascular innervation also has demonstrated that sympathetic nerves reach the heart between days 11 and 12 of incubation. These studies have provided information on the anatomical integrity of the system, but the functional integrity of the sympathetic system was not addressed.

Pappano and colleagues (1974) first addressed this deficiency demonstrating that adrenergic activity could be induced via atrial field stimulation on day 19 of chicken incubation. Thus, during the later 15% of development sympathetic regulatory systems have the necessary intracellular components for activity. As noted in Chapter 2, adrenergic influence could originate from either neural and/or humoral sources that could not be separated utilizing the pharmacological approach employed in this study. Thus, sympathetic integrity acting primarily on peripheral vasculature may be established much earlier than previously determined with cardiac effects developing later.

Considering the gap between field stimulation data (Pappano 1976) and pharmacological assessment, the present work was undertaken to determine the origins of adrenergic actions on the embryonic cardiovascular system. Tyramine, was used during the later half of embryonic development: 1) to establish the origin of adrenergic tone, 2) determine the onset of sympathetic integrity and 3) determine maturation patterns during the development of chickens. In addition,

chemical sympathectomy with 6-hydroxydopamine was utilized to clarify the source of tyramine-induced cardiovascular change.

Materials and Methods

Eggs of the domestic chicken, *Gallus gallus*, were shipped by bus in lots of 10 dozen from the University of Texas A&M poultry farm over the period February 1999 to April 1999. A total of 90 dozen eggs were shipped to the University of North Texas, Department of Biological Sciences, and set in incubation in a Lyon electronics ProfHi incubator. Eggs were set at 38±0.5 °C with a relative humidity of 60% to 70% and automatically rotated 90° at a three hour interval to ensure maximal viability.

Catheterization Procedure

Eggs were selected from incubation on days 12,15,18,19,20 and 21 of a 21-day embryonic period. Three day intervals were used early is the study based on anatomical integrity of the sympathetic innervation to the heart with successive days utilized during late development to detect changes coordinated with the onset of lung ventilation. Prior to experimentation, eggs were candled to locate a major chorioallontoic membrane (CAM) artery. Once located, eggs were placed in a water-jacketed temperature control chamber to maintain an environment close to incubation conditions. A small hole was then made in the shell with a 21-gauge needle, allowing the removal of a 1-cm square section of shell. A branch of an underling artery was then encircled with three 6-00 silk ligatures to act as catheter anchors. The distal ligature was then tightened

occluding blood flow followed by proximal ligature closure limiting flow. Micro-fine scissors were then used to cut the vessel to allow anterograde insertion of a saline filled polyethylene catheter. Catheters were constructed of PE-90 (1.27 mm OD / 0.86 mm ID, Clay-Adams) heat pulled to a tip outer diameter smaller than 0.5 mm. Following successful cannulation the intermediate ligature was tightened and the proximal ligature was removed. The catheter was then fixed to the shell with cyanoacrylic glue, plugged with a pin occluder, and the egg then moved to an experimental chamber.

Each experimental chamber consisted of a water-jacketed 300-ml glass container fitted with a glass lid. Each lid contained three holes allowing gas exposure as well as catheter connection to WPI type disposable blood pressure transducers. Following the set up of four eggs, chambers were continually flushed from the bottom up with water saturated room air. Transducers were then connected to Adinstruments MacLab data acquisition system with signals processed with software on an 8500 Power Macintosh computer. Pressure transducers were calibrated against a static column of water, with the relative zero determined as described in Chapter 3.

Study procedure

After a 30 min control period, a dosage of tyramine corrected for embryonic mass (0.1 $mg*kg^{-1}$) was injected via a T joint in the pressure catheter. Cardiovascular responses were assumed to occur within five minutes after initial treatment (based on preliminary studies) and were allowed to recover for 10 min

following return to control levels. This procedure was repeated for dosages of 1.0 $mg_{*}kg^{-1}$and 10 $mg_{*}kg^{-1}$each followed by a recovery period. Embryos were then treated with the sympathectomizing agent 6-hydroxydopamine (6-OH) at a dosage of 1 $mg_{*}kg^{-1}$ followed by a second dosage of 20 $mg_{*}kg^{-1}$. A recovery period of 20 min after return to control levels was allowed between each dosages of 6-OH. Upon completion of sympathectomy treatment, eggs were again allowed to recovery for 20 min. Tyramine injection of 1 $mg_{*}kg^{-1}$ was then repeated followed by repeated dosage of 20 $mg_{*}kg^{-1}$ with recovery periods allowed as indicated for pre 6-OH treatment. Total time elapsed between the 20 $mg_{*}kg^{-1}$ 6-OH treatment and the second dosage of 10 $mg_{*}kg^{-1}$ tyramine averaged 68 min throughout the study. This protocol was repeated for a minimum of 5 eggs on day 12, 15,18, 19, 20 and 21 of a 21 day-incubation period.

Statistical Analysis

A paired Student t-test was used to access significant differences between pre- and post- drug infusion for all variables measured on each day of development. A one-way ANOVA was conducted on the arcsine transformed percent difference between control and treatment levels in all variables to determine significant changes between days of incubation. Fisher's LSD post-hoc comparison was used to isolate significant differences between days of incubation as well as to determine differences in dosage. The fiduciary level of significance for all tests was taken at $p<0.05$. All data are presented as mean ± 1

s.e.m.. For each day of study, 5 eggs were used to determine cardiovacular responses.

Results

Given that individual pressure parameters were consistently altered by a given dosage of tyramine, further discussion will present mean arterial pressure as representative of presser actions. Systolic and diastolic pressure responses to each dosage of tyramine are presented in Table 4.1. Tyramine produced minimal responses at the lowest dosage, 0.1 $mg_{*}kg^{-1}$. Mean arterial pressure (Map) was significantly elevated by an average of 0.8, 0.5 and 2.on days 12, 18 and 19 respectively (Fig. 4.2).

General Tyramine Effects

Treatment with 1.0 $mg_{*}kg^{-1}$ and 10.0 $mg_{*}kg^{-1}$ tyramine produced similar cardiovascular responses on all days of study ($p<0.6$, $p<0.9$ for Map and f_H) and will be described collectively. Mean arterial pressure rose an average of 2.3 mmHg from days 18 to 21 following tyramine injection (Fig 4.3 & 4.4). In addition, heart rate (f_H) also rose following injection averaging an increase of 9 $beat_{*}\ min^{-1}$ on all days tested with the exception of day 20 (Fig. 4.3 & 4.4). Responses of Map to tyramine treatment on days 18 through 21 were consistent (Table 4.2 a & b). Mean arterial pressure responses during the final days of incubation were significantly different from those on days 12 and 15 with the exception of day 20 (Table 4.2 a & b). Heart rate responses were constant throughout development in the chicken embryo as shown in Table 4.2 a & b.

Effects of Sympathectomy

Treatment with 20 $mg_{*}kg^{-1}$ of 6-hydroxydopamine (6-OH) altered pressure throughout embryonic development in chickens (Table 4.3). Following injection of 6-OH, Map exhibited an initial rise ranging from 0.3 to 8.0 mmHg on days 12 to 21 respectively (Table 4.3). Heart rate was also elevated by treatment with 20 $mg_{*}kg^{-1}$ 6-OH on day 18 to 21, with increases ranging from 7 to 19 $beat_{*}min^{-1}$. All changes were significant with the exception of day 20 (Table 4.3). All responses dissipated over time and control values had recovered within 30 min of treatment with 20.0 $mg_{*}kg^{-1}$ of 6-OH. Treatment with 1.0 $mg_{*}kg^{-1}$ produced similar effects on days 19 and 21 of incubation, but all reactions were dampened in comparison to treatment with 20.0 $mg_{*}kg^{-1}$.

A comparison of acute responses to 6-OH treatment between days of incubation revealed cardiovascular reactions matured with development. Map responses were significantly elevated ($p<0.0001$) in the final 3 days of development (Table 4.4). Heart rate response was consistent throughout the majority of incubation, with a significant increase on day 21 that differed from reaction on day 12 and 15 (Table 4.4).

Post 6-OH tyramine response

Heart rate reactions to tyramine injection (1.0 $mg_{*}kg^{-1}$) were dampened by sympathectomy on all days of incubation. This reduction was significantly ($p<0.05$) different on days 12 and 15 which averaged an increase of 10 $beats_{*}min^{-1}$ pre-sympathectomy treatment with no change following sympathetic

removal (Fig. 4.5). All other cardiovascular variables responded similarly to tyramine before and after 6-OH treatment throughout the days of study.

Following sympathectomy Map was unchanged by the initial treatment with 10 $mg_{*}kg^{-1}$ tyramine (Table 4.8). Following the second injection of 10 $mg_{*}kg^{-1}$ tyramine Map increase an average of 0.85 mmHg on days 18-21 (Fig. 4.6). This response differed from the 4.6 mmHg increase in pressure exhibited during pre 6-OH tyramine treatment (Fig. 4.6). Heart rate exhibited a similar reaction to the initial dosage of tyramine, with a non-significant reduction in rate following the first post 6-OH tyramine injection (Table 4.8). The second dosage of tyramine produced an average increase in heart rate of 2.5 $beats_{*}\ min^{-1}$ on days 19 and 20 of incubation. This response was different than pre-6-OH treatment value, which averaged an increase of 16 $beats_{*}\ min^{-1}$ on these days (Fig. 4.6).

Discussion

Classical studies of cardiovascular regulation during chicken development have determined that the sympathetic division of an adult regulatory mechanism is immature until day 19 of chicken incubation (Pappano and Loffelholz 1974). In the present study, a clear positive chronotropic as well as hypertensive response followed intra-arterial injection of the known adrenergic activator tyramine on 12 to 21 days of incubation. This indicates that all sympathetic terminal components necessary to induce changes in arterial pressure and heart rate are present over the final 40% of development in chickens. In addition, sympathetic response to

tyramine activation increases until day 18, plateauing for the final 15% of development.

Critique of Method

Conclusions drawn from this study have been developed under the assumption that tyramine induces release of noradrenaline from postganglionic terminals in chicken embryos. While it is acknowledged that this study did not determine the primary site of drug action, prior work in fetal and adult mammals has established that post-ganglionic terminals are the sites of tyramine action. In addition, previous study has demonstrated that noradrenaline stores in the adrenal medulla are not effected by tyramine, indicating that changes in CVS function are strictly due to catecholamine release from sympathetic nerve terminals.

Sympathectomy with 6-hydroxydopamine (6-OH) had been previously conducted on a limited number of isolated preparations from newly hatched chickens (Bennett and Malmfors 1974). The current study was the first to characterize cardiovascular responses of chicken embryos to *in vivo* treatment with 6-OH. Therefore, the present study's dosage and timeframe required to achieve some degree of sympathetic removal was based on fetal lamb literature (Tabsh et al. 1982). While it is acknowledged that assuming 6-OH dosages known to be effective at eliminating fetal mammalian sympathetic action would also be sufficient in embryonic chickens could be flawed, a number of observations justify its use. Given the diverse protocols used in fetal sheep, all of

which have resulted partial or total sympathectomy (Schuijers et al. 1986, Lewis and Sischo 1985, Iwamoto et al. 1983, Tabsh et al. 1982, Lewis et al. 1984), the 6-OH dosage utilized in this work should be sufficient in chicken embryos as well. Further, assessment of sympathectomy in the current study was with conducted changes in the response to tyramine used as the indicator of sympathectomy by 6-OH treatment, as in studies on fetal sheep (Schuijers et al. 1986, Lewis and Sischo 1985, Iwamoto et al. 1983, Tabsh et al. 1982, Lewis et al. 1984). It should be noted that total 6-OH dosage used in this work was below that demonstrated to achieve total sympathectomy in 2 to 6 week old chickens (Bennett and Malmfors 1974). Thus, while this procedure may not have achieved complete sympathectomy, the alteration in cardiovascular reaction to tyramine would suggest at least partial depletion of noradrenaline.

In addition to determining correct dosages, time intervals needed to achieve complete or partial sympathectomy in chicken embryos needed to be assumed. In fact, the interval between final treatment with 6-OH and tyramine assessment used in the present study was far shorter than that used in adult chickens and fetal sheep (Bennett and Malmfors 1974, Schuijers et al. 1986, Lewis and Sischo 1985, Iwamoto et al. 1983, Lewis et al. 1984). Yet, a previous study in fetal sheep has also demonstrated that this protocol was sufficient to eliminate the cardiovascular response to tyramine for up to 7 days after treatment (Tabsh et al. 1982). In addition, study in adult mammals has indicated that 6-OH is taken into post-synaptic terminals via a membrane pump, suggesting a rapid

action (Jonsson and Sachs 1975). Hence, the potential speed of terminal uptake as well as the sympathetic assessment with tyramine, assures that the protocol used was sufficient for at least partial sympathetic terminal destruction.

Tyramine Responses

Tyramine injection of both 1.0 $mg_{*}kg^{-1}$ and 10 $mg_{*}kg^{-1}$ dosages caused a consistent increase in Map and f_H over the period of study, while the lowest dosage (0.1 $mg_{*}kg^{-1}$) resulted in sporadic attenuated reactions (Fig 4.2,4.3 &4.4). The latter findings differed from those found in a prior study in which 0.1 $mg_{*}kg^{-1}$ induced an increase in both inotropic and chronotropic action in isolated chicken hearts from day 16 of incubation until hatch (Michal et al. 1967). Fundamental differences between isolated preparations and *in vivo* studies could account for this discrepancy. Further, as suggested in a previous study (Pappano 1976), vascular responses to the lowest tyramine dosage on day 12 could be the result of catecholamine release from sites other than adrenergic nerve terminals. Thus, the present study reports maximal cardiovascular responses with an injection of 1 $mg_{*}kg^{-1}$ tyramine with vascular reactions to treatment beginning between day 15 and 18 of chicken incubation.

Pressure rises caused by treatment with either 1 $mg_{*}kg^{-1}$ or 10 $mg_{*}kg^{-1}$ of tyramine were significant over the later four days of embryonic development (Fig 4.3, 4.4). This agrees with prior work on isolated heart preparations that demonstrated a stimulatory action of tyramine beginning on day 16 of incubation (Michal et al. 1967). Thus, the cardiovascular system is innervated by potentially

functional sympathetic efferent nerves between days 15 and 18 of chicken incubation. In addition, pressure response to tyramine remained constant over the final four days of chicken incubation for each dosage (Table 4.2), indicating a lack of further maturation of sympathetic terminals. This finding differed from those determined in previous studies in which tyramine treatment exhibited an increase in action with development (Michal et al. 1967, Pappano 1976). These studies while informative, utilized isolated heart preparations which could partially account for differences from the current study. This point is illustrated when comparisons are conducted between responses found in earlier days of development. Michal et al. (1967) found a negative inotropic action of tyramine on isolated embryonic chicken hearts on day 14 of incubation, a finding absent in this study (Fig 4.3 & 4.4). Thus, while previous data support the onset of positive inotropic action over the later stages of chicken development *in vivo*, the current study found no significant change in pressure response during this period.

Chronotropic responses to treatment with either 1 $mg_{*}kg^{-1}$ or 10 $mg_{*}kg^{-1}$ of tyramine were constant throughout the period of study, further indicating maximal cardiovascular reactions are induced by 1 $mg_{*}kg^{-1}$ of tyramine (Table 4.2 a & b). In addition, 1 $mg_{*}kg^{-1}$ tyramine elevated heart rate significantly on day 12 and 15 suggesting that cardiac sympathetic innervation is operational soon after reaches the embryonic heart. Importantly, heart rate was unaffected by either dosage of tyramine on day 20, a finding markedly different from that demonstrated in prior studies (Pappano 1974, Michal et al. 1967). Again, this may reflect innate

differences between isolated preparations and *in vivo* studies in which peripheral vascular and cardiac responses interact to produce the measured change. Why this transient loss of chronotropic sensitivity occurs on day 20 is unclear. However this pattern mirrors that previously determined for sino-atrial adrenergic sensitivity which peaks on day 18 and then falls on days 19 to 20 of chicken embryo incubation (Pappano and Loffelholz 1974). Sensitivity then recovered on day 21(Loffelholz and Pappano 1974) as confirmed in the present work from tyramine actions. Loffelholz and Pappano (1974) attributed their findings to an overall hypoxic state in embryos during the last 3 days of incubation, resulting in an acidosis and decrease in cardiac sensitivity (Girard 1973). Additionally, a peak in cardiac catechol-O-methyltransferase activity on days 19 and 20 (Ignarro and Shideman 1968) has been suggested to account for the decrease in cardiac response on day 20 of incubation (Loffelholz and Pappano 1974). Thus, numerous changes may account for the transient loss of chronotropic action on day 20 and further study is needed to definitively determine the origin of this change.

6-Hydroxydopamine

The acute actions of 6-hydroxydopamine on heart function further suggest sympathetic integrity is established by day 18 of chicken development (Table 4.3). In addition, cardiac response to 6-OH was constant over the final 20% of development, indicating that sympathetic components are unchanged a finding also suggested by tyramine treatments. The lack of a significant chronotropic

response on days 12 and 15 was unexpected given the previously established actions of tyramine on these days (Fig 4.3). However, each embryo was exposed to tyramine prior to sympathectomy, possibly accounting for this finding due to an inability of sympathetic terminals to regenerate noradrenaline. Regardless, these findings validate the apparent integrity by day 18 of cardiac sympathetic regulatory components as illustrated by tyramine treatment (Fig 4.3). The selective mechanism responsible 6-OH transport into post-synaptic terminals further assures that noradrenaline released from sympathetic efferents causes the positive chronotropic action (Jonsson and Sachs 1975, Kostrzewa and Jocobowitz 1974). Thus, noradrenaline released from sympathetic terminals can augment cardiac function by day 18 on chicken development.

Sympathetic terminal release of noradrenaline may be capable of alteration of peripheral resistance as early as day 12 of chicken incubation (Table 4.3). As suggested by Table 4.4, pressure response is constant until day 18 of incubation, followed by an increase over the final three days of incubation, both events suggesting a late maturation of function. These data, in conjunction with pressure responses to tyramine on day 12 suggest that sympathetic action on peripheral resistance is possible by 60% of chicken embryo incubation. In view of this possibility, the lack of cardiac responses on day 12 and 15 following 6-OH treatment are difficult to explain. Further experimentation is needed to clarify sites of 6-OH action on developing chicken cardiovascular systems.

Tyramine Post 6-OH

Injections with 1 $mg_{*}kg^{-1}$ of tyramine produced cardiovascular reactions similar to pre sympathectomy values. This finding could be due to the time frame needed to achieve maximal sympathectomy following the initial treatment. Alternatively, it is possible that this dosage of 6-OH was insufficient to achieve the needed degree of catecholamine depletion. Prior research has demonstrated that catecholamine must be depleted to extreme levels to produce detectable differences (Ungerstedt and Marchall, 1975). However, the second dosage of 10 $mg_{*}kg^{-1}$ tyramine did exhibit a decrease in ability to increase cardiovascular function, indicating that time period is a principle factor in conducting chemical sympathectomy is time period.

Dampening of pressure responses following sympathectomy treatment were first evident following the second dosage of 10 $mg_{*}kg^{-1}$ tyramine on day 18 of incubation (Fig. 4.6). Therefore, the protocol used was sufficient to produce partial or total sympathectomy in chicken embryos. In addition, this verifies that cardiovascular responses to tyramine over the final four days of incubation were due to noradrenaline release from sympathetic terminals.

Chronotropic responses following the secondary dosage of tyramine indicated that while peripheral sympathectomy is possible over the final 4 days of incubation, cardiac innervation is partially resistant. Chronotropic responses to tyramine were altered only on days 18 and 19 (Table 4.5). However, given that significant chronotropic responses to tyramine were absent on day 20, sympathectomy would be expected to produce no change in reaction. Therefore,

clear cardiac denervation was achieved only on days 18 and 19, suggesting that either a long time interval or a greater 6-OH dosage is needed to achieve cardiac sympathectomy.

Conclusion

While past study has demonstrated that both adrenergic receptors and sympathetic innervation of the heart are present on day 12 of incubation, little had been determined about sympathetic activity (Pappano 1977). Utilizing isolated preps and field stimulation, Pappano and colleagues (1974) have determined that sympathetically derived increase in cardiac function was present as of day 19. The present study has expanded the window of potential sympathetic regulation of the cardiovascular system to include day 18 of chicken incubation. In addition, day 20 represents a critical period when cardiac tissue becomes desensitized to sympathetic stimulation. Thus, adrenergic regulation mediated by the sympathetic nervous system undergoes a series of transformations during the internal pipping period in chicken embryos. Anticipated augmentation of cardiovascular responses were absent during late development, indicating a significant portion of regulatory maturation must take place during the neonatal period and that sympathetic regulation is quiescent until hatching.

Table 4.1. Systolic (Sys) and diastolic (Dia) pressures responses to 0.1, 1.0 and 10 $mg_{*}kg^{-1}$ tyramine during development. Asterisk indicates treatment (T) was significantly ($p< 0.05$) different from control (C) as determined by a paired T-test. Data are presented as mean ± sem. N=5 for all analyses unless noted.

		0.1 $mg_{*}kg^{-1}$				1.0 $mg_{*}kg^{-1}$				10 $mg_{*}kg^{-1}$			
Day		Sys		Dia		Sys		Dia		Sys		Dia	
12	C	14.9±1.2		9.9±0.8		14.5±1.0		9.8±0.8		13.0±0.9		8.9±0.9	
	T	15.8±1.1	*	10.2±0.9		14.8±1.0	*	9.7±0.7		13.4±0.9		8.7±0.8	
15	C	20.9±1.6		14.0±1.0		22.3±2.1		14.5±1.6		20.8±2.0		13.8±1.6	
	T	21.7±1.7		14.0±1.1		22.2±2.3		14.5±1.8		22.4±2.3		14.3±1.6	
18	C	23.5±1.1		13.6±0.8		22.2±0.9		13.4±0.8		22.2±0.7		12.9±0.9	
	T	24.4±1.1	*	14.2±0.9	*	23.6±1.0	*	14.5±0.8		23.7±1.0	*	14.1±0.8	*
19	C	28.1±1.2		18.2±1.1		27.1±1.3		16.6±0.9		27.0±1.3		16.9±1.2	
	T	29.6±1.1	*	19.3±0.9		30.4±1.5	*	19.4±1.3	*	30.4±1.4	*	20.4±1.3	*
20	C	31.7±0.8		21.5±0.9		31.7±0.8		21.7±0.9		29.0±1.3		20.4±1.3	
	T	33.6±1.1		23.6±1.2		34.7±1.1	*	24.9±1.3	*	34.6±2.3	*	25.1±1.5	*
21	C	41.0±2.4		26.0±2.0		39.6±2.2		26.0±1.9		39.8±2.5		25.8±1.9	
	T	42.3±1.8		28.8±1.7		42.3±2.4	*	28.7±2.1	*	44.5±3.0	*	30.9±2.2	*

Table 4.2. A comparison of mean arterial pressure (Map) and heart rate (f_H) response between days of incubation of both 1.0 mg_*kg^{-1} and 10.0 mg_*kg^{-1} tyramine injection. Lettering indicates days reacting similarly to a gives dosage of tyramine. Significant differences ($p<0.05$) as determined by ANOVA conducted on the arcsine transformed percentage changes are represented as days with a different letter. N=5 for all analyses unless noted.

1.0 mg_*kg^{-1}

Day	Map	f_H
12	BC	A
15	C	A
18	A	A
19	A	A
20	AB	A
21	A	A

10.0 mg_*kg^{-1}

Day	Map	f_H
12	C	A
15	BC	A
18	A	A
19	AB	A
20	A	A
21	A	A

Table 4.3. Differences in mean arterial (Map), systolic (Sys) diastolic (Dia) pressures and heart rate (f_H) response to two dosages of 6-hydroxydopamine 1 $mg_{*}kg^{-1}$ (1) and 20 $mg_{*}kg^{-1}$ (20). Asterisk indicates significant difference in parameter from control values (C). Data are presented as mean ± sem. N=5 for all analyses unless noted.

Day		Map		Sys		Dia		f_H	
12	C	11.1±0.8		13.6±0.9		9.1±0.7		211±9	
	1	11.1±0.8		13.7±0.8		9.0±0.8		208±9	
	C	11.0±0.8		13.3±1.0		9.1±0.8		211±8	
	20	11.3±0.8	*	13.9±0.9	*	9.2±0.8	*	210±8	
15	C	16.1±1.4		19.9±1.6		13.1±1.2		225±14	
	1	16.4±1.5		20.1±1.9		13.4±1.3		227±13	
	C	16.0±1.5		19.9±1.8		12.7±1.3		222±12	
	20	16.5±1.5	*	20.7±1.7	*	13.1±1.3		223±13	
18	C	18.3±1.0		22.9±1.2		13.9±0.8		245±4	
	1	19.1±1.1		24.0±1.2	*	14.5±0.9		251±4	
	C	18.0±1.2		23.0±1.5		13.6±0.9		242±4	
	20	19.0±1.1	*	23.6±1.2		14.7±1.0	*	249±4	*
19	C	22.7±1.0		27.8±1.1		18.1±1.0		239±6	
	1	23.5±1.2	*	28.5±1.4		18.6±1.3		243±6	
	C	22.2±1.0		27.1±1.1		17.7±1.1		239±5	
	20	24.2±1.2	*	29.5±1.4	*	19.1±1.2	*	248±5	*
20	C	24.4±2.1		29.0±2.1		20.1±2.2		260±7	
	1	26.0±2.3		31.0±2.3		21.5±2.4		265±5	
	C	24.1±2.4		28.9±2.5		19.6±2.4		256±6	
	20	28.9±2.1	*	34.8±1.8	*	24.1±2.3	*	267±6	
21	C	29.9±2.1		36.0±2.7		24.6±1.7		290±17	
	1	31.8±2.1	*	38.5±2.4	*	25.7±2.0	*	297±15	*
	C	28.7±1.5		35.0±1.5		22.9±1.4		289±14	
	20	36.7±2.6	*	45.2±3.1	*	29.6±1.4	*	308±12	*

Table 4.4. Comparison of acute mean arterial (Map), systolic (Sys) diastolic (Dia) pressures and heart rate (f_H) r responses to intravenous injection of 20 $mg_{*}kg^{-1}$ 6-OH. Days with a like letter exhibited responses that were similar. Unlike letters indicate significantly differing responses ($p< 0.05$) as indictated by a one way ANOVA conducted on arcsine transformed percent changes. N=5 for all analyses unless noted.

Day	Map	Sys	Dia	f_H
12	A	AB	A	A
15	A	AB	A	A
18	AB	AB	B	AB
19	B	B	B	AB
20	C	C	C	AB
21	C	C	C	B

Table 4.5. Change in mean arterial (Map), systolic (Sys) diastolic (Dia) pressures and heart rate (f_H) following injection of 10 $mg*kg^{-1}$ tyramine pre (C) post (10I) and second post (10II) 6-OH treatment. Asterisk indicates significance of at least ($p<0.05$) between pre and post one and two treatment as indictated by a one way ANOVA conducted on arcsine transformed percent changes. N=5 for all analyses unless noted.

Day		Map		Sys		Dia		f_H	
12	C	0.1±0.2		0.4±0.2		-0.3±0.2		2±6	
	10 I	0.3±0.1		0.6±0.3		0.1±0.1		6±2	
	10II	0.0±0.2		0.3±0.4		-0.1±0.1		0±4	
15	C	1.1±0.4		1.6±0.7		0.5±0.3		14±6	
	10 I	3.0±2.5		0.9±0.2		0.5±0.3		4±5	
18	CII	4.1±1.3		4.3±1.7		4.0±1.2		11±4	
	10 I	2.0±0.4		2.2±0.6		1.8±0.6	*	12±3	
	10II	0.7±0.2	*	0.8±0.2		0.8±0.3	*	3±2	
19	C	3.3±0.3		3.3±0.6		3.5±0.4		21±2	
	10 I	2.0±0.3		2.4±0.3		1.8±0.4	*	13±3	
	10II	0.8±0.5	*	0.9±0.5	*	0.9±0.7	*	4±4	*
20	C	5.2±1.6		5.7±1.8		5.0±1.6		11±3	
	10 I	3.4±0.6		4.2±0.8		3.2±0.3		11±4	
	10II	0.2±0.6	*	-0.3±1.2		0.3±0.5	*	1±2	*
21	C	5.9±1.4		4.9±1.8		5.4±1.3		15±4	
	10 I	4.8±1.2		5.9±1.4		4.0±1.0		12±5	
	10II	1.7±0.8	*	2.5±0.9		1.3±0.9	*	5±2	

Figure legend

Figure 4.1

Representative raw data traces illustrating the cardiovascular responses following injection of 10 $mg_{*}kg^{-1}$ tyramine (arrow). Each panel shows arterial pressure and heart rate responses on day 18 (A), 20 (B) and 21 (C) of incubation.

Figure 4.2

The average cardiovascular reaction to 0.1 $mg_{*}kg^{-1}$ tyramine injection in chicken embryos during development. Heart rate (f_H) and mean arterial pressure are presented as control (open bar) and treatment response (closed bar). Asterisks indicate significant difference ($p<0.05$) from control following tyramine injection. Data are presented as mean ± s.e.m. with an n=5 for each mean.

Figure 4.3

The cardiovascular response to 1 $mg_{*}kg^{-1}$ tyramine injection in chicken embryos during development Heart rate (f_H) and mean arterial pressure are presented as control (open bar) and treatment response (closed bar). Asterisk indicates significant difference($p<0.05$) from control following tyramine injection. Data are presented as mean ± s.e.m. with an n=5 for each mean.

Figure 4.4

The cardiovascular response to 10 $mg_{*}kg^{-1}$ tyramine injection in chicken embryos during development. Heart rate (f_H) and mean arterial pressure are presented as control (open bar) and treatment response (closed bar). Asterisk indicates

significant difference ($p<0.05$) from control following tyramine injection. Data are presented as mean ± s.e.m. with an n=5 for each mean.

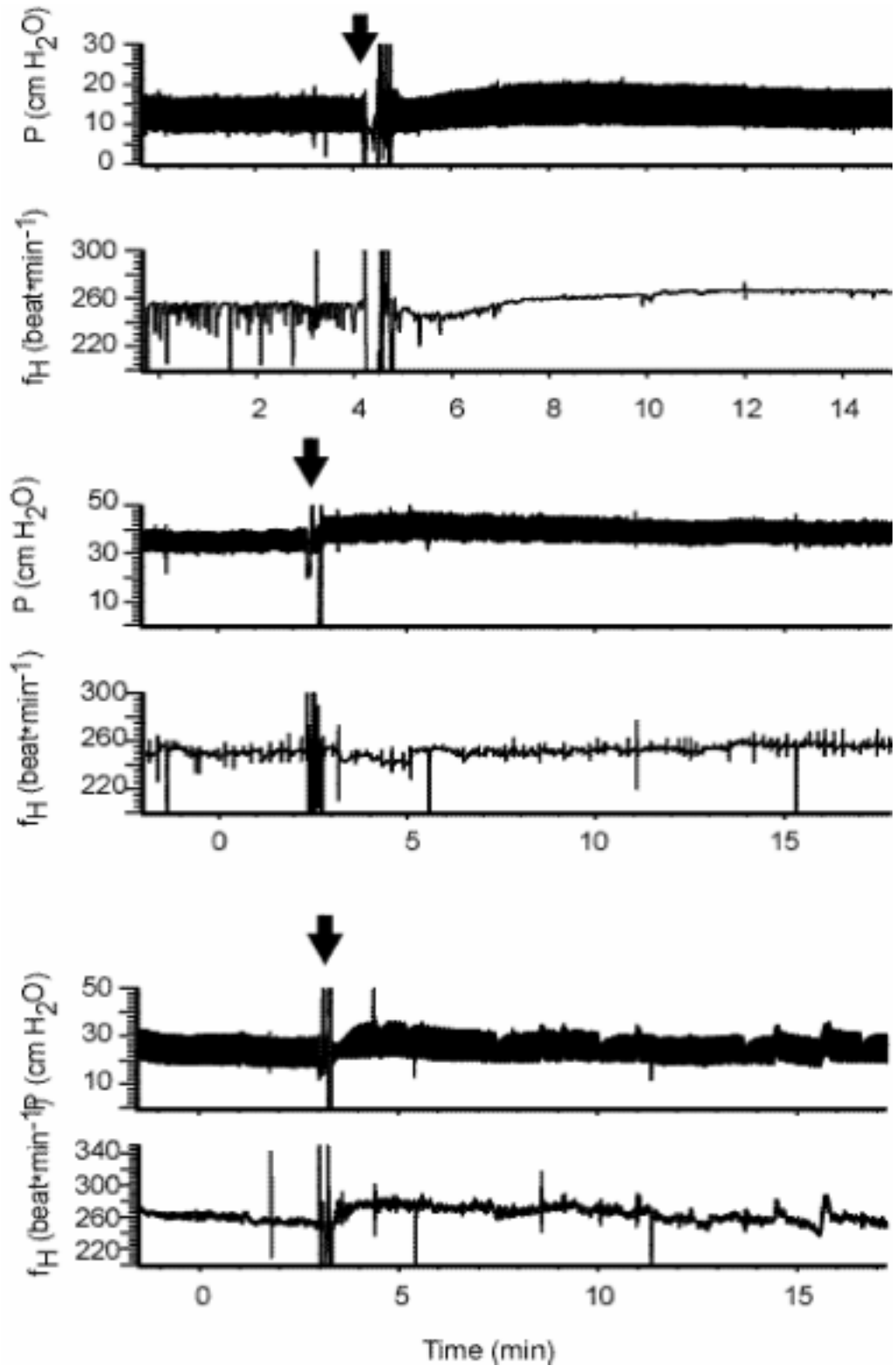
Figure 4.5

The cardiovascular response to 1 $mg_{*}kg^{-1}$ tyramine injection in chicken embryos during development pre and post 6-hydroxydopamine treatment. Data are presented as control (first set open and closed bar) and post-treatment, (second set open and closed bar) for both heart rate (f_H) and mean arterial pressure (Map). Asterisk indicates significant difference ($p<0.05$) in tyramine response from pre 6-hydroxydopamine treatment. Data are presented as mean ± s.e.m. with an n=5 for each mean.

Figure 4.6

The cardiovascular response to 10 $mg_{*}kg^{-1}$ tyramine injection in chicken embryos during development pre and post 6-hydroxydopamine treatment. Data are presented as control (first set open and closed bar) and post-treatment, (second set open and closed bar) for both heart rate (f_H) and mean arterial pressure (Map). Asterisk indicates significant difference ($p<0.05$) in tyramine response from pre 6-hydroxydopamine treatment. Data are presented as mean ± s.e.m. with an n=5 for each mean.

Figure 4.1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fH (beat•min-1)
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260
220
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8
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12
14
P (cm H2O)
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fH (beat•min-1)
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220
0
5
10
15
P (cm H2O)
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fH (beat•min-1)
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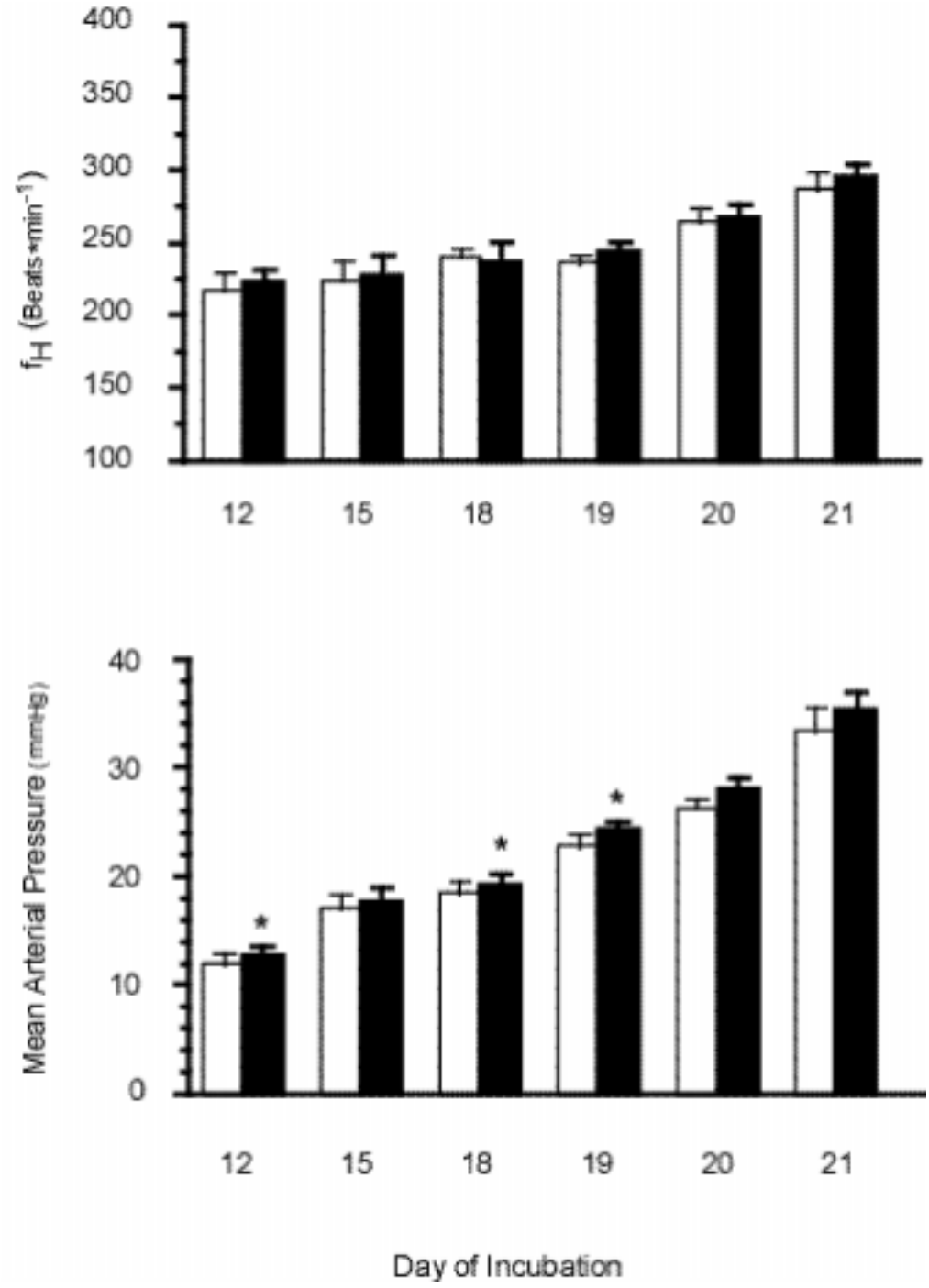
Time (min)

Figure 4.2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Mean Arterial Pressure (mmHg)
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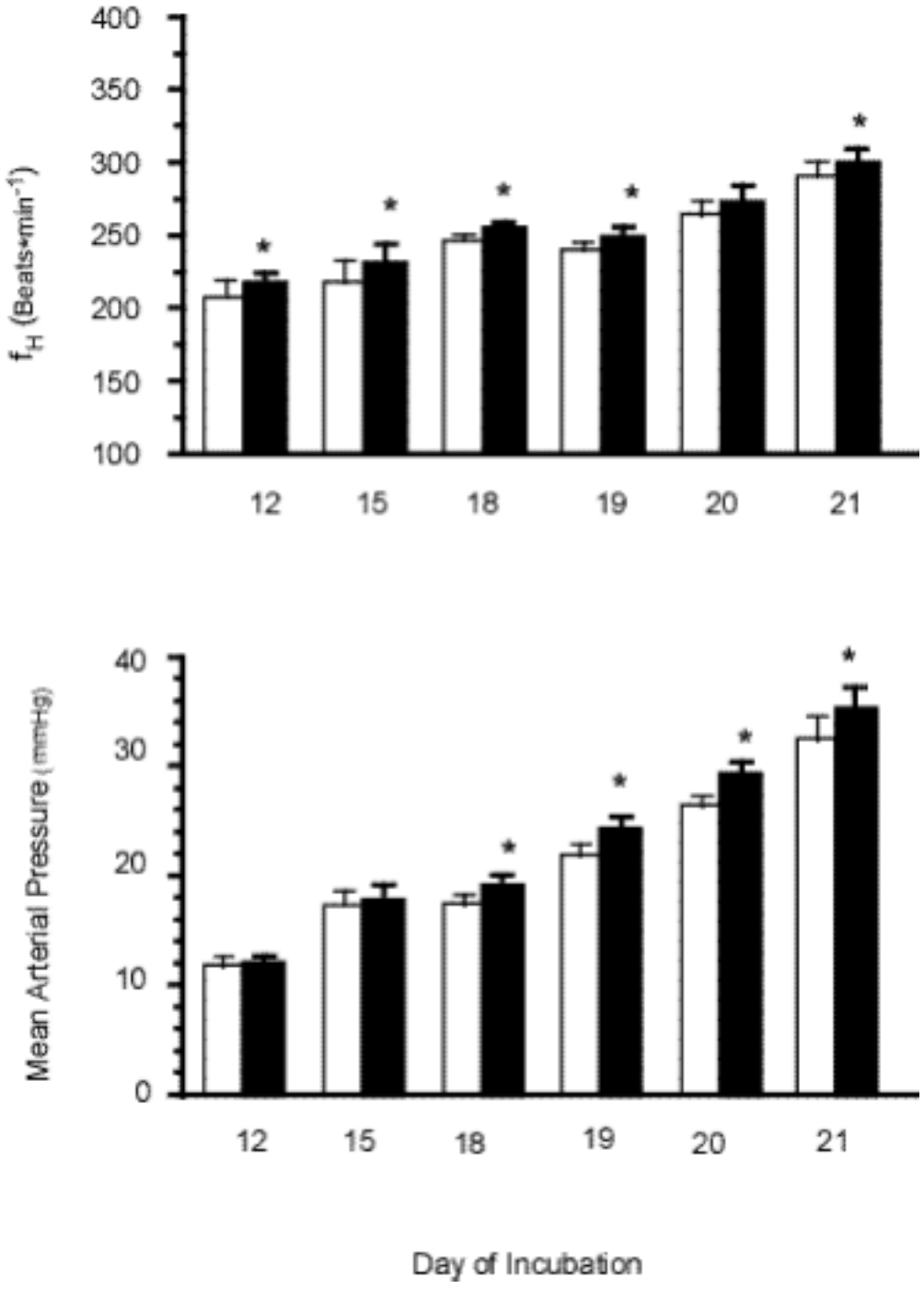
Day of Incubation

Figure 4.3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Mean Arterial Pressure (mmHg)
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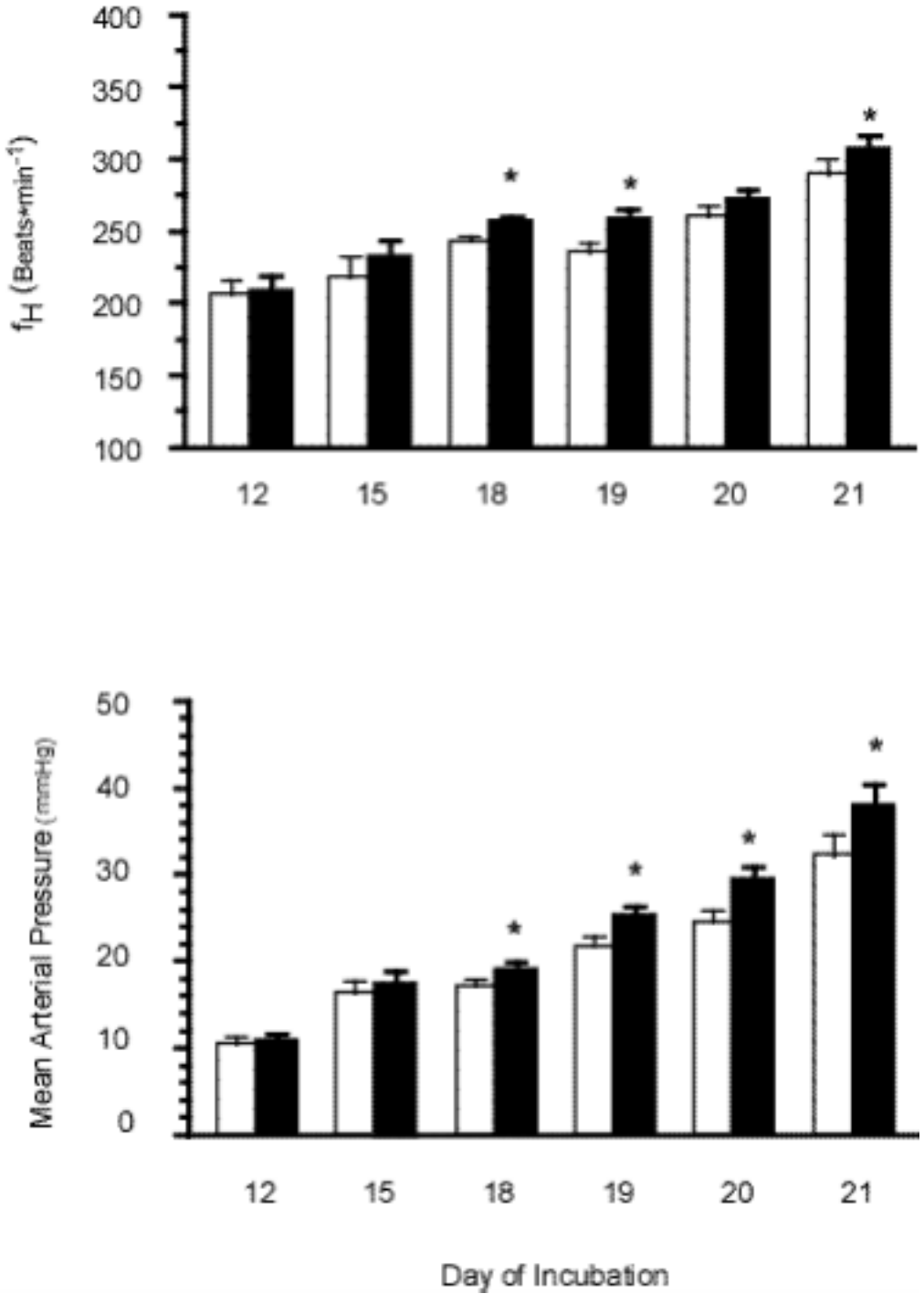
Day of Incubation

Figure 4.4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Mean Arterial Pressure (mmHg)
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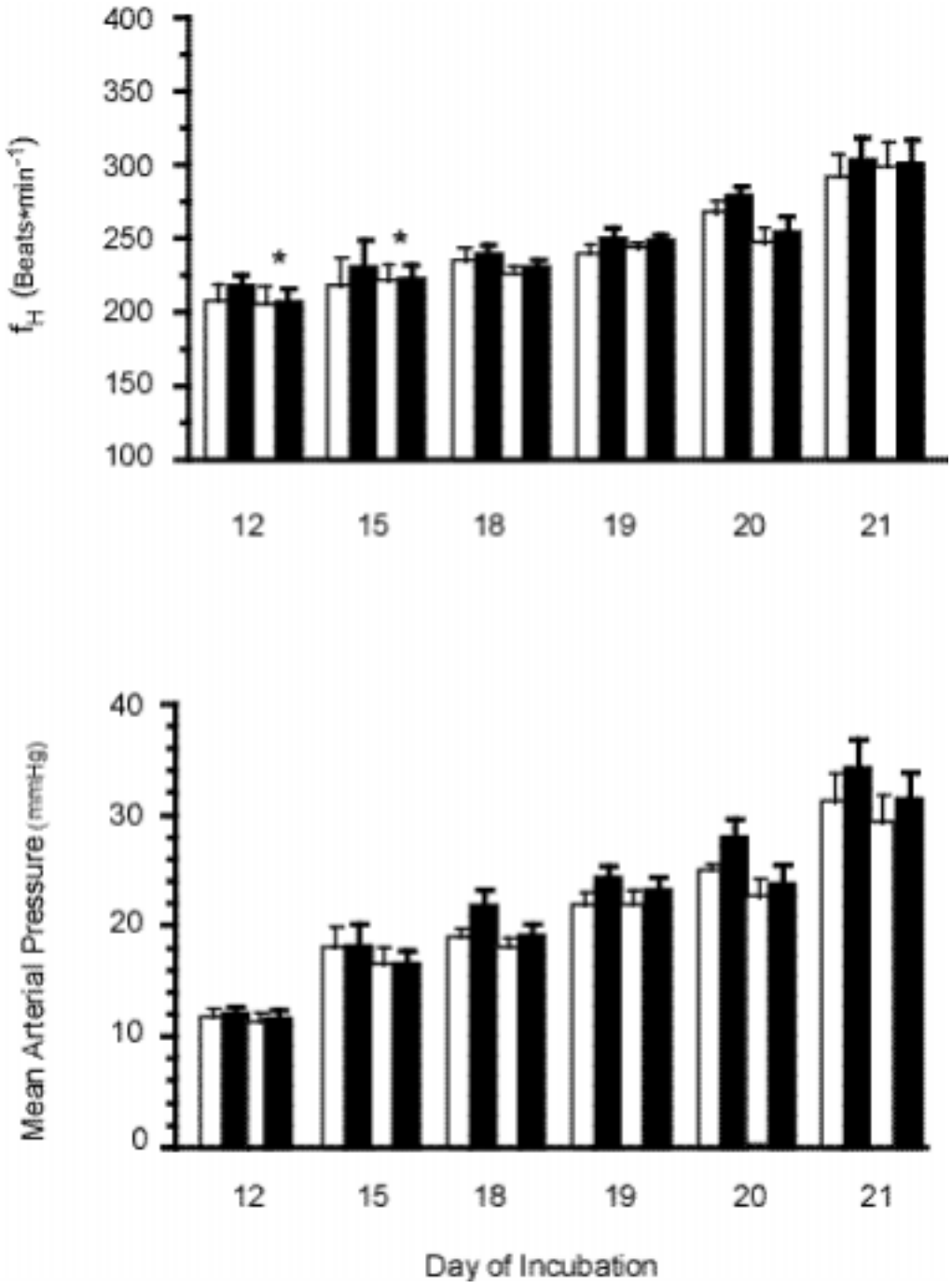
Day of Incubation

Figure 4.5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Mean Arterial Pressure (mmHg)
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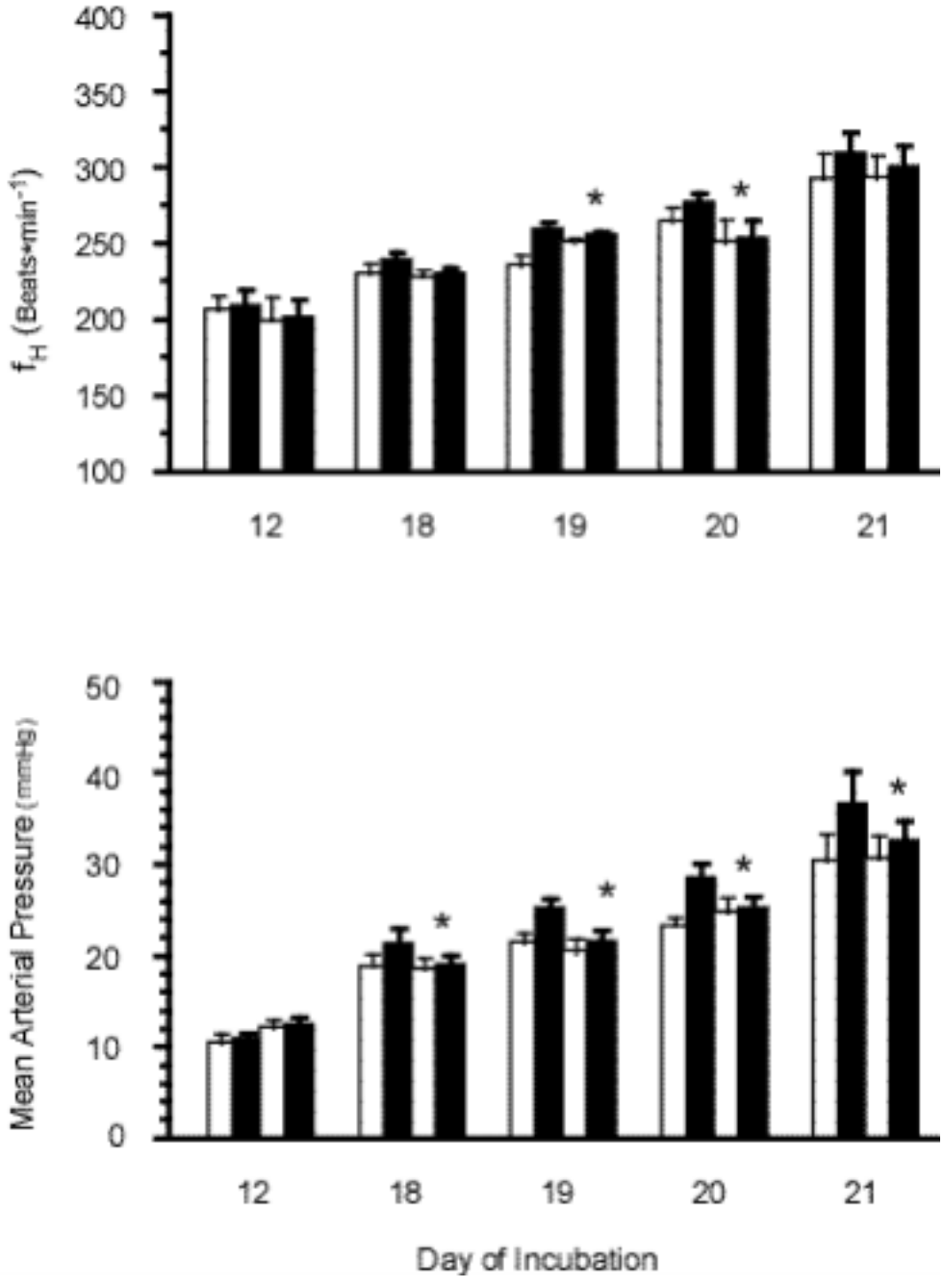
Day of Incubation

Figure 4.6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Mean Arterial Pressure (mmHg)
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Day of Incubation

CHAPTER V

THE DEVELOPMENT OF CARDIOVASCULAR CHEMOREFLEX ACTION IN CHICKENS: RESPONSE TO ACUTE CHANGE IN AMBIENT GAS STRESS.

Introduction

The homeostatic balance of gas transfer to and from peripheral tissues is mainly dependent on cardiovascular reflexes, with contributions from local regulatory processes. Among the neural components, the chemoreflex plays an important role in matching supply to demand by the different organs. In adult vertebrates, a myriad of reactions occurs in response to chemoreceptor activation, with hyperventilation and blood flow redistribution comprising the main components. While these adult responses have been well characterized in several vertebrate groups, fetal development of this reflex in terrestrial vertebrates has been addressed in relatively few studies utilizing primarily the fetal lamb.

The fetal lamb has been the vertebrate model used in determining the importance of chemoreflex function as well as its maturational pattern during fetal development. In fetal sheep, cardiovascular responses to alteration of blood gas composition are due, in part, to a chemoreflex component. Under hypoxic conditions a chemoreflex results in a transient bradycardia followed by a delayed

progressive increase in arterial pressure. These responses are dependent on central and local mechanisms respectively to alter cardiovascular function in fetal sheep (Lewis and Sischo1982). Hypercapnic stress produces primarily positive chronotropic action with little pressure change in fetal sheep (Wood et al. 1990). These reactions differ from those found in embryonic chickens, which exhibit a general cardiovascular depression during hypoxia as well as hypercapnia, suggesting fundamental regulatory differences between embryonic and adult chickens (Tazawa 1981b, Van Golde et al. 1998, Mulder et al. 1997).

In embryonic chickens, acute exposure to low environmental oxygen severly challenges to the cardiovascular system. Hypoxia causes a reduction in both heart rate and blood pressure with redistribution of cardiac output to the brain, heart and chorioallantoic membrane (Mulder et al. 1998, Tazawa 1981b). The possible adaptive significance of a hypercapnic cardiovascular reaction in chicken embryos has not been previously suggested. However, hypoxic alterations of cardiovascular functions are implicated in the protection of embryonic chickens during periods of sub-ambient oxygen tension (Mulder et al. 1998). Prior work has determined that autonomic control of the cardiovascular system is inactive until day 16 of incubation (Pappano 1977). Thus, under resting conditions, central regulation is absent prior to day 16 of chicken development. However, acute periods of stress may induce activation of compensatory regulation systems.

In this study I sought to test the hypothesis that chemoreflex-driven changes in cardiovascular function are important during embryonic development of chickens. A chemoreflex would allow the embryo to accommodate for periods of reduced O_2 or elevated CO_2 levels. In addition, I hypothesized that the response intensity would increase in parallel with the maturation of the autonomic nervous system.

Materials and Methods

Domestic chicken eggs, *Gallus gallus*, of the white leghorn strain were purchased from Texas A&M and shipped overnight to the University of North Texas Department of Biological Sciences. On arrival, eggs were placed in incubation at 38 ± 0.5 °C, 60-70% relative humidity and turned automatically every 3 hrs. For the purposes of the study, experimental manipulation was conducted on days 9, 12, 15 and18-21 of a 21-day incubation period.

Surgical Procedures

On the day of study, eggs were removed from the incubator, candled to locate a chorioallantoic artery and placed in a temperature-controlled chamber at 38±0.5 °C. A portion of the eggshell was removed exposing the previously located artery, which was temporarily occluded up stream with a 6 zero silk ligature and a second ligature was placed downstream to eliminate retrograde flow following cannulation. A small cut was then made in the artery and a heat-pulled saline-filled catheter was then used to occlusively cannulate the vessel. All the procedures were carried out under a dissection microscope (Wild M3Z).

Once cannulation was completed the catheter was fixed to the shell with cyanoacrylic glue and the egg was placed in the experimental chamber (see Ch 4 for description).

Signal recording and calibration

Each catheter was attached to a pressure transducer (WPI, type BLPR), which in turn was connected to a bridge amplifier (CB Sciences, model ETH-400) and the pressure trace stored in a computer using PowerLab data acquisitions software. Heart rate was continuously calculated from the pressure signal via an acquisition tachograph. In all cases, zero was initially set at the top of the experimental bath, and all values were corrected after the experiment as described in Chapter 2.

Experimental Protocol

Once were placed in the experimental chambers they were allowed 30 min to reach recovery from possible reactions to the canulation. Once this time period was complete a 5 min control period was recorded for comparisons to experimental treatments. Following this control period eggs were exposed to 15% O_2 for 5 min and then returned to normoxia for 30 min. This procedure was repeated for 10 as well as 5% O_2 exposures, with changes in heart rate and arterial pressure recorded. This protocol was repeated for 3, 5 and 10% CO_2 mixed with normoxic air concentration of normoxic hypercapnia as well as a single exposure to 5% CO_2 with 10% O_2. To limit possible adative effects of multiple gas exposures, different experimental groups of eggs were used for

hypoxic and hypercapnic studies. Once the protocol was finished, embryos were euthanized with an overdose of xylocaine and KCl and frozen for later determination of the pressure correction factor.

Statistical Analysis

A paired Student t-test was used to access significant differences between control and hypoxic exposure for all levels. A one-way ANOVA was conducted on the arcsine transformed percent difference between control and hypoxic exposure levels in all variables to determine significant changes between days of incubation. A similar statistical analysis was conducted within day of incubation to determine differences in reactions to changing levels of hypoxia. Fisher's LSD post-hoc comparison was used to isolate significant differences between days of incubation as well as to determine differences in hypoxia. The fiduciary level of significance for all tests was taken at $p<0.05$. All data are presented as mean ± 1 s.e.m.. A sample number of five eggs was used for all experimental procedures, unless otherwise specified.

Results

Response to Hypoxia

Hypoxic exposure caused chorioallantoic pressure and f_H to fall in embryos from day 9 to 19 (Fig. 5.1a). Heart rate continued to exhibit this pattern on day 20, but pressure was relatively unaffected by hypoxia at the late point in development (Fig. 5.1b and Fig. 5.1c).

Systolic and diastolic pressure responses to different degrees of hypoxia are presented in Table 5.1. Given that all pressure parameters responded similarly during hypoxic exposure, further discussion will present mean arterial pressure (Map) as representative of presser actions. Five-minute exposures to 15% O_2 result in significant change in all cardiovascular parameters on all days, with the notable exception of days 20 and 21. Map fell an average of 1.7 mmHg. This reduction was significantly different on days 9 to 19 as well as day 21 ($p < 0.05$; Fig. 5.2). In addition, heart rate decreased significantly on days 9,15,18 and 19 ($p<0.05$).

Exposure to 10% O_2 induced a general depression of cardiovascular function on all days of incubation studied. Map was significantly affected by hypoxia on days 9 to 19 with an average fall in pressure of 2.3 mmHg ($p< 0.05$; Fig. 5.3). Heart rate was consistently affected, falling significantly an average of 34 beats$\cdot$min^{-1} on all days of incubation (Fig. 5.3).

Embryonic exposure to 5% O_2 also produced general depression of all cardiovascular parameters measured. Map fell an average of 4.2 mmHg during exposure to 5% O_2 compared to control levels (Fig.5.4). In addition, heart rate fell an average of 65 beats$*$min^{-1} below control levels during exposure to 5% O_2 as indicated throughout incubation (Fig.5.4).

Developmental differences

No differences between developmental stages were observed during exposure to 15% and 5% hypoxia. However, some differences between days of

incubation appeared during exposure to 10% O_2. Map change to 10% hypoxia peaked on day 19, with a maximal decrease of 5.6 mmHg (Table 5.2). This response was significantly different than that exhibited on days 18, 20 and 21 during hypoxia. Heart rate exhibited a different pattern of response, with a relatively constant reduction throughout incubation until days 20 and 21 (Table 5.2). On day 20-21 the response was significantly dampened, with a change of 20 beats$*$min^{-1} on days 20 and 21 versus 39 beats$*$min^{-1} on average on days 9 to 19 (Table 5.2).

Difference between degree of hypoxia

A comparison of responses to each level of hypoxia revealed that the f_H decreased significantly more during exposure to 5% O_2 (-67 beats$*$min^{-1}) than during 15% O_2 (-13 beats$*$min^{-1}) (Table 5.3). Chronotropic responses to 5% O_2 were also significantly different than those exhibited during 10% O_2 on days 15 and 18 of incubation (Table III). In addition, f_H reductions produced by 10% O_2 were significantly different than those during 15% O_2, on the majority of incubation days studied (Table 5.3).

Map changes induced during 5% O_2 were significantly greater than those produced by 15% O_2 exposure on the majority of incubation days tested (Table 5.3). In addition, 5% O_2 responses differed from those found during 10% O_2 exposure on day 12 of incubation (Table 5.3). Pressure responses to 10% O_2 and 15% O_2 were similar on all days with the exception of day 20 (Table 5.3).

Response to Hypercapnia

Cardiovacular reactions to various hypercapnic levels were inconsistant, with only a limited effect on arterial pressure (Fig 5.5). As indicated for hypoxic exposures systolic and diastolic pressure changes essentially mirrored those demonstrated for Map during graded hypercapnic treatments (Table 5.4). Initial exposure to 3% CO_2 produced sporadic responses with significant changes in Map on days 18 (0.8 mmHg) and 21 (2.2 mmHg) only (Fig 5.6). A weak chronotropic response was also exhibit during 3% CO_2 exposure with a significant decrease on day 9 (18 beats$*$min^{-1}) (Fig 5.6). Intermediate concentrations of CO_2 also induced slight cardiovascular responses with arterial pressure remaining unchanged during exposures to 5% CO_2 (Fig 5.7). However, this concentration did elicit a negative chronotropic reaction, with a significant average fall of 16 beats$*$min^{-1} on the majority of days studied (Fig 5.7). Again, arterial pressure responses during the extreme CO_2 level tested (10%) were inconsistent with significant alterations during exposure on days 12 (-0.4 mmHg) and 21 (2.4 mmHg) of incubation (Fig 5.8). However, chronotropic responses to 10% CO_2 exposure were substantial on all tested days of study with significant reactions on days 9, 18, 19 and 21 (Fig 5.8).

Between Day Comparison

Arterial pressure responses to 3% and 10% CO_2 exposures were significantly ($p< 0.05$) different between each day of study (Table 5.5). In general, pressure fell during hypercapnic exposure early development, but increased from days 15 to 21, (Table 5.5). Maximal pressure change of 2.3

mmHg was evident on day 21 when embryos were exposed to 3% or 10% CO_2 elevated pressure (Table 5.5). Maximal f_H reductions to 3% CO_2 occurred on day 9 of incubation (18 beats*min^{-1}) a finding significantly different than all other days (Table 5.5). In addition, 10% CO_2 exposure on day 21 produced a minimal but significant reduction in heart rate, a finding that differed from the majority of responses during development (Table 5.5).

Hypercapnic Comparison within Incubation Day

On each incubation day studied, pressure responses to all three CO_2 levels were constant (Table 5.6). Heart rate reactions to hypercapnia were accentuated as CO_2 concentration was elevated, with the exception of day 9 (Table 5.6). While on day 9 of incubation there was a constant effect of hypercapnia regardless of degree, 10% CO_2 produced negative chronotropic action which differed from 3% CO_2 on days 15 to 21 (Table 5.6). Responses to 5% CO_2 differed from 3% reactions on days 12, 15 and 19 (Table 5.6).

Hypoxic Hypercapnia

The combined exposure of 5% CO_2 with 10% O_2 produced results similar to those caused by hypoxia alone (Fig 5.9). This was indicated by the results of a two way ANOVA on arcsine transformed percentages which produce p values of 0.06 and 0.75 for changes in Map and f_H, respectively. In addition, a between day comparison revealed that all embryos reacted similarly to the combination of gases with the exception of day 9. Heart rate on day 9 of development was

significantly ($p<0.01$) depressed in comparison to all other days of incubation during combined exposure to 5% CO_2 and 10% O_2 (Fig 5.9).

Discussion

Chemoreflex control of cardiovascular function is an important homeostatic mechanism for the maintenance of proper gas transport to and from peripheral tissues in adult chickens (Butler 1967, Ray and Fedde 1969). Despite the critical role this mechanism plays in adults, a chemoreflex is immature or absent during the majority of chicken development (Van Golde et al 1997, Tazawa 1981a&b, Girard 1973). The present study has extended the analysis into the final days of incubation including the onset of lung ventilation in an effort to detect maturation of a cardiovascular chemoreflex reaction. Cardiovascular responses characteristic of an adult chemoreflex during hypoxia and hypercapnia were absent throughout the latter half of chicken development. Additionally, pressure responses to intermediate hypoxia were maximal over the final three days of incubation while heart rate effects were minimized. Hypercapnia primarily altered heart rate while arterial pressure exhibited a relative tolerance to increase in CO_2 levels. Therefore, those cardiovascular responses characteristics of an adult chemoreflex are absent during embryonic development in chickens.

Adult Responses

Adult chickens rely on chemoreflex action for the alteration of cardiovascular function during periods of acute hypoxia or hypercapnia to

maintain proper gas transport (Butler 1967, Butler and Taylor 1974, Ray and Fedde 1969). In adults, acute hypoxic exposure produces a hypotensive tachycardia with rate changes ascribed to β-adrenergic action, while pressure reduction is due to the direct response of vessels to a reduced O_2 (Butler 1967). Further, adult chickens are relatively tolerant of hypercapnia, but as levels intensify, a hypertensive tachycardia develops (Ray and Fedde 1969). Given the pronounced nature of these adult cardiovascular responses it was hypothesized that during chicken development a chemoreflex would become evident and mature until hatching. This hypothesis must be rejected as embryonic chickens at no time demonstrate reactions to hypoxia or hypercapnia that would typify an adult chemoreflex.

Hypoxia

All control cardiovascular parameters were within the range established previously during the ontogeny of chickens (Girard 1973; Tazawa 1981a). In addition, with the notable exception of days 20 and 21 all levels of hypoxia produced quantitatively similar responses with an accentuation at 5% O_2 (Table 5.3).

Acute hypoxia resulted in a pronounced bradycardia during the developmental period studied (Table 5.3). While at extreme levels of hypoxia (5% O_2) this reaction could be the result of reduced O_2 delivery to the heart, bradycardia at the remaining two hypoxic levels suggests possible central regulation. Data from fetal sheep indicate normal cardiac function can be

maintained at a P_{O2} of 50% normoxic levels, further suggesting that responses in the embryonic chicken could be mediated via central influences (Fisher et al. 1982). Atrial field stimulation studies have demonstrated previously that parasympathetic depression of heart rate is possible on day 12 of chicken incubation (Pappano 1976, Higgins and Pappano 1981). Thus, functional components are present midway through embryonic development, with the possibility for a central nervous system mediated change in heart rate during hypoxia. However, while regulatory components are known to be present, this work indicates that during hypoxia they remain inactive in developing chickens.

Prior studies have suggested that the negative chronotropic actions of hypoxia are due to direct inhibition of cardiac contraction as well as possible release of vasoactive substances altering venous return (Tazawa 1981b, Van Golde et al. 1997). In addition, the present study has shown that negative chronotropic responses were evident on day 9 prior to cardiac innervation by either division of the autonomic nervous system. Further, previous work has noted a clear negative chronotropic reaction to hypoxia prior to day 9 of chicken incubation, which was assumed to be mediated via mechanisms other than parasympathetic action (Altimiras and Phu 1999). Therefore, negative chronotropic reactions during hypoxia in this study were caused by direct action on the heart and/or alterations of venous return due to vasoactive substances, with little action attributed to the parasympathetic innervation.

Arterial pressure responses mimicked the chronotropic reactions during the majority of incubation for all hypoxic levels used, with 5% O_2 augmenting the response. These findings were similar to those demonstrated in previous studies with the exception of pressure change on day 20 of incubation (Tazawa 1981b; Girard 1973). Despite this apparent discrepancy, all studies have indicated that chicken embryos respond to acute hypoxia with a general hypotension, which is a reaction similar to that shown in adults (Butler 1967, Ray and Fedde 1969). Thus, embryonic chickens have a hypoxic pressure response similar to that found in adults. The majority of avian adult responses are due to direct inhibition of peripheral vessels with no central mechanisms mediating hypoxic hypotension (Butler 1967). While the possibility of a fundamentally different regulatory system during embryonic development exists, embryonic pressure responses appear to be the result of direct action of hypoxia on the vessels. Hence, humoral and local vasoactive substances, as well as the direct affect of low O_2 on vascular smooth muscle, may account for hypoxic hypotension in embryos.

It is important to recognize that both the present and past studies have attempted to determine hypoxic effects on the total vascular system. This approach, while informative, limits the ability to determine reaction of specific vascular beds to hypoxia. Thus, important centrally mediated changes in vascular tone that may differ between organ systems are indiscernible using the present measurement technique. This point is illustrated in adult chickens in which hypoxia produces an overall hypotension, but there is a noted α–

adrenergic induced pulmonary vasoconstriction which accompany this hypotension (Butler 1967, Besch and Kadono 1977). Therefore, given the importance of adrenergic cardiovascular tone during the latter half of chicken development, differential vascular reaction may occur as in adults (see Ch 2). The possibility of an adrenergic contribution to hypoxic hypotension in embryonic chickens is further addressed in Chapter 6.

Hypercapnia

The negative chronotropic actions of graded hypercapnia demonstrated in the present study were similar to those found in prior studies on chicken embryos from days 14 to 16 of incubation (Tazawa 1981b, Girard 1973). In addition, the aforementioned studies did not report the change in arterial pressure during hypercapnia demonstrated in this study, which again differed from the reported hypertension found in adults (Tazawa 1981b, Girard 1973). Thus, embryonic protective mechanisms may be absent or fundamentally different from adult systems.

Negative chronotropic responses were evident during 5% and 10% CO_2 exposures, suggesting chicken embryos are tolerant to low levels of hypercapnia. While the lack of reaction to low levels of hypercapnia may be attributed to possible buffering capacity of embryonic blood, mechanisms which underline the negative chronotropic actions at higher CO_2 levels are unknown. As stated for hypoxic exposure, parasympathetic function can be induced via field stimulation by day 12 of incubation, implying the ability for centrally mediated change in

heart rate (Pappano 1977). However, negative chronotropic responses to hypercapnia were evident prior to day 12 of incubation and, with the exception of day 21, remained constant over incubation (Table V). Tazawa (1981b) had previously attributed depressive hypercapnic responses to direct action on cardiac tissue of embryos from 14 to 16 days of incubation. Hence, direct actions of hypercapnia over the latter half of incubation, as suggested for hypoxic responses, may explain the findings in the present study. Release of vasoactive substances, resulting in reduced venous return as well as increased filling time could also account for chronotropic reactions found in this study. However, additional pharmacological study is necessary to make definitive statements as to the origin of hypercapnic action of embryonic heart rate.

Unlike embryonic heart rate, arterial pressure remained constant during hypercapnic exposures throughout the majority of chicken incubation. This finding was similar to that previously shown in chicken embryos from 14 to 16 days of incubation (Tazawa 1981a). Therefore, unlike adult chickens, which exhibit a hypertension during hypercapnia, embryonic chickens maintain constant arterial pressure alteration of CO_2 levels. This pattern was evident during the majority of developmental days studied, but day 21 embryos exhibited a significant hypertension during hypercapnia (Fig 5.8). Mechanisms that underlie this alteration of pressure responses are unclear. However, the onset of an adult chemoreflex may partially account for this change.

Hypoxic Hypercapnia

As stated previously cardiovascular responses to 10% O_2 were similar to those determined during chick development (Tazawa 1981, Tazawa et al. 1985, and Girard 1973). Combined hypercapnic hypoxic exposures lack the anticipated augmentation of cardiovascular reaction to hypoxia (Fig 5.9). In adult chickens acute exposure to hypoxia coupled with an elevated CO_2 level produces both hypertension as well as hypotension, with the precise nature of the responses depending on technique and severity of exposure (Butler 1967, Scheid and Piiper 1970). Thus, direct comparisons between adult and embryonic hypoxic hypercapnic cardiovascular responses are difficult. However, given that combined gas exposure produced reactions similar to hypoxia alone, it is unlikely that addition of CO_2 activated other regulatory mechanisms.

Chemoreflex Onset

Levels of both hypoxic and hypercapnic stress used in the present study were equivalent to those previously determined to induce chemoreflex-driven cardiovascular changes in adult chickens (Butler 1967, Ray and Fedde 1969, Besch and Kadono 1977). Thus, the absence of an adult-like reaction throughout the majority of chicken incubation must be attributed to fundamental differences between the two life stages. However, as demonstrated for both hypoxic and hypercapnic exposures, a transition to an adult regulation mechanism may occur in the final days of chicken incubation.

As stated previously, embryonic arterial blood pressure was relatively unaffected by exposure to 10% O_2 while negative chronotropic actions were

significantly dampened over the final two days of chicken incubation. In addition, cardiovascular responses to 10% CO_2 on day 21 differed from all other days of incubation. Collectively, this information indicates that a critical period in chicken development occurs in the final 2 days of incubation, during which an adult chemoreflex may become functional.

Hypercapnic exposure on day 21 produced an increase in blood pressure, a response that differed from all other days of incubation. This reaction typifies an adult cardiovascular response to elevated levels of CO_2 implying that a chemoreflex is operational on day 21. However, pressure responses to hypoxia were unchanged on days 20 and 21 lacking the hypotension characteristic of both embryonic and adult reactions. Under both experimental conditions, chronotropic responses on day 20 and 21 were unlike those characterized for adult suggesting a chemoreflex is absent. Therefore, the functional status of a cardiovascular chemoreflex remains in question. An adult chemoreflex requires peripheral and central chemoreceptors as well as intact efferent connections to the developing cardiovascular system. During the final two days of chicken incubation functional efferent connections to the cardiovascular system are well established, thus this component of the adult chemoreflex is present in the embryo (Pappano 1977). While the operational status of chemoreceptors during chicken incubation has not been well characterized, preliminary data with NaCN suggest peripheral receptors are active on day 21 of incubation (Crossley and Burggren in prep). Collectively the data suggest that a hypercapnic chemoreflex

becomes operational on day 21 of chicken incubation, but further study is needed to determine how hypercapnia modulates this response.

Conclusion

Alteration of ambient gas composition produces dramatic physiological and morphological changes in developing chicken embryos (Grabowski et al. 1969, Altimiras and Phu In press, Tazawa 1986). Despite the potential deleterious influences of low O_2 or elevated CO_2, chicken embryos lack an adult cardiovascular chemoreflex during the majority of incubation. It is important to recognize that adult cardiovascular responses are accompanied by respiratory changes. Clearly, embryonic chickens lack the respiratory component throughout the majority of development. Thus, during development regulatory mechanisms and cardiovascular responses may differ from those found in adult chickens. The final days of development may represent a period of “system crossover” or transition, as embryonic regulation fades and adult regulation becomes operational. However, further pharmacological study is needed to determine the mechanisms that modulate embryonic cardiovascular responses to alteration of ambient gas composition.

Table 5.1. Systolic (Sys) and diastolic (Dia) pressure response to different levels of hypoxia. Asterisk indicates significance ($p<0.05$) as indicated by a paired Student t-test of treatment (T) compared to control (N) levels on a given day of incubation. Data are presented as mean ± sem. In all cases n = 5.

		15% O_2		10% O_2		5% O_2	
Day		Sys	Dia	Sys	Dia	Sys	Dia
9	N	5.3±0.4	3.2±0.3	5.2±0.4	2.6±0.2	5.0±0.4	2.8±0.4
	T	4.8±0.4 *	2.6±0.2	4.3±0.3 *	2.4±0.3	4.0±0.3 *	2.4±0.3
12	N	10.5±0.8	4.9±0.4	10.4±0.9	5.0±0.5	10±1.3	4.8±0.7
	T	9.7±0.9 *	4.6±0.4	8.8±0.8 *	4.1±0.5 *	7.8±1.2 *	3.4±0.6 *
15	N	16.9±1.6	8.1±1.1	16.7±1.6	8.6 ±1.1	16.8±1.7	9.0±1.2
	T	15.5±1.3 *	7.4±1.0	13.9±1.1 *	6.8 ±0.8 *	12.7±1.1 *	6.00±0.6 *
18	N	22.7±1.1	12.0±1.2	21.2±1.2	12.4±0.9	20.9±1.3	12.7±1.0
	T	19.8 ±1.0 *	11.0±1.2	19.9±1.0	10.5±0.7 *	17.8±0.5 *	7.4±0.6 *
19	N	26.9±1.1	16.4±1.1	27.4±1.1	17.1±1.2	27.9±1.4	16.8±1.3
	T	22.2±1.3 *	13.2±0.9	20.9 ±1.0 *	12.1±1.2 *	22.9±1.9 *	11.0±1.1 *
20	N	33.4±1.8	21.4±1.6	35.4±2.7	21.7±1	33.6±2.6	23.1±1.8
	T	32.2±2.0	20.3±1.7	32.4±2.6	18.1±1.2	27.0±1.9 *	16.5±1.7 *
21	N	35.5±2.4	21.9±1.2	34.0±2.7	22.9±2.1	35.0±2.5	22.6±1.1
	T	32.1±1.8	19.8±1.3	29.7±2.0	19.6±1.2	30.1±2.2 *	16.4±1.2 *

Table 5.2. Difference in the effect of 10% O_2 effects on mean arterial (Map) systolic (Sys), diastolic (Dia) pressures and heart rate (f_H) between days of development. All days with significantly different hypoxic response are identified with a like letter. As determine with a two way ANOVA conducted on arcsine transformed percentage changes ($p<0.05$). In all cases n= 5.

Day	Map		Sys		Dia		f_H	
9	-0.5±0.5	AB	-0.7±0.6	AC	-0.1±0.4	A	-30±20	AB
12	-1.4±0.1	A	-1.6±0.2	A	-0.9±0.1	A	-45±8	A
15	-2.5±0.5	AB	-2.9±0.7	AB	-1.8±0.3	A	-43±5	A
18	-1.6±0.7	B	-1.3±0.7	B	-1.9±0.8	A	-38±8	AC
19	-5.6±0.9	A	-6.5±1.0	A	-5.0±0.7	A	-39±5	A
20	-3.9±1.9	BD	-4.4±2.0	AB	-3.3±1.9	A	-20±7	BC
21	-3.2±1.6	BD	-3.0±1.7	BC	-3.6±1.5	A	-20±7	B

Table 5.3. A comparison of hypoxic treatments effects on mean arterial (Map) systolic (Sys), diastolic (Dia) pressures and heart rate (f_H) within a specific day of incubation. Deltas indicate difference from other exposures as determine with a two way ANOVA conducted on arcsine transformed percentage changes ($p<0.05$). ΔΔΔ Differs from 15% only; ΔΔ differs from 15% and 10%; Δ 10% and 5% differ from 15%. In all cases n = 5.

Day		Map		Sys		Dia		f_H	
9	15	-0.4±0.5		-0.4±0.7		-0.5±0.4		-13±13	
	10	-0.5±0.5		-0.7±0.6		-0.1±0.4		-30±20	Δ
	5	-0.8±0.2		-1.0±0.2		-0.5±0.2		-78±13	Δ
12	15	-0.6±0.1		-0.8±0.1		-0.3±0.1		-21±10	
	10	-1.4±0.1		-1.6±0.2		-0.9±0.1		-45±8	
	5	-2.1±0.4	ΔΔ	-2.7±0.6	ΔΔΔ	-1.2±0.3	ΔΔ	-65±16	Δ
15	15	-1.1±0.3		-1.4±0.5		-0.7±0.2		-20±6	
	10	-2.5±0.5		-2.9±0.7		-1.8±0.3		-43±5	Δ
	5	-3.9±0.6	ΔΔΔ	-4.1±0.9	ΔΔΔ	-3.0±0.6	ΔΔ	-78±7	ΔΔ
18	15	-2.0±0.6		-2.9±1.0		-1.0±0.4		-13±4	
	10	-1.6±0.7	Δ	-1.3±0.7		-1.9±0.8	Δ	-38±8	Δ
	5	-4.4±1.0	Δ	-3.1±1.1		-5.3±1.1	Δ	-77±8	ΔΔ
19	15	-3.7±0.8		-4.7±1.0		-3.2±1.0		-18±5	
	10	-5.6±0.9		-6.5±1.0		-5.0±0.7		-39±5	
	5	-5.2±1.2		-5.1±1.5		-5.8±0.7		-53±6	Δ
20	15	-1.1±0.8		-1.2±1.1		-1.1±0.6		-3.0±3	
	10	-3.9±1.9	Δ	-4.4±2.0		-3.3±1.9	Δ	-20±7	Δ
	5	-7.0±1.3	Δ	-6.6±2.1	ΔΔΔ	-6.6±0.9	Δ	-65±15	Δ
21	15	-2.5±0.9		-3.4±1.6		-2.1±0.8		-7.0±7	
	10	-3.2±1.6		-3.0±1.7		-3.6±1.5		-20±7	Δ
	5	-6.0±1.7		-4.9±2.0		-6.2±1.5		-53±8	Δ

Table 5.4. The effect of CO_2 exposure (3%, 5% and 10%) on systolic (Sys) and diastolic (Dia) pressures throughout development. Data is presented as control (N) treatment (T). Days during which hypercapnia significantly ($p<0.05$) altered pressure are indicated with asterisk. In all cases n = 5.

Day		3% CO_2		5% CO_2		10% CO_2	
		Sys	Dia	Sys	Dia	Sys	Dia
9	N	6.6±0.9	4.7±0.8	6.0±0.8	4.1±0.8	5.9±0.8	4.1±0.8
	T	6.4±0.8	4.4±0.8 *	6.0±0.8	4.1±0.8	5.9±0.8	4±0.9
12	N	8.7±0.9	5.1±1.0	8.6±1.2	5.3±1.1	8.5±1.2	5±0.9
	T	8.6±1.1	5.4±1.0	8.4±1.1	5.0±0.8	8.1±1.2 *	4.9±0.9
15	N	19.9±1.2	10.6±0.7	18.8±1.4	10.9±1.1	18.9±1.6	10.6±1.2
	T	20.1±1.3	10.9±1.0	19.0±1.4	10.3±1.3	19.3±1.6	9.4±1.0
18	N	22.6±1.9	13.2±1.1	22.6±1.3	13.3±1.0	22.5±1.4	14.2±0.9
	T	24.0±1.9	13.9±1.2 *	23.3±1.5	13.5±1.3	23.5±1.5	14±1.0
19	N	24.2±1.0	14.3±0.5	24.3±1.2	14.7±0.7	24.7±1.2	15.5±0.8 *
	T	25.1±1.2	14.6±0.7	24.0±1.1	14.0±0.6	24.8±1.5	14.7±0.8
20	N	28.2±2.7	19.1±1.8	30.1±2.5	19.2±1.5	28.9±2.5	19.2±1.3
	T	30.4±2.5	19.6±1.0	30.6±2.2	19.8±1.2	27.7±1.2	19.7±0.7
21	N	31.5±1.9	19.3±0.9	34.4±1.8	20.3±0.7	32.0±1.2	19±0.4 *
	T	34.4±2.0 *	20.6±0.9	35.4±1.6	19.8±0.5	34.0±1.3 *	20.5±0.6

Table 5.5. Difference in the effect of CO_2 exposures (3%, 5% and10%) between days of development on mean arterial pressure (Map) and heart rate (f_H). All days of incubation which reacted similarly to CO_2 are indicated by like lettering, as determined with a two way ANOVA conducted on arcsine transformed percentage changes ($p<0.05$). In all cases n= 5.

	3%				5%				10%			
Day	Map		f_H		Map		f_H		Map		f_H	
9	-0.3±0.1	A	-18±4	A	-0.1±0.1	A	-22±6	A	0±0.1	A	-30±4	A
12	-0.1±0.2	AE	-3±7	AB	-0.3±0.3	A	-23±11	A	-0.3±0.1	A	-18±8	AB
15	0.1±0.4	AC	1±6	B	-0.3±0.4	A	-14±4	A	-0.4±0.3	AC	-25±1	A
18	0.9±0.3	CD	-3±3	B	0.7±0.4	A	-9±2	AB	0.4±0.2	BC	-21±1	AB
19	0.5±0.3	CE	-6±3	B	-0.6±0.4	A	-19±3	A	-0.4±0.5	A	-34±5	A
20	0.9±0.8	CDE	0±3	B	0.5±0.3	A	-14±11	AB	-0.4±1.5	AB	-22±8	AB
21	2.3±0.1	D	5±2	B	0.8±0.7	A	-4±6	B	2.4±0.7	B	-12±4	B

Table 5.6. The differences in mean arterial pressure (Map), systolic pressure (Sys), diastolic pressure (Dia) and heart rate (f_H) during exposure to three levels of hypercapnia. Deltas indicate significant ($p<0.05$) difference from other exposures, ΔΔΔ differs from 3% only; ΔΔ differs from 5% and 3%; Δ deltas 5% and 10% differ from 3%. In all cases n = 5.

Day		Map	Sys	Dia		f_H	
9	3	-0.3±0.1	-0.2±0.1	-0.3±0.1		-18±4	
	5	-0.1±0.1	0±0.1	0±0.1		-22±6	
	10	0.0±0.1	0±0.1	0±0.1		-30±4	
12	3	-0.1±0.2	-0.1±0.2	0.2±0.3		-3±7	
	5	-0.3±0.3	-0.1±0.0	-0.2±0.3		-23±11	Δ
	10	-0.3±0.1	-0.4±0.1	-0.1±0.1		-18±8	
15	3	0.1±0.4	0.2±0.3	0.3±0.4		1±6	
	5	-0.3±0.4	0.3±0.5	-0.6±0.3		-14±4	Δ
	10	-0.4±0.3	0.4±0.6	-1.2±0.3	ΔΔΔ	-25±1	Δ
18	3	0.9±0.3	1.4±0.6	0.7±0.2		-3±3	
	5	0.7±0.4	0.8±0.4	0.2±0.5		-9±2	
	10	0.4±0.2	1±0.4	-0.2±0.3		-21±1	ΔΔΔ
19	3	0.5±0.3	0.9±0.5	0.3±0.3		-6±3	
	5	-0.6±0.4	-0.2±0.7	-0.6±0.4	Δ	-19±3	Δ
	10	-0.4±0.1	0.1±0.9	-0.8±0.3	Δ	-34±5	Δ
20	3	0.9±0.8	2.3±0.1	0.4±0.9		0±3	
	5	0.5±0.3	0.4±0.8	0.6±0.8		-14±11	
	10	-0.4±1.5	-1.2±2.0	0.5±1.1		-22±8	ΔΔΔ
21	3	2.3±0.1	2.9±0.6	1.3±0.6		5±2	
	5	0.8±0.7	1.1±0.7	-0.5±1.5		-4±6	
	10	2.4±0.7	3.3±1.0	1.8±0.6		-12.5±4	ΔΔΔ

Table 5.7. The effect of 10% O_2 exposure (10) and 5% CO_2 with 10% O_2 (5/10) on systolic (Sys) and diastolic (Dia) pressures throughout development. Data is presented as control (N) treatment (T). Days during which treatment altered cardiovascular function compared to control are indicated with Asterisk ($p<0.05$). In all cases n= 5.

		10% O_2				5 CO_2 10% O_2			
Day		Sys		Dia		Sys		Dia	
9	N	6±0.7		4.1±0.8		6±0.7		4.3±0.8	
	T	5.1±0.7	*	3.5±0.8	*	4.8±0.6	*	3.5±0.9	*
12	N	9±1.2		5.7±1.0		9±1.3		5.9±1.1	
	T	7.4±0.9	*	4.9±0.9	*	6.8±0.9	*	5±0.9	
15	N	19.8±1.5		11.2±1.1		20.1±1.4		12.3±1.2	
	T	15.5±1	*	8.7±1.2	*	15.9±0.9	*	9.3±1.6	*
18	N	23.6±1.8		14±1.4		23±1.6		14.6±1	
	T	19.6±1.6	*	11.2±0.8		20.3±1.3	*	12.1±1	*
19	N	25.6±1		15.3±0.6		26.5±0.9		17.2±0.5	
	T	22.9±1.2	*	10.6±0.5	*	23.6±0.7		13±0.7	*
20	N	27.3±2.2		18.5±0.9		30.5±3.1		21±1.8	
	T	26.1±1.9		16.3±0.5		26±1.6		17.5±0.7	
21	N	32.3±2.2		18.6±1.2		32.5±2.2		20.2±1.3	
	T	30.1±2.4		16.1±1.5		31.2±2.6		19.1±2	

Figure Legend

Figure 5.1

Representative raw data traces depicting arterial pressure (P) and heart rate (f_H) responses to 10% O_2 from embryos of 19 (A), 20 (B) and 21 (C) days of incubation. Bar indicates a 5-min hypoxic exposure.

Figure 5.2

The cardiovascular reaction to 15% O_2 in chicken embryos during development. Heart rate(f_H) and mean arterial pressure are presented as control (open column) and treatment (filled column) response. Asterisk indicates significant difference ($p<0.05$) in parameter from control. Data are presented as mean ± sem. In all cases n= 5.

Figure 5.3

The cardiovascular reaction to 10% O_2 in chicken embryos during development. Heart rate (f_H) and mean arterial pressure are presented as control (open column) and treatment (filled column) response. Asterisk indicates significant difference ($p<0.05$) in parameter from control. Data are presented as mean ± sem. In all cases n= 5.

Figure 5.4

The cardiovascular reaction to 5% O_2 in chicken embryos during development. Heart rate (f_H) and mean arterial pressure are presented as control (open column) and treatment (filled column) response. Asterisk indicates significant

difference ($p<0.05$) in parameter from control. Data are presented as mean ± sem. In all cases n= 5.

Figure 5.5

Representative traces demonstrating arterial pressure (P) and heart rate (fH) responses to 5% CO_2 from embryos of 19 (A), 20 (B) and 21 (C) days of incubation. Bar indicates a 5-min hypercapnic exposure.

Figure 5.6

The cardiovascular reaction to 5-min of 3% CO_2 in chicken embryos during development. Heart rate (f_H) and mean arterial pressure are presented as control (open column) and treatment (filled column) response. Asterisk indicates significant difference ($p<0.05$) in parameter from control. Data are presented as mean ± sem. In all cases n= 5.

Figure 5.7

The cardiovascular reaction to 5-min of 5% CO_2 in chicken embryos during development. Heart rate (f_H) and mean arterial pressure are presented as control (open column) and treatment (filled column) response. Asterisk indicates significant difference ($p<0.05$) in parameter from control. Data are presented as mean ± sem. In all cases n= 5.

Figure 5.8

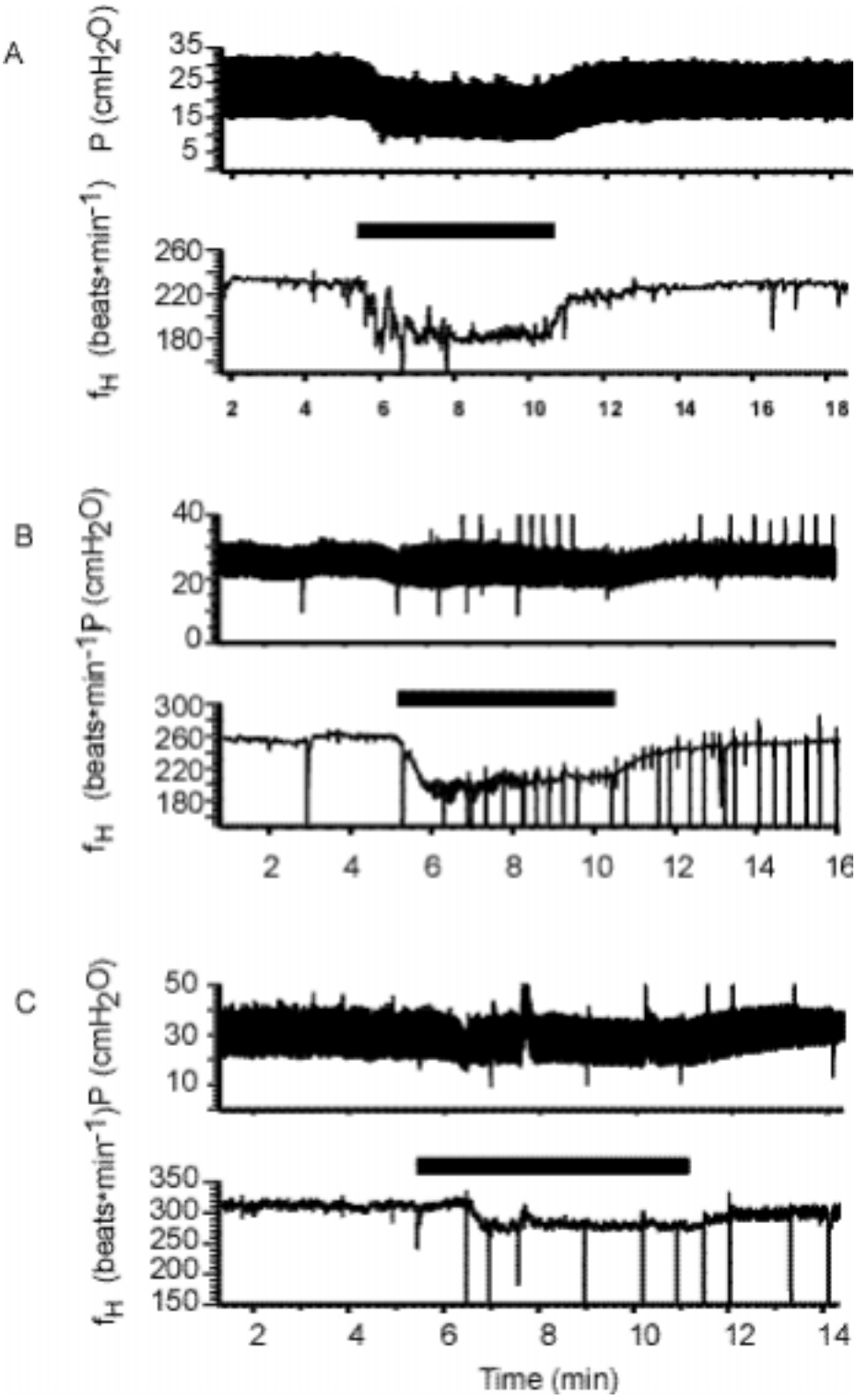
The cardiovascular reaction to 5-min of 10% CO_2 in chicken embryos during development. Heart rate(f_H) and mean arterial pressure are presented as control (open column) and treatment (filled column) response. Asterisk indicates

significant difference ($p<0.05$) in parameter from control. Data are presented as mean ± sem. In all cases n= 5.

Figure 5.9

The cardiovascular reaction to 5-min of 5% CO_2 and 10% O_2 in chicken embryos during development. Heart rate (f_H) and mean arterial pressure are presented as control (open column) and treatment (filled column) response. Asterisk indicates significant difference ($p<0.05$) in parameter from control. Data are presented as mean ± sem. In all cases n= 5.

Figure 5.1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5
P (cmH2O)
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220
180
fH (beats•min-1)
2
4
6
8
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12
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B
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P (cmH2O)
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fH (beats•min-1)
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10
P (cmH2O)
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fH (beats•min-1)
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4
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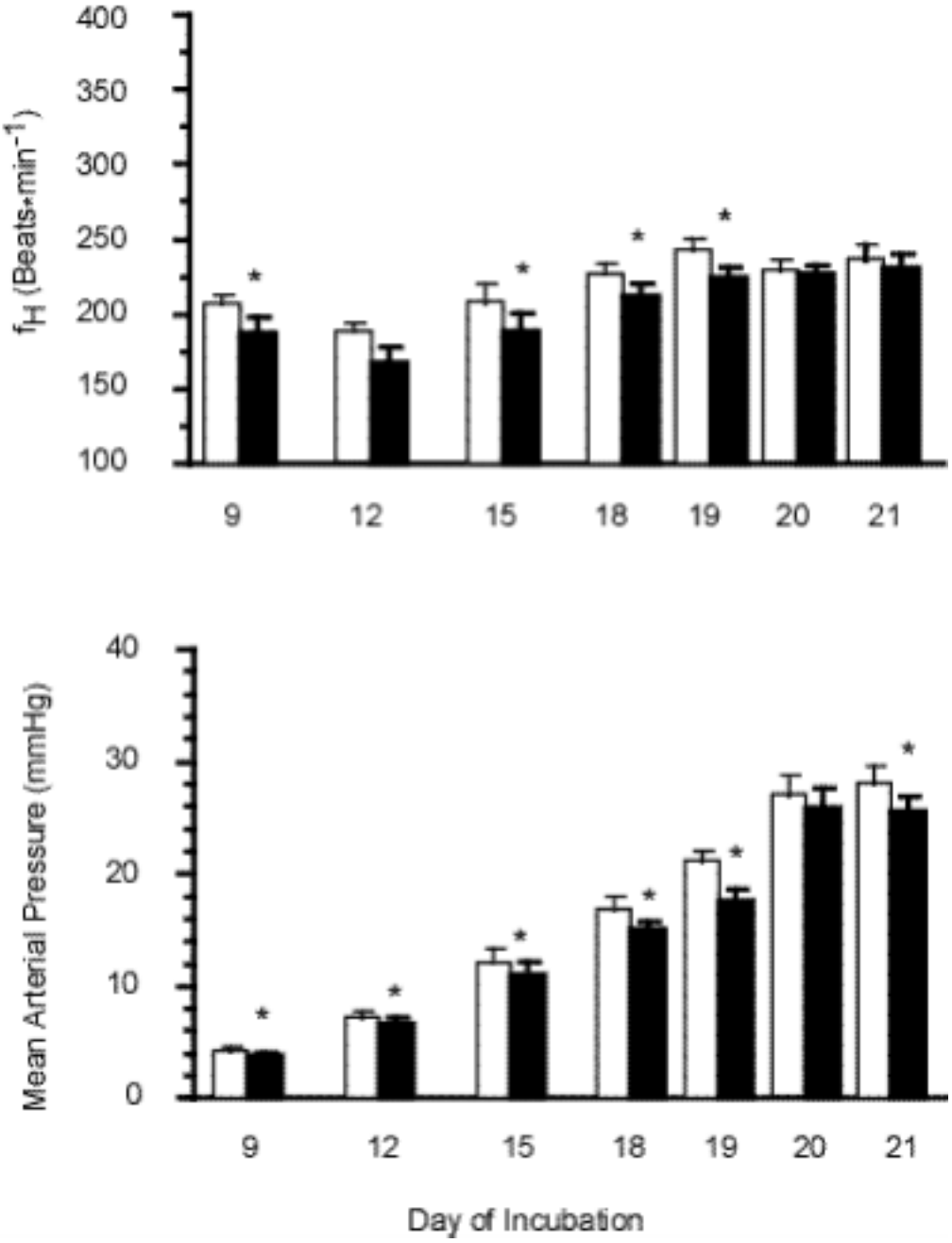
12
14
Time (min)

Figure 5.2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100
f_H (Beats•min^{-1})
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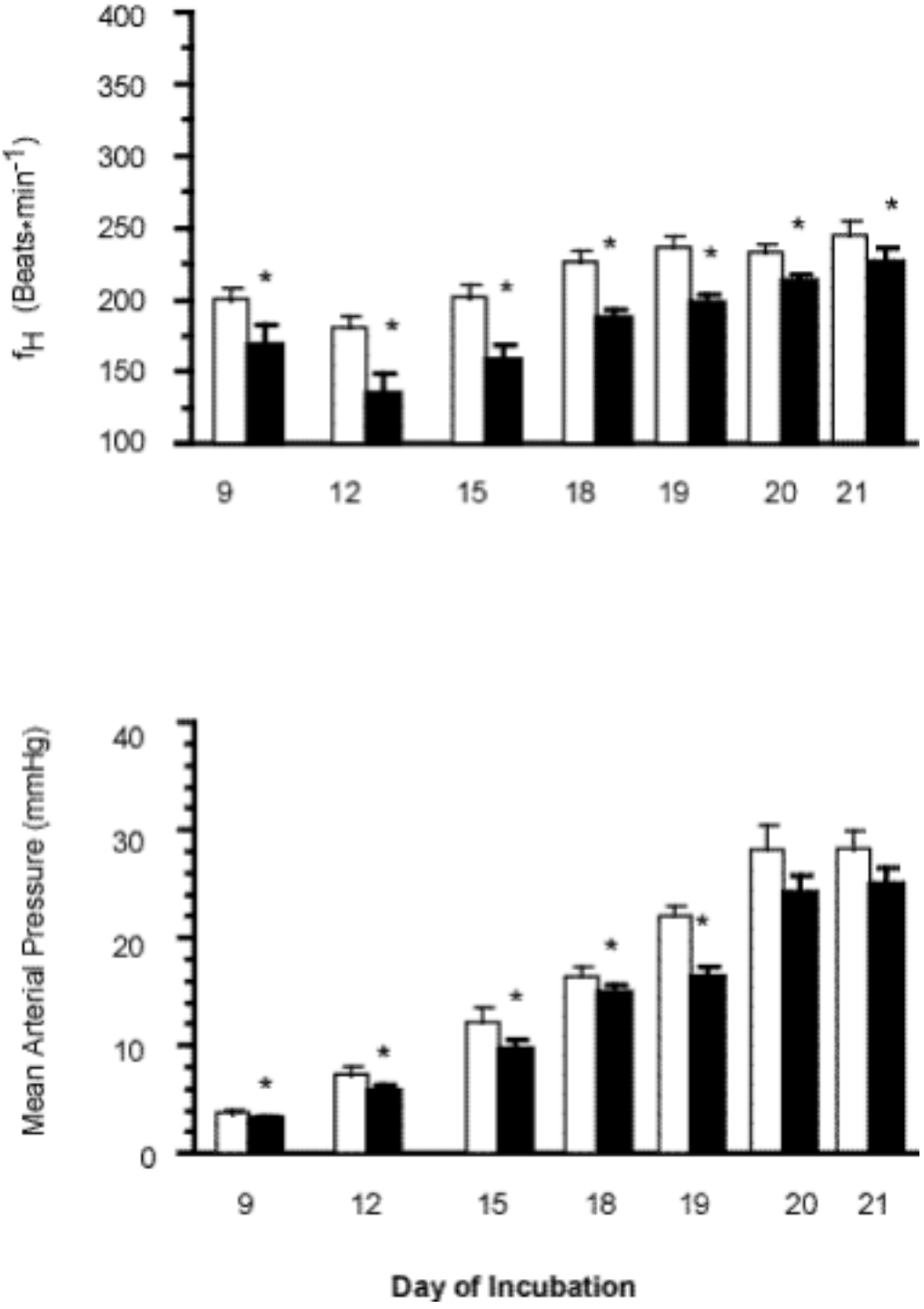
10
0
Mean Arterial Pressure (mmHg)
Day of Incubation

Figure 5.3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f_H (Beats•min^{-1})
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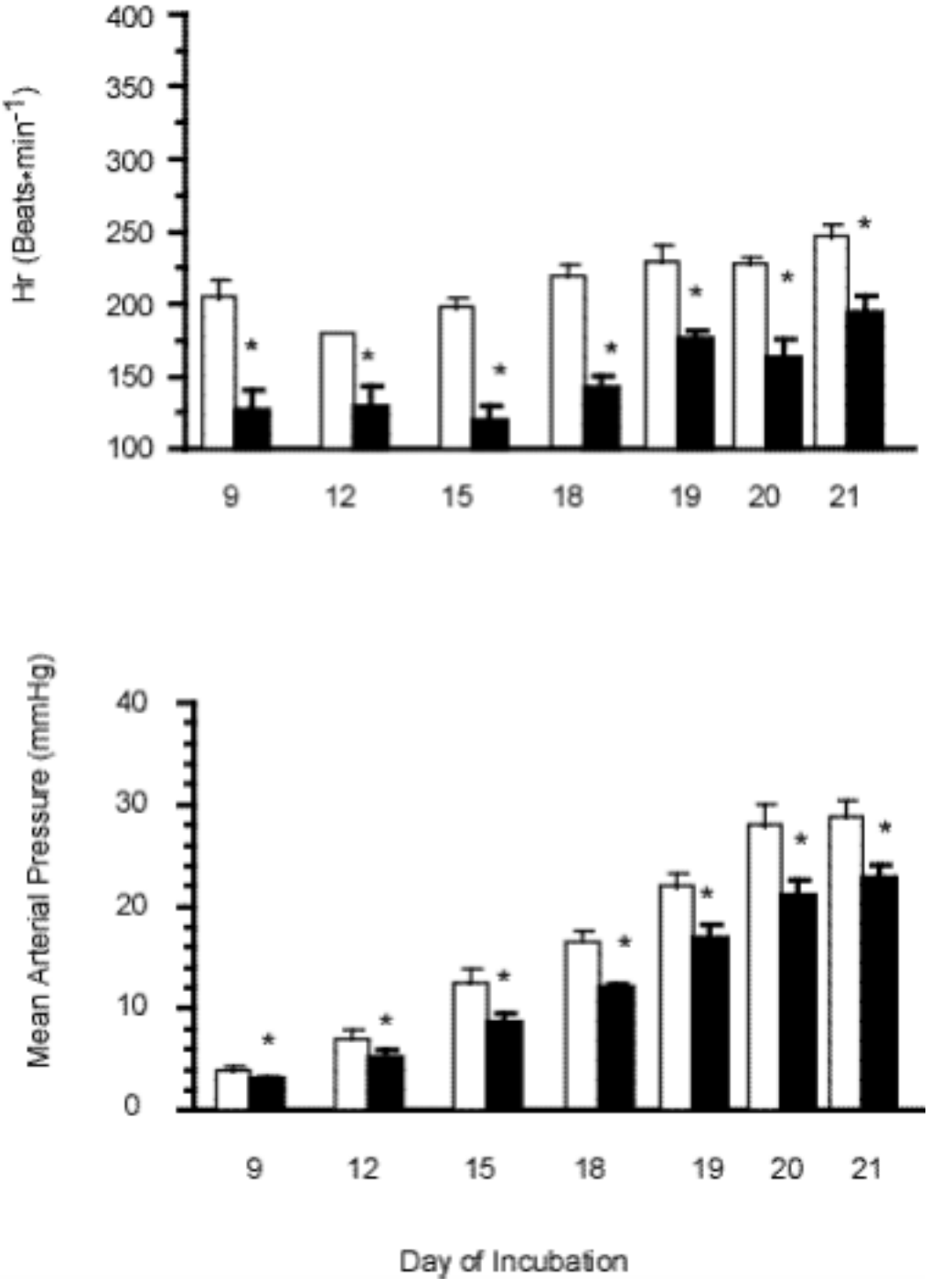
Mean Arterial Pressure (mmHg)
Day of Incubation

Figure 5.4


Hr (Beats•min^{-1})
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100
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20
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Mean Arterial Pressure (mmHg)
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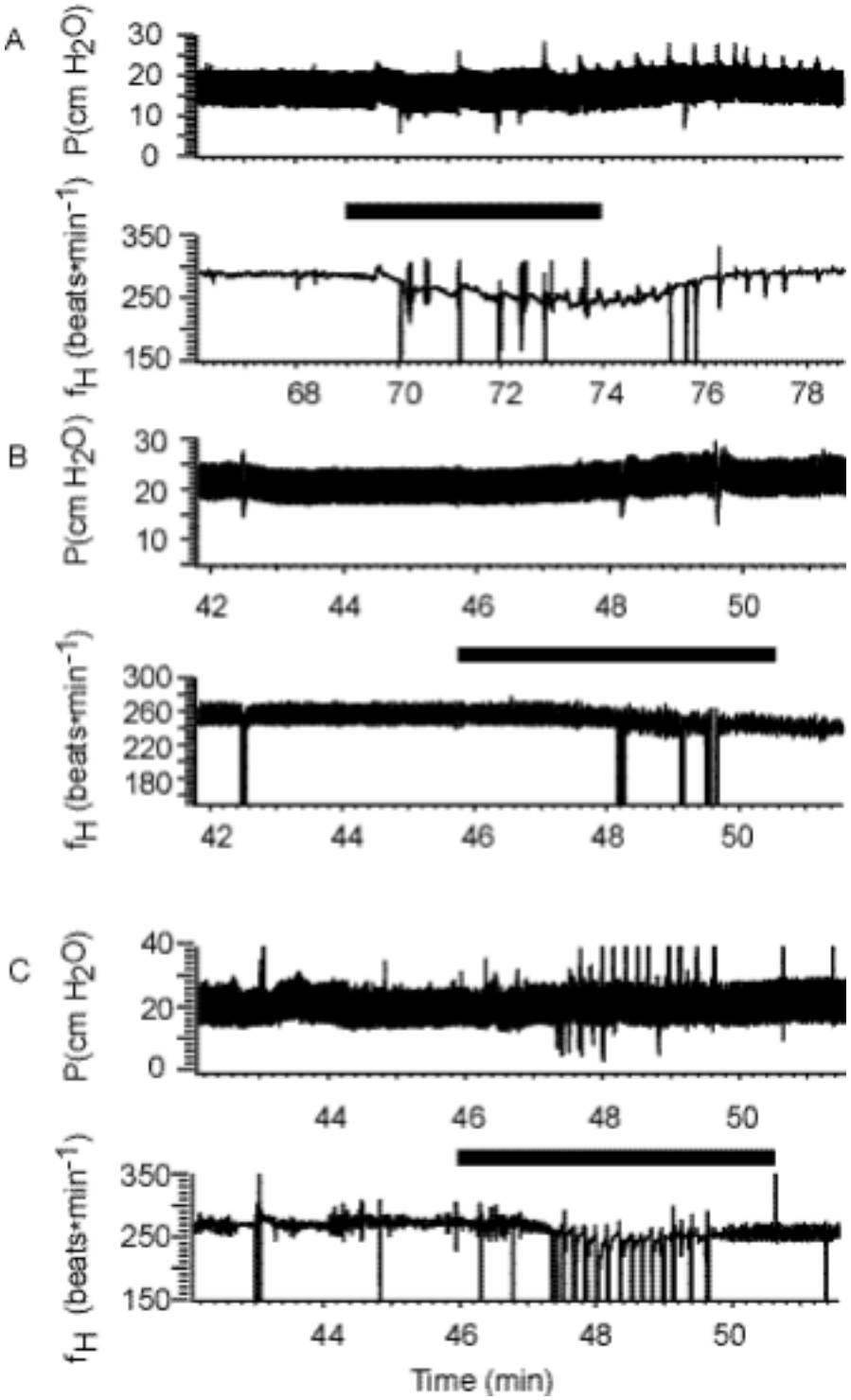
30
20
10
0
Day of Incubation

Figure 5.5


A
B
C
P(cm H2O)
fH (beats•min-1)
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20
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350
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68
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74
76
78
42
44
46
48
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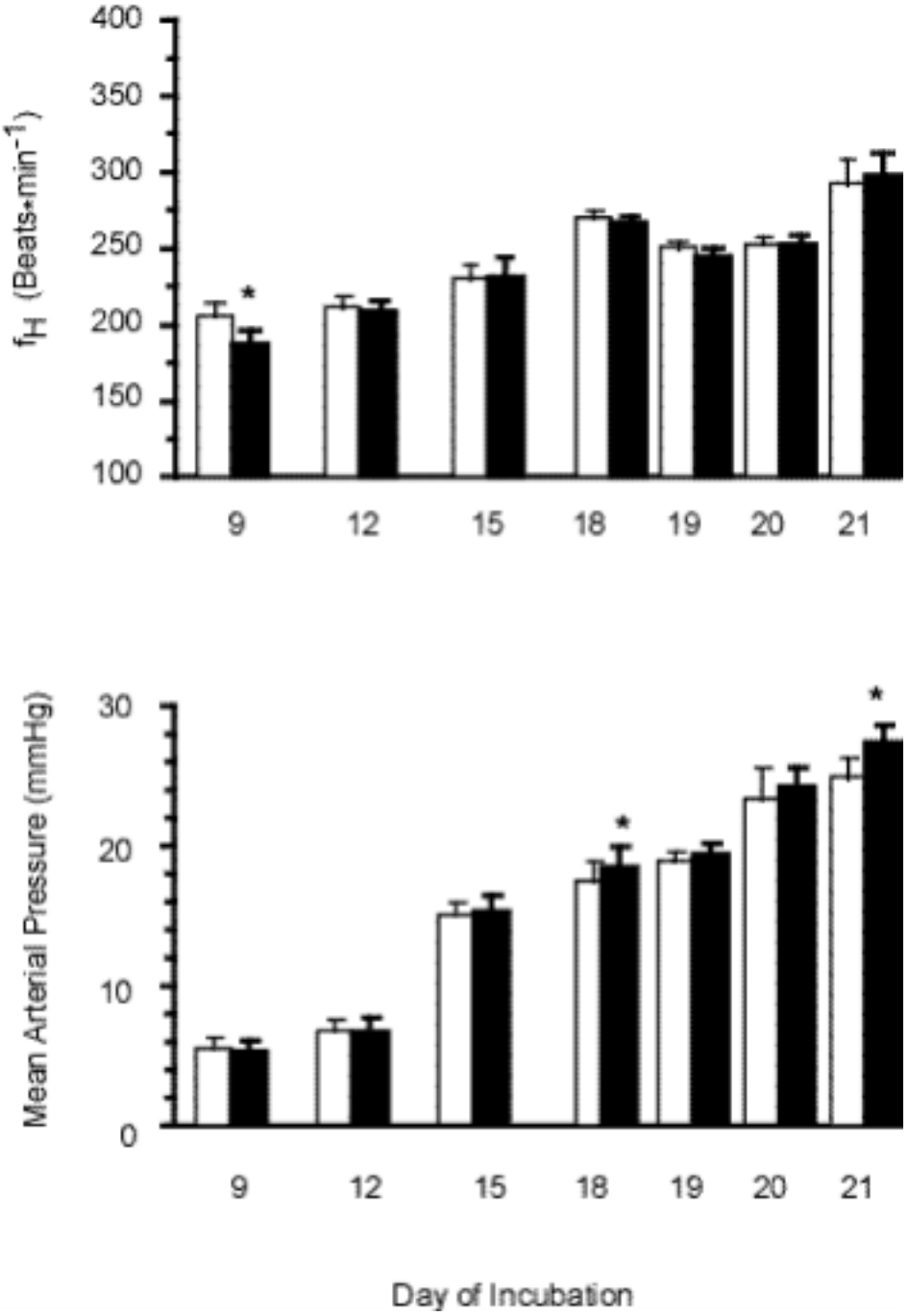
40
Time (min)

Figure 5.6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Mean Arterial Pressure (mmHg)
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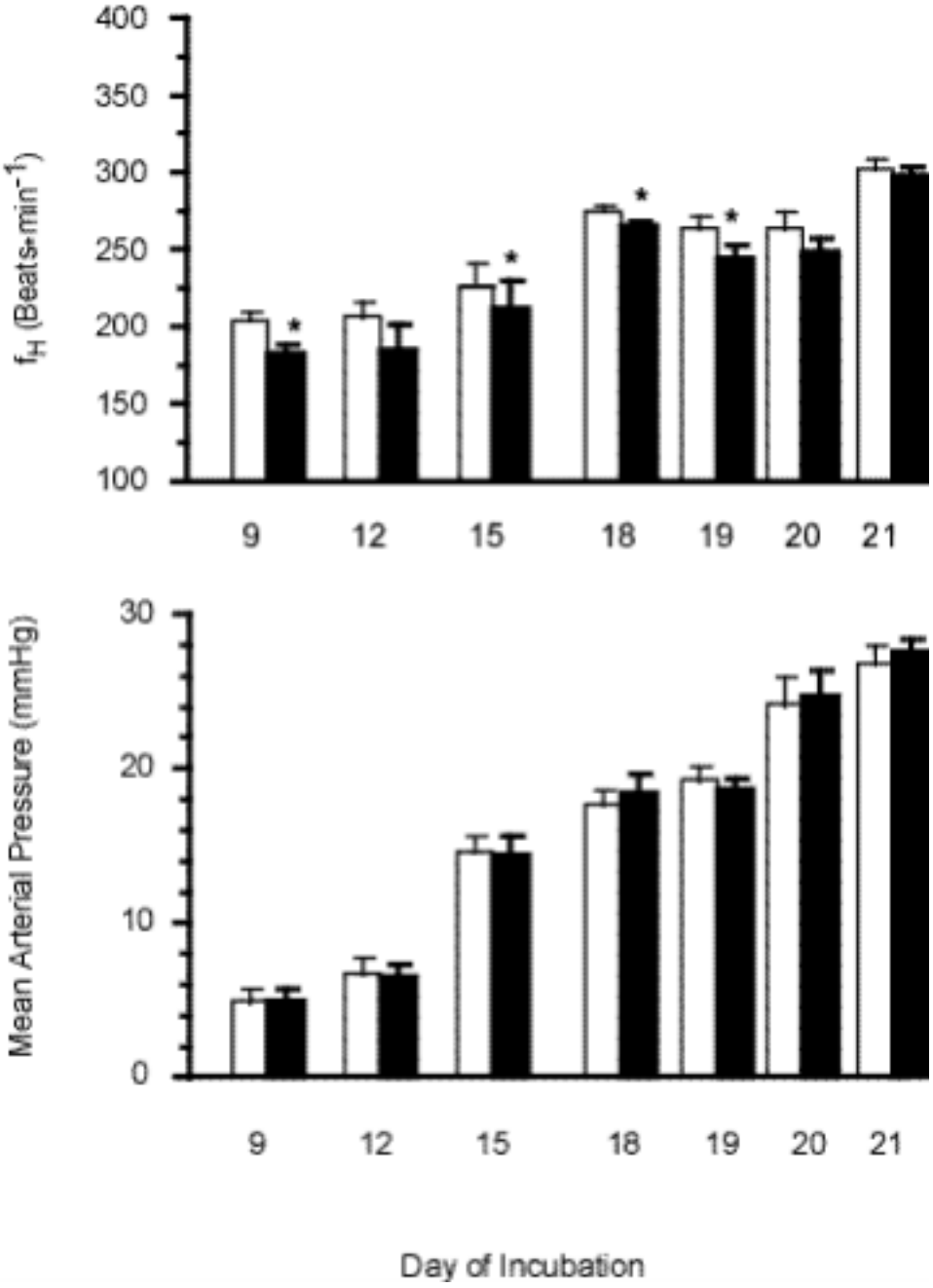
21
Day of Incubation

Figure 5.7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Mean Arterial Pressure (mmHg)
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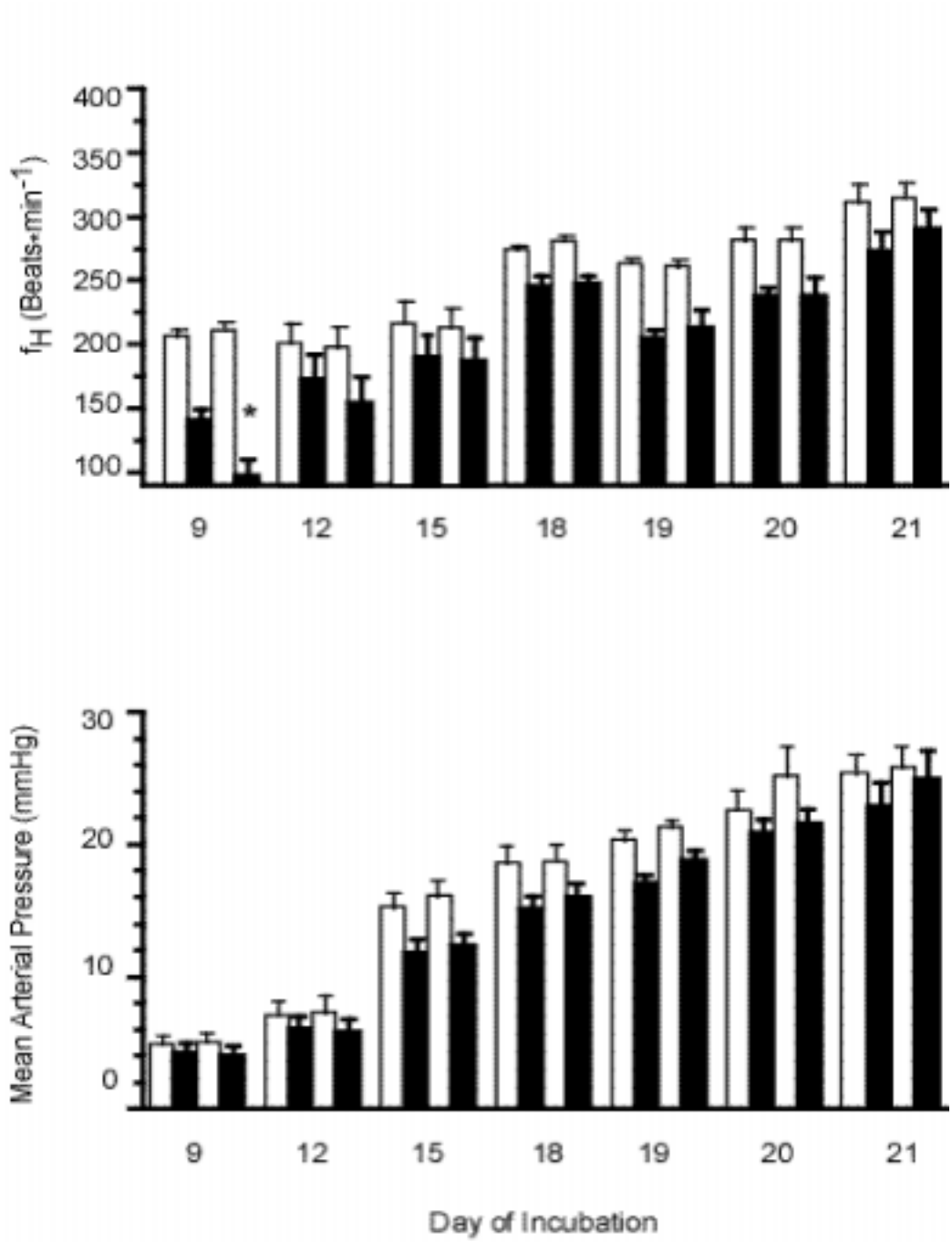
20
21
Day of Incubation

Figure 5.8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Mean Arterial Pressure (mmHg)
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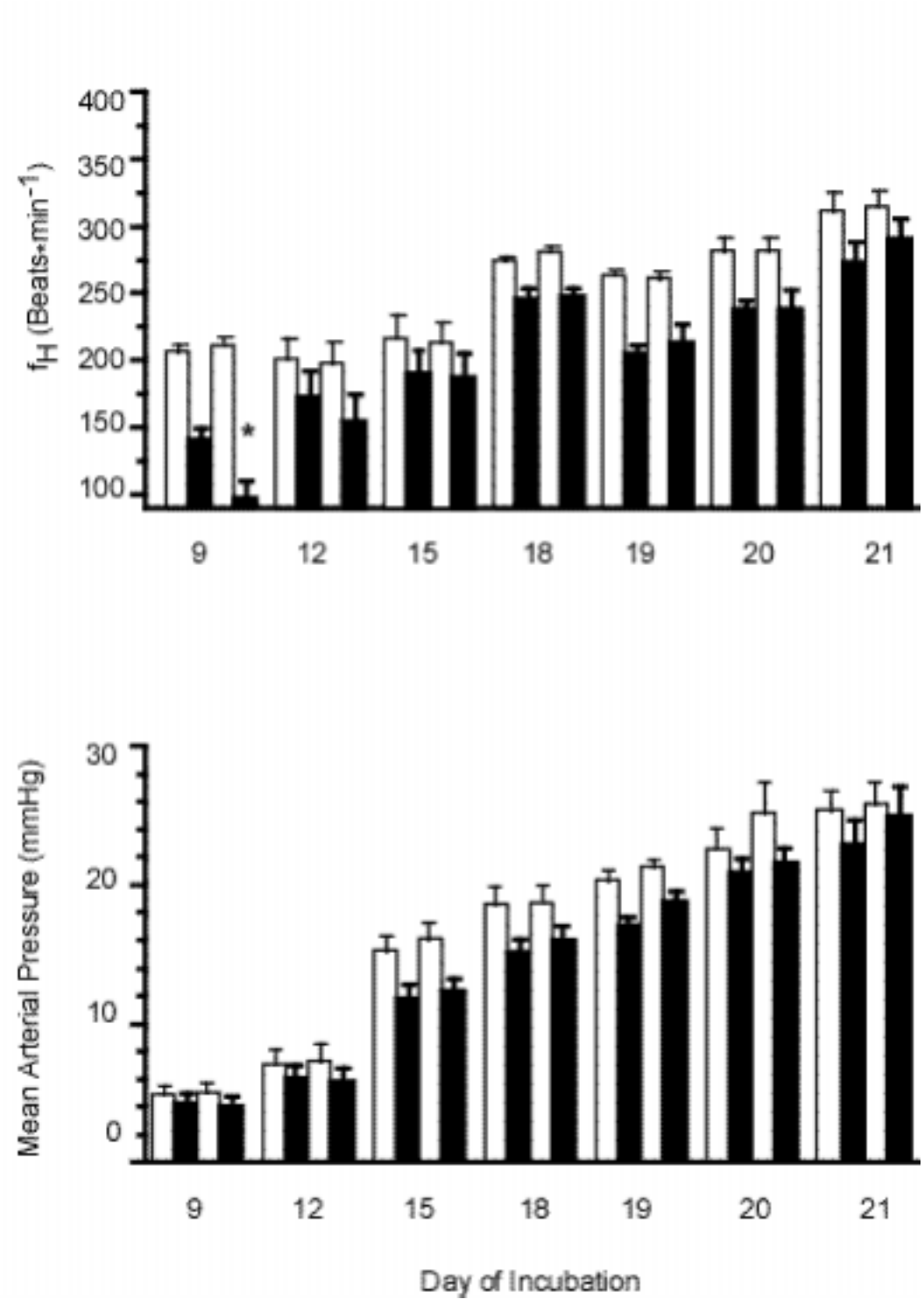
20
21
Day of Incubation

Figure 5.9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f_H (Beats•min^{-1})
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Mean Arterial Pressure (mmHg)
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21
Day of Incubation

CHAPTER VI

THE MATURATION OF AUTONOMIC ALTERATION IN CARDIOVASCULAR FUNCTIONS DURING BOUTS OF ACUTE HYPOXIC STRESS IN THE DOMESTIC CHICKEN *Gallus gallus.*

Introduction

In adult chickens, cardiorespiratory responses to hypoxic conditions have been thoroughly characterized (Butler 1967, Ray and Fedde 1969). Normocapnic hypoxia induces a marked tachycardia in adult chickens which is coupled with a hypotension, as well as an increase in ventilation rate and amplitude when they are exposed to 10% or lower O_2 concentrations (Butler 1967; Ray and Fedde 1969). This tachycardia is mediated via peripheral chemoreceptors, which in turn produces a chemoreflex resulting in an alteration of cardiovascular function. Embryonic chickens lack cardiovascular reactions typified in adults during the majority of incubation (Tazawa 1981a, Girard 1973, Van Golde 1997). During hypoxic exposure, a general depression of embryonic cardiovascular function has been shown in response to O_2 levels previously utilized in adults (Tazawa 1981a, Girard 1973, Van Golde 1997, see Chapter 5 this thesis). While these studies have characterized general reactions to hypoxic stress, mechanisms that underlie cardiovascular changes have been left to speculation. Mulder et al. (1998) suggest the maturation of a protective

mechanism is present during the second half of development which maintains blood flow to the brain, heart and chorioallontoic membrane during hypoxia. Further, Van Golde et al. (1998) suggest that circulating catecholamines may play an important role in modulating control of this system early when exposed to hypoxia early in development. Their data suggest a maturation of cardiovascular responses to hypoxia with development implying activation of central control and or chemoreflex regulation of the system. Thus, while indirect evidence indicates the central nervous system (CNS) may be involved in cardiovascular (CV) responses to hypoxia, study of the how the CNS may change CV function has not been conducted.

The sympathetic and parasympathetic arms of the autonomic nervous systems reach the heart of embryonic chickens between days 11 to13, in addition, cholinergic as well as adrenergic receptors are present on days 3 to 4, (Pappano 1977). Therefore, the physical components necessary for central cardiovascular regulation are established at the midpoint of incubation and could account for the hypoxic cardiovascular depression shown in Chapter 5.

The objective of this study was to establish the source of cardiovascular response to hypoxia in chicken embryos. Selective blockade of adrenergic and cholinergic receptors would assess the ability of autonomic system to modulate changes in cardiovascular function during periods of stress. It was hypothesized that the decrease in arterial pressure and rise in heart rate during hypoxia will be

the result of simulation of cholinergic and adrenergic receptors iin the cardiovascular system.

Materials and Methods

Domestic chicken eggs, *Gallus gallus*, of the White Leghorn strain were purchased from Texas A&M and shipped overnight to the University of North Texas Department of Biological Sciences. On arrival, eggs were placed in incubation at 38 ± 0.5 °C, 60-70% relative humidity and turned automatically every 3 hrs. For the purpose of the study, experimental manipulation was conducted on days 9, 12, 15 and18-21 of a 21-day incubation period.

Surgical Procedures

On the day of the study, eggs were removed from the incubator, candled to locate a chorioallantoic artery and placed in a temperature-controlled chamber. A portion of the eggshell was removed exposing the previously located artery. The artery was temporarily occluded up stream with a 6 zero silk ligature and a second ligature was placed downstream to eliminate retrograde flow following cannulation. A small cut was then made in the artery and a heat-pulled saline-filled catheter was then used to occlusively cannulate the vessel. All the procedures were carried out under a dissection microscope (Wild M3Z). Once cannulation was completed the catheter was fixed to the shell with cyanoacrylic glue and the egg was placed in the experimental chamber (described in Chapter 4). Four embryos were studied simultaneously.

Signal recording and calibration

Each catheter was attached to a pressure transducer (WPI, type BLPR), which in turn was connected to a bridge amplifier (CB Sciences, model ETH-400) and the pressure trace stored in a computer using PowerLab data acquisitions software. Heart rate was continuously determined from the arterial pressure signal via an acquisition tachograph. In all cases, ther arterial pressure calibration zero was initially set at the top of the experimental bath, and all values were corrected after the experiment as described in Chapter 2.

Experimental Protocol

Once placed in the experimental setup eggs were allowed to recover for 30 min followed by a 5 min control measurement period. Following the control period, eggs were exposed to a 5-min bouts of 10% O_2, then returned to normoxia for 30-min. Following initial hypoxic exposure eggs where allowed an addition 10-min to recover then 1 $mg*kg^{-1}$ of the cholinergic blocker atropine was infused using the method as described in Chapter 2. Arterial pressure and heart rate were allowed to reach stable values followed by the onset of a new 30-min control period. Embryos were then exposed to 5 min of 10% O_2 with cardiovascular responses recorded. This process was repeated for β-adrenergic blocker 3 mg*kg-1 propranolol and the α–blocker phentolamine. In each case changes in arterial pressure and heart rate were monitored. Following the completion of the study, eggs were euthanized with an overdose of xylocaine and KCl then quickly frozen for later analysis.

Statistical Analysis

A paired Student t-test was used to access significant differences between control and hypoxic exposure for all levels. This procedure was repeated for the analysis of drug effects as well as the difference in pre- and post- drug hypoxia. A two way ANOVA of the arcsine transformed percent changes with day of incubation and before/after drug injection as independent variables. The fiduciary level of significance for all tests was taken at $p<0.05$. All data are presented as mean ± 1 s.e.m.. For all experimental procedures, a sample number of five eggs was used unless otherwise specified.

Results

Cardiovascular reactions to 10% O_2 exposures (that is, hypoxia without drug treatment) where similar to those discussed in Chapter 4. In addition, cardiovascular responses to each antagonist were similar to those previously discussed in Chapter 2. For the purposes of this study, changes in mean arterial pressure (Map) and embryonic heart rate (f_H) will reflect cardiovascular responses (Fig 6.1).

As previously established, atropine had little effect on cardiovascular function throughout the period of study (Fig 6.1). Propranolol treatment caused a significant reduction in f_H throughout the study, similar to the finding established in chapter 2 (Fig 6.1). Mean arterial pressure rose following injection of propranolol, a reaction that was significant ($p<0.05$) on days 19 and 21(Fig 6.1). Further phentolamine treatment also produced a fall in embryonic f_H throughout incubation with significant reductions caused on days 12, 18, 19 and 20 (Fig 6.1).

These responses differed from those established in Chapter 2 and will be addressed later. Mean arterial pressure was significantly reduced following injection of phentolamine on days 12 to 20 of incubation (Fig 6.1) a pattern also established in earlier works.

Post-Drug Hypoxic Responses

Following treatment with 1 $mg_{*}kg^{-1}$ atropine, 10% O_2 continued to compromise cardiovascular function as established in earlier works (Fig 6.2). Pressure responses were also unaltered by atropine treatment during the period of study with the exception of day 18 in which atropine intensified the hypoxic hypotension by 10% (Table II). In addition, chronotropic hypoxic reactions were unaffected by pre-treatment with atropine during the period of study (Table 6.2).

Pre-treatment with propranolol altered embryonic hypoxic cardiovascular reactions late in development (Fig 6.3). Mean arterial pressure response was significantly altered by propranolol treatment on days 18 to 21 (Fig 6.3). Over the early period of study hypoxia with or without propranolol treatment reduced Map an average of 16% (Fig 6.3). From 18 to 21 days of incubation Map fell an average of 13% during hypoxia allow while post propranolol treatment hypoxia caused Map to rise 6% (Fig 6.3). Heart rate response to hypoxia was unaltered by propranolol treatment with significantly different response occuring on day 20 only (Fig 6.3).

Phentolamine treatment significantly altered cardiovascular responses to hypoxia in the final days of chicken incubation, as demonstrated following

propranolol treatment. Mean arterial pressure reductions during hypoxia were significantly elevated ($p < 0.05$) on days 19, 20 and 21 of incubation (Fig 6.4). During this developmental interval, Map changed from an 11% increase in pressure during hypoxia to a 28% decrease following phentolamine treatment (Fig 6.4). Chronotropic hypoxic reactions were unaffected by treatment with phentolamine, with the exception of day 21 in which the negative action of hypoxia were intensified (Fig 6.4).

Comparison Between Days of Incubation

Changes in cardiovascular response to hypoxia following cholinergic blockade were statistically identical (Map and f_H $p<0.5$) on all days of chicken incubation studied. In addition, a between-day comparison of chronotropic responses following propranolol treatment revealed that all embryo changed similarly ($p<0.6$) in response to hypoxia. Mean arterial pressure responses to hypoxia following β-blockade were also statistically similar on all days of incubation. However, the p value ($p<0.059$) and the marked difference within day of incubation pressure responses (Fig 6.3) suggests that different days of incubation may differ.

As indicated by the interaction between treatment and day of development, Map response to hypoxia after phentolamine treatment changed with incubation as older embryos exhibited a greater fall in pressure than early embryos ($p<0.0001$) (Table 6.3). Heart rate responses to hypoxia following

phentolamine injection were not significantly altered as embryos progressed in development.

Discussion

The cardiovascular system of developing chickens performs a number of functions most notable of which is the maintenance of proper gas transfer to and from peripheral tissues. Hypoxic challenges dramatically stress cardiovascular function in developing chickens (Tazawa et al. 1985, Tazawa 1981a, Girard 1973, Van Golde et al. 1997, Grabowski et al. 1969). While the general depressive impact of hypoxia on cardiovascular function is well characterized, the mechanisms which underlie these changes remain poorly understood. The present study has established that an adrenergic mechanism is present during chicken development that alters cardiovascular function in response to acute hypoxic stress. The mechanism becomes operational between days 15 and 18 of incubation and acts primarily on arterial pressure with an intensity that is unchanged over the final 4 days of incubation. Cholinergic mechanisms, which play an important role in fetal lamb hypoxic responses, are absent throughout the development of chickens suggesting fundamental differences between the two species. Further, regulatory mechanisms that are responsible for hypoxic cardiovascular changes in embryos differ from those present in adult chickens.

Cholinergic Response

Cholinergic regulatory systems are of little importance to cardiovascular function during acute hypoxia exposures in embryonic chickens (Fig 6.2). In

adult chickens, cholinergic action, specifically vagal activation, is a major mechanism augmenting cardiovascular function during hypoxic exposure (Duree and Sturkie 1963, Butler 1967). Hypoxic stress is adult chickens elevates f_H and respiratory frequency both mediated via vagal influences (Duree and Sturkie 1963, Butler 1967). Thus, two scenarios must exist during embryonic development: 1) embryonic chickens rely on unique non-adult systems during bouts of hypoxia to maintain cardiovacular function or 2) embryonic chickens lack regulatory system during development. While these findings were unexpected, Chapter 2 has demonstrated that cholinergic tone on the cardiovascular system is also absent in development in chickens. Further, as previously established in Chapter 3, cholinergic system are also inactive during bouts of hypertensive stress during development in chicken embryos. Collectively these data suggest the parasympathetic arm of the autonomic regulation of cardiovascular function is quiescent throughout chicken development, becoming functional during the post-hatch period.

β-Adrenergic Response

During hypoxic exposure, β-adrenergic systems are important in regulating the cardiovascular system over the final 4 days of chicken incubation, unlike cholinergic systems (Fig 6.3). Following β-blockade, hypoxia resulted in a dramatic hypertension from day 18 until hatch in chicken embryos (Fig 6.3). This cardiovascular response differed from that known to occur in adult chickens during exposure to hypoxic stress. As ambient O_2 falls, adult chickens exhibit a

hypotension, as well as a pronounced tachycardia (Durfee and Sturkie 1963, Butler 1967, Richard and Sykes 1967, Ray and Fedde 1969). The aforementioned tachycardia was abolished in animals treated with the β-blocker inderal, an antagonistic behavior which was absent during chicken development (Fig 6.3) (Butler 1967). Thus, as was suggested for cholinergic action, β-adrenergic action in embryos during hypoxia differs from that known to occur in adult chickens.

This difference in β-adrenergic action between embryonic and adult animals could be attributed to additional vascular beds that are present in embryos and lacking in adult chickens. The chorioallontoic membrane (CAM) serves as the gas exchange organ for developing birds and contains a substantial portion of the overall embryonic blood volume (Romanoff 1967). β-Adrenergic systems could potentially vasodialate blood vessels in the CAM during hypoxia to maintain perfusion pressure, thus countering the compromising actions of low oxygen. Umbilical blood flow in fetal sheep, which has been suggested to be analogous to the CAM circulation, is dependent upon β-adrenergic action during bouts of hypoxia to maintain blood flow (Cohn et al. 1978, Metcalfe and Stock 1993). Therefore, chorioallantoic vascular beds of chicken embryos may also rely on β-receptor induced vasodilatation during hypoxia. Without measuring accompanying changes in blood flow it is difficult to determine resistance changes in the CAM vasculature during hypoxia with and without β-blockade. Preliminary study has demonstrated that the vasculature of

the CAM vasodialates in response to the β-agonist isoproterenol while it remains relatively unaffected by 10% O2 (Crossley, Altimiras and Burggren in prep). This would suggest that during hypoxia chicken embryos in the final four days of development alter CAM vascular resistance via, in part, β-adrenergic mechanisms to maintain perfusion pressure. While it appears this systems is integral in producing the characteristic cardiovascular hypoxic response late in development, the reason for a lack of action prior to day 18 in unclear.

An assessment of cardiac output distributions during severe hypoxia has previously indicated that regulatory mechanisms may become operational during a developmental interval untested in the present study (Mulder et al. 1998). Hypoxia caused a redistribution of cardiac output to the CAM, heart and brain in the interval from 14 to16 days and to the heart and brain from 17 to 19 days of chicken incubation (Mulder et al. 1998). This redistribution was absent in embryos from day 10 to 13 possibly suggesting the system was immature (Mulder et al 1998). Therefore, an interval from day 15 to 18 may exist when CAM vasculature becomes dependent on β-adrenergic systems during hypoxia stress. This interval was not tested in the present study and cannot be verified. β-adrenergic or other vasoactive substances may account for this redistribution during hypoxia, but further study is needed to determine the origin cardiac output alteration.

While the vascular responses to hypoxia consistently relied on β-adrenergic stimulation, chronotropic actions were relatively independent of β-

influences. This reaction differed from that characterized in adult chickens that exhibit a marked hypoxic tachycardia that is eliminated by β-blockade (Butler 1967). An increase in f_H was evident on day 20 of incubation, but hypertension that accompanied hypoxia following β-blockade may account for this change. Thus, the importance of β-adrenergic system in overall cardiovascular responses to hypoxia differed dramatically between late stage embryos and adult chickens.

α–Adrenergic Response

The noted hypoxic hypotension during the final four days of incubation is dependant on β-receptor stimulation, but the source of the post β-blockade hypertension is clearly due to unopposed α–adrenergic action on peripheral vessels (Fig 6.4). Post β-blockade hypoxic hypertension can be attributed, in part, to an α–adrenergic activation in embryonic chickens from day 19 to 21. While systemic vasculature in hypoxic adult chickens is unaffected by α–blockade hypoxic pulmonary vasoconstriction is eliminated by treatment with an α–antagonist (Jones and Johansen 1972). Therefore, total embryonic pressure responses to hypoxia post β-blockade are similar to those of the adult pulmonary circulation over the final 3 days of incubation. However, given the protocol used in the present investigation, it is difficult to localize specific embryonic vascular beds that response to α–stimulation.

As suggested for β-adrenergic stimulation, a potential vascular bed for α–adrenergic action during hypoxia could be the arteries of the CAM. Without

accompanied changes in blood flow, assessment of *in vivo* changes in vessel resistances is impossible. However, preliminary study on perfused CAM vessels has demonstrated a clear increase in resistance following infusion of the α–agonist phenylephrine suggesting this organ contain α–ρeceptors. Thus, α–stimulation of CAM vessels may contribute to the overall embryonic hypoxic hypertension during the final days of chicken development.

Hypoxic induce α–σtimulated chronotropic changes were absent, as illustrated for β-stimulation, during chicken development, with the exception of day 21 (Fig 6.4). Since α–receptors play no role in the adult chronotropic reaction to hypoxia and that the negative responses evident on day 21 were coupled with a marked hypotension, it is conceivable that this reaction was indirectly mediated. Thus, as determined for β-adrenergic systems, α–adrenergic systems exhibit no influence on embryonic chronotropic reactions to hypoxia.

Conclusion

During chicken development, cardiovascular response to acute hypoxia differed dramatically for those present in adult animals. In addition, this study has demonstrated that neural and or humoral mechanisms that coordinate these changes also differ between life phases.

Cholinergic systems are non-responsive to hypoxic stress throughoutchicken development, so they must mature in the neonatal period. This apparent inactivity during hypoxia in chicken embryos differs from that

demonstrated in the fetal lamb. Upon initiation of hypoxic exposure, late stage fetal sheep exhibit a transient bradycardia that has been attributed to vagal activation (Cohn et al. 1978, Berman et al. 1976, Giussani et al. 1993, Martin 1985). This characteristic, which is also present during the development of llamas, suggests there is a fundamental difference in the onset of regulatory components between birds and mammals (Giussani 1996).

In contrast to the noted lack of cholinergic response to hypoxia, adrenergic actions are pronounced over the final four days of incubation producing primarily vascular specific response. While the vascular beds affected were undetermined in the current study, previous studies in fetal sheep, which exhibit similar hypoxic adrenergic importance, may provide some insight. Vascular resistance within the carcass, lungs and liver of fetal sheep are elevated during hypoxia, a response that was eliminated by pre-treatment with an α–antagonist (Reuss et al. 1982). The essential nature of this system to fetal sheep is evident in the 43% mortality that occurs during hypoxia following α–blockade (Giussani et al. 1993). If one assumes that the hypoxic redistribution of cardiac output in embryonic chickens can be attributed to α–adrenergic systems as in fetal sheep, then adrenergic components may be essential for distributing flow in the late stage chicken embryo. While there are differences between the two systems, this provides a possible explanation for the observed hypertension during late ontogeny in chickens. Thus, the combined actions of β– and α– adrenergic mechanisms act in an effort to maintain gas transport during bouts of embryonic hypoxia. While

the source of adrenergic action was undetermined in this work, both neural as well as humoral catecholamines may contribute to observed responses

Table 6.1. Systolic (Sys) and diastolic (Dia) pressure response to 10 % O_2 pre (C) and post (T) drug injection. Asterisks represent significant ($p<0.05$) difference between normoxic (N) and hypoxic (C or T) exposure as indicated by the results of paired Student t-test. Data are presented as mean ± sem. In all cases n = 5 unless noted.

		Atropine				Propranolol				Phentolamine			
Day		Sys		Dia		Sys		Dia		Sys		Dia	
12	N	11.4±1.6		5.5±1		11.6±1.6	*	6.2±0.9	*	11.3±1.4		6.3±0.5	
	C	9.6±1.4	*	4.5±0.9	*	8.8±1.3		4.9±0.9		9.1±1.5	*	5.3±0.4	*
	N	11.9±1.9		5.4±1		11.5±1.7	*	6±1	*	10.1±1.9		6±0.6	
	T	9.1±1.7	*	4.1±0.9	*	9±1.8		5.1±0.9		7.1±1.1	*	4.7±0.5	
15	N	18±1.8		9.6±1.2		23.6±1.9		14.2±1.3		23.3±1.7		12.8±1.3	
	C	14.4±1.5	*	7.5±0.9	*	18.6±2.1	*	10.9±1.4	*	21.7±2.1	*	10.2±1.4	*
	N	18.9±2.3		10.4±1.6		22.7±2.9		13.6±1.6		20.9±1.8		11±1.6	
	T	13.8±1.9	*	7.3±1.2	*	19±2.5	*	11.7±1.4		16.4±1.8	*	8±1.4	*
18	N	21.2±1.2		12.4±0.9		24.4±2.6		14.3±1.5		25.6±0.8		13.4±0.6	
	C	19.9±1.0		10.5±0.7		19±1.1	*	9.1±0.8	*	24.9±1.6		11.9±0.6	
	N	23.6±1.2		13.4±1.2		25.3±1.6		13.5±1.3		16.3±2.2		6.6±0.9	
	T	18.2±1.0	*	8.6±0.7	*	23±2.5		11.5±1.5		12±1.2	*	4.8±1	*
19	N	27.1±1.3		16±1.3		29.7±1.4		18.1±1.3		30.2±1.4		17.8±1	
	C	21.8±1	*	11.8±1.6	*	22.9±1.7	*	12.4±1.2	*	32±1.2		18.4±1	
	N	29.7±1.4		18.1±1.3		31.1±1		17.7±1.1		22.4±1.7		11.4±1.7	
	T	22.9±1.7	*	12.4±1.2	*	30.9±1.7		17.1±1.4		15.8±1.1	*	7.7±0.7	*
20	N	34±2.7		22.9±2.1		31.8±3.3		22±1.5		37.1±2.4		21.6±1.8	
	C	29.7±2		19.6±1.2		30.8±2.5		20.3±1.4		43.5±3.1	*	26.4±2.1	*
	N	33.9±2.3		21.7±1.8		36.7±2.7		23.5±1.8		31.5±2.5		18.4±1.9	
	T	30.5±1.9	*	18.5±1.5	*	40±2.7	*	27.3±2.1	*	23.6±3.3	*	14.2±2.2	*
21	N	34.5±2.2		21.5±1		38.4±2.4		21.91.6		39.2±3		20.9±2.4	
	C	30.4±1		17.4±1.4		34.5±2.4		19.5±1.4	*	43.7±4.4		25.1±3.6	*
	N	36.3±2.3		20.3±1.4		42.2±2		25.3±2.5		38.1±5.8		22±4	
	T	32.4±2.9		17.7±1.8		46.7±3.3		28.6±2.3		28.8±3.8	*	16.4±0.5	

Table 6.2. Difference in change of mean arterial (Map), systolic (Sys) diastolic (Dia) pressures and heart rate (f_H) responses to10% O_2 following drug treatment. Significance difference values of the ANOVA conducted on the arcsine transformed percentage change between pre- and post- atropine (Atrop), propranolol (Prop) and phentolamine (Ph) during 10% hypoxic exposure. Deltas indicate level of significance Δ = $p<0.05$, $\Delta\Delta$ = $p<0.001$ and $\Delta\Delta\Delta$ = $p<0.0001$. In all cases n = 5.

	1mg*kg^{-1} Atrop				3 mg*kg^{-1} Prop				3 mg*kg^{-1} Ph			
Day	Map	Sys	Dia	f_H	Map	Sys	Dia	f_H	Map	Sys	Dia	f_H
12												
15												
18	Δ	Δ	Δ		Δ		Δ			Δ		
19					$\Delta\Delta$	Δ	$\Delta\Delta$		$\Delta\Delta\Delta$	$\Delta\Delta\Delta$	$\Delta\Delta\Delta$	
20					$\Delta\Delta$	Δ	$\Delta\Delta$	Δ	$\Delta\Delta\Delta$	$\Delta\Delta\Delta$	$\Delta\Delta\Delta$	
21					$\Delta\Delta$	$\Delta\Delta$	$\Delta\Delta$		$\Delta\Delta\Delta$	$\Delta\Delta\Delta$	$\Delta\Delta\Delta$	Δ

Table 6.3. Maturation of 3 $mg_{*}kg^{-1}$ phentolamine affects on mean arterial (Map), systolic (Sys) diastolic (Dia) pressures and heart rate (f_H) reactions to 10% O_2. Days of incubation with like letters were similar in response to 10% post hypoxia those days which differ significantly ($p < 0.05$) from others are indicated with different letters. Data are presented as mean ± sem. In all cases n = 5.

Day		Map		Sys		Dia		f_H	
12	N	8.1±1.2		10.1±1.9		6±0.6		182±11	
	10	5.9±0.5	A	7.1±1.1	A	4.7±0.5	A	146±5	A
15	N	15.6±1.7		20.9±1.8		11±1.6		180±9	
	10	11.3±1.7	AB	16.4±1.8	AB	8±1.4	AB	135±18	A
18	N	10.6±1.5		16.3±2.2		6.6±0.9		131±27	
	10	7.4±1.1	AB	12±1.2	AB	4.8±1	AB	84±24	A
19	N	16.1±1.6		22.4±1.7		11.4±1.7		194±19	
	10	10.9±0.9	CB	15.8±1.1	CB	7.7±0.7	CB	164±19	A
20	N	24.4±2.2		31.5±2.5		18.4±1.9		204±11	
	10	18.2±2.8	C	23.6±3.3	C	14.2±2.2	C	160±33	A
21	N	29.3±4.5		38.1±5.8		22±4		242±21	
	10	22.1±2	CB	28.8±3.8	CB	16.4±0.5	CB	167±28	A

Figure Legend

Figure 6.1

Collective cardiovascular responses to antagonist treatments. Heart rate (A) and pressure (B) responses to atropine (Atropine), propranolol (Prop) and phentolamine (Phento). Asterisk indicate significant difference ($p<0.05$) between control (open column) and treatment (filled column) over the developmental period studied.

Figure 6.2

Heart rate (f_H) and mean arterial pressure (Map) comparison between control (open column) and response to 10% O_2 (filled column) before (first column set) and following (second column set) treatment with 1 mg_*kg^{-1} atropine. Asterisk indicated significantly ($p<0.05$) different responses to hypoxia following drug treatment as determined by the ANOVA conducted on the arcsine transformed % change.

Figure 6.3

Heart rate (f_H) and mean arterial pressure (Map) comparison between control (open column) and response to 10% O_2 (filled column) before (first column set) and following (second column set) treatment with 3 mg_*kg^{-1} propranolol. Asterisk indicated significantly different responses ($p<0.05$) to hypoxia following drug treatment as determined by the ANOVA conducted on the arcsine transformed % change.

Figure 6.4

Heart rate (f_H) and mean arterial pressure (Map) comparison between control (open column) and response to 10% O_2 (filled column) before (first column set) and following (second column set) treatment with 3 mg_*kg^{-1} phentolamine. Asterisk indicated significantly different responses ($p<0.05$) to hypoxia following drug treatment as determined by the ANOVA conducted on the arcsine transformed % change.

Figure 6.1

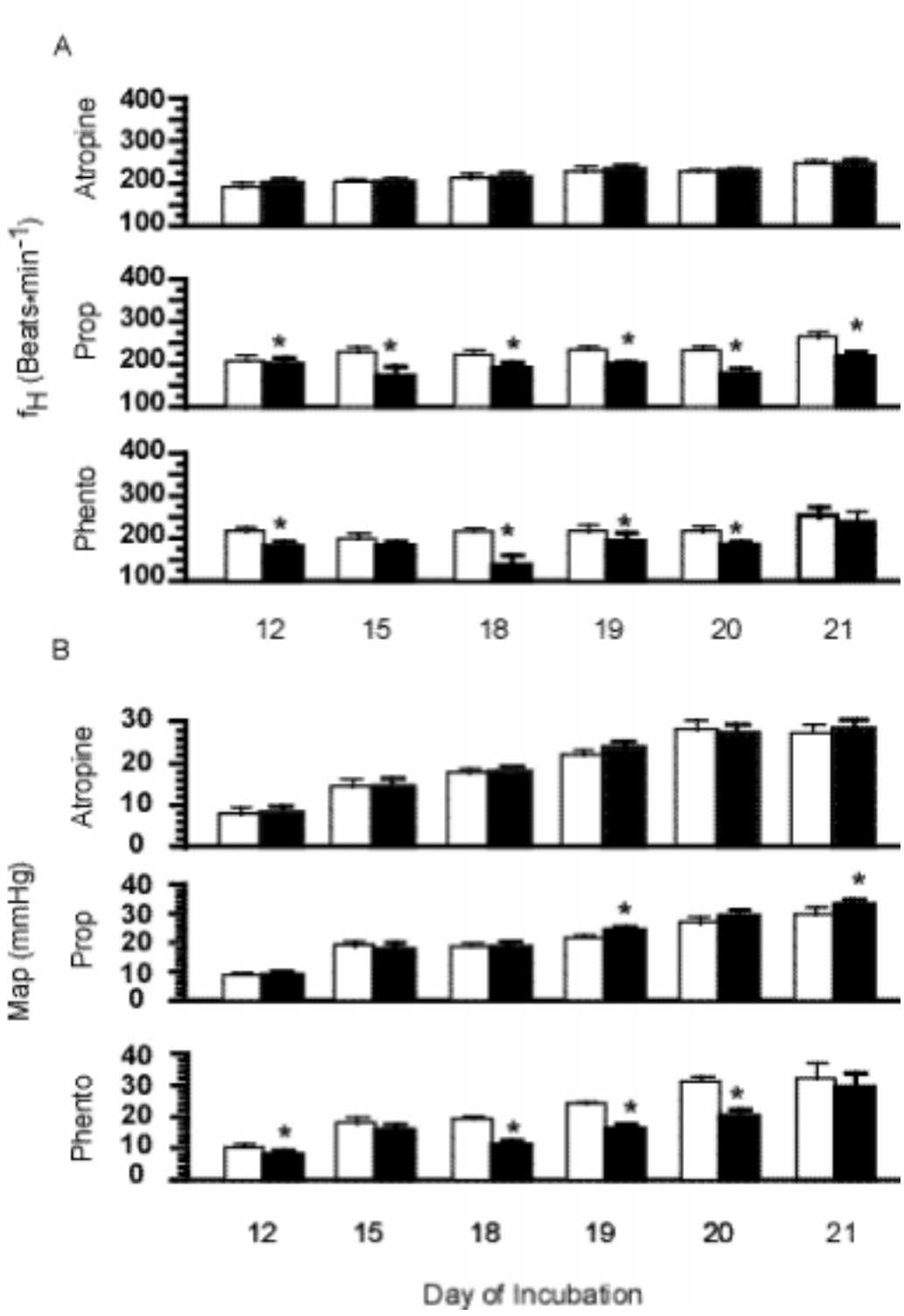

A
Atropine
Prop
Phento
400
300
200
100
f_H (Beats·min^{-1})
12
15
18
19
20
21
B
Atropine
Prop
Phento
30
20
10
0
40
30
20
10
0
Map (mmHg)
Day of Incubation

Figure 6.2

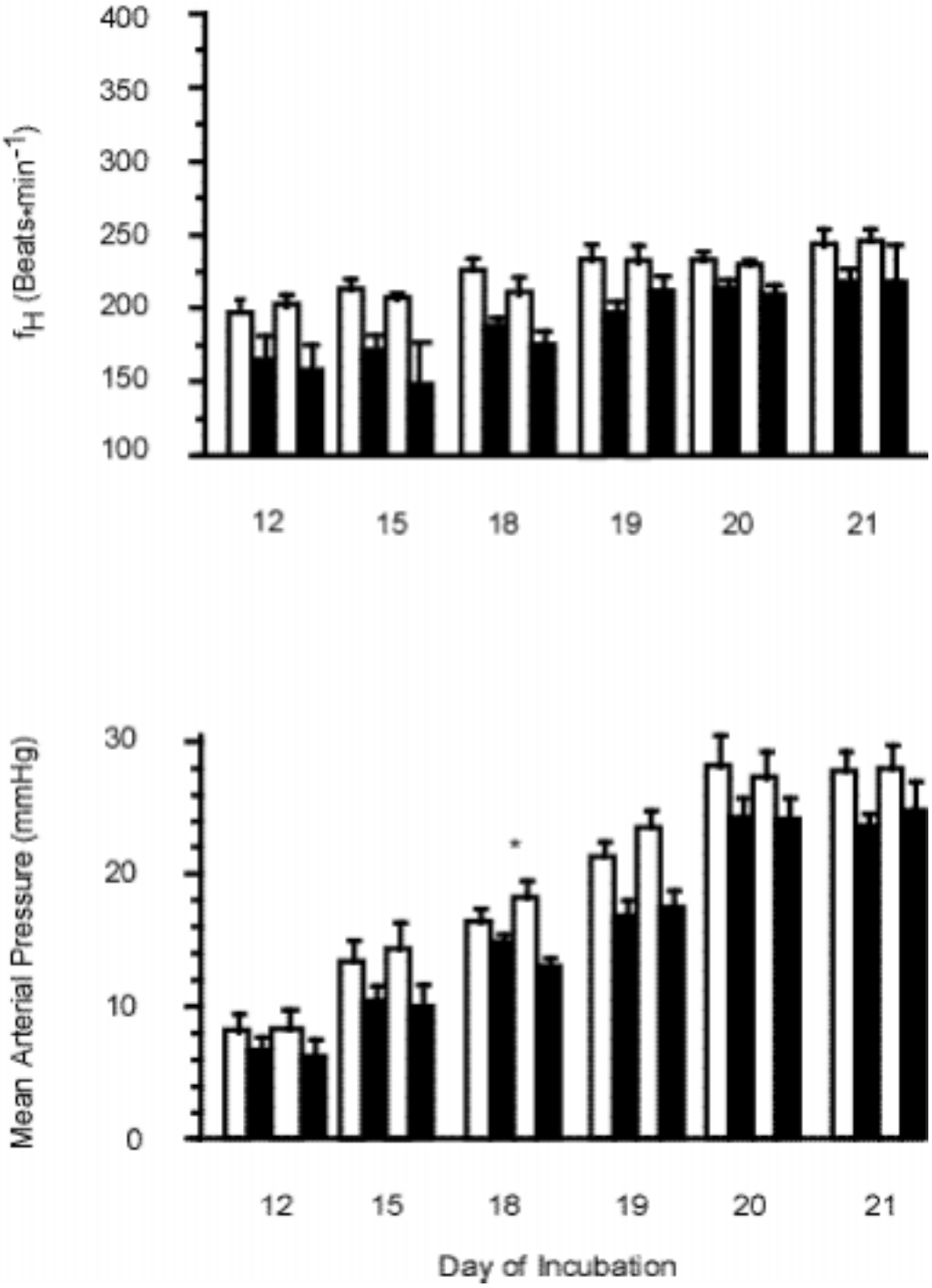
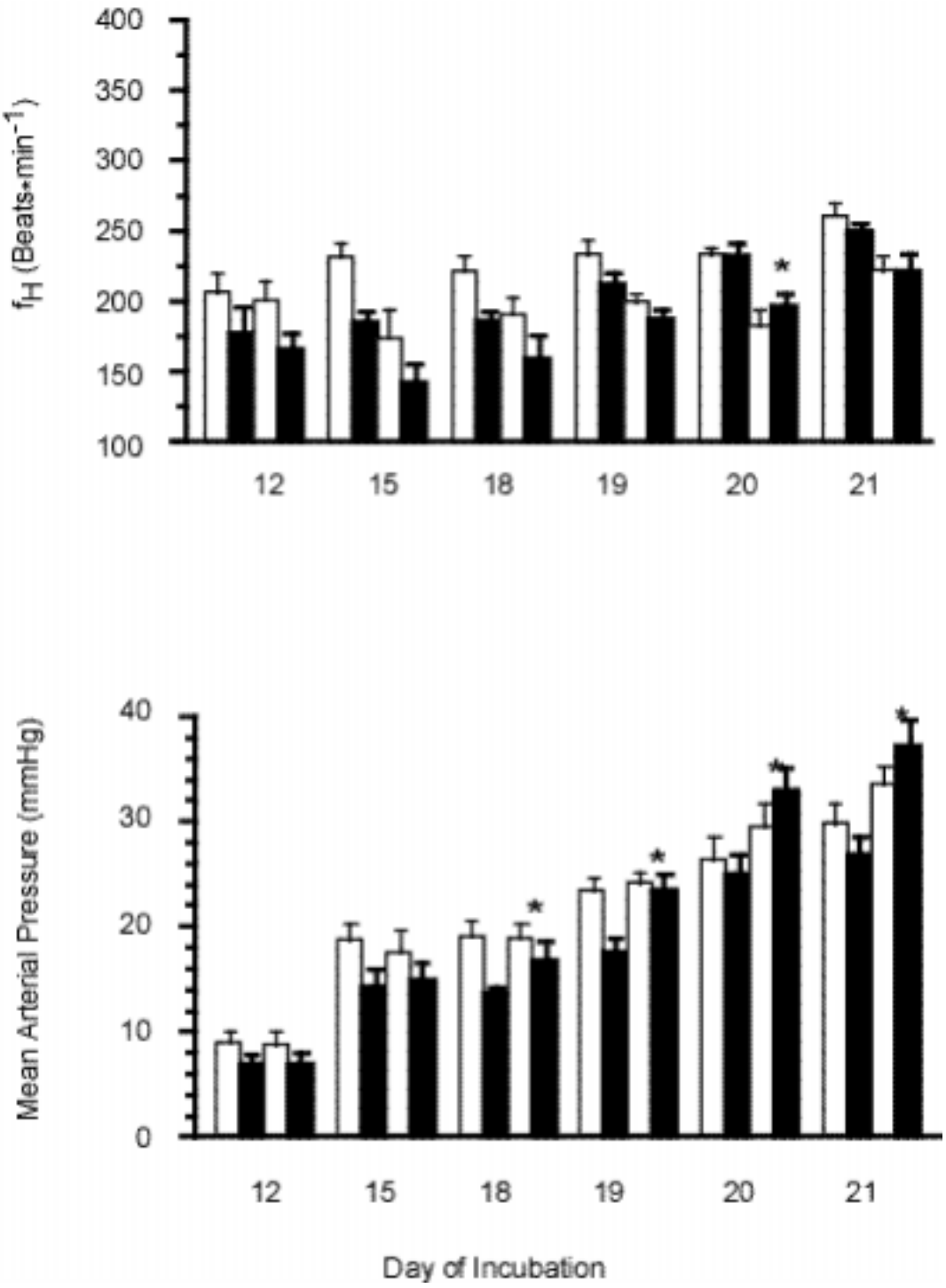

400
350
300
250
200
150
100
f_H (Beats•min^{-1})
12
15
18
19
20
21
30
20
10
0
Mean Arterial Pressure (mmHg)
*
12
15
18
19
20
21
Day of Incubation

Figure 6.3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f_H (Beats•min^{-1})
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*
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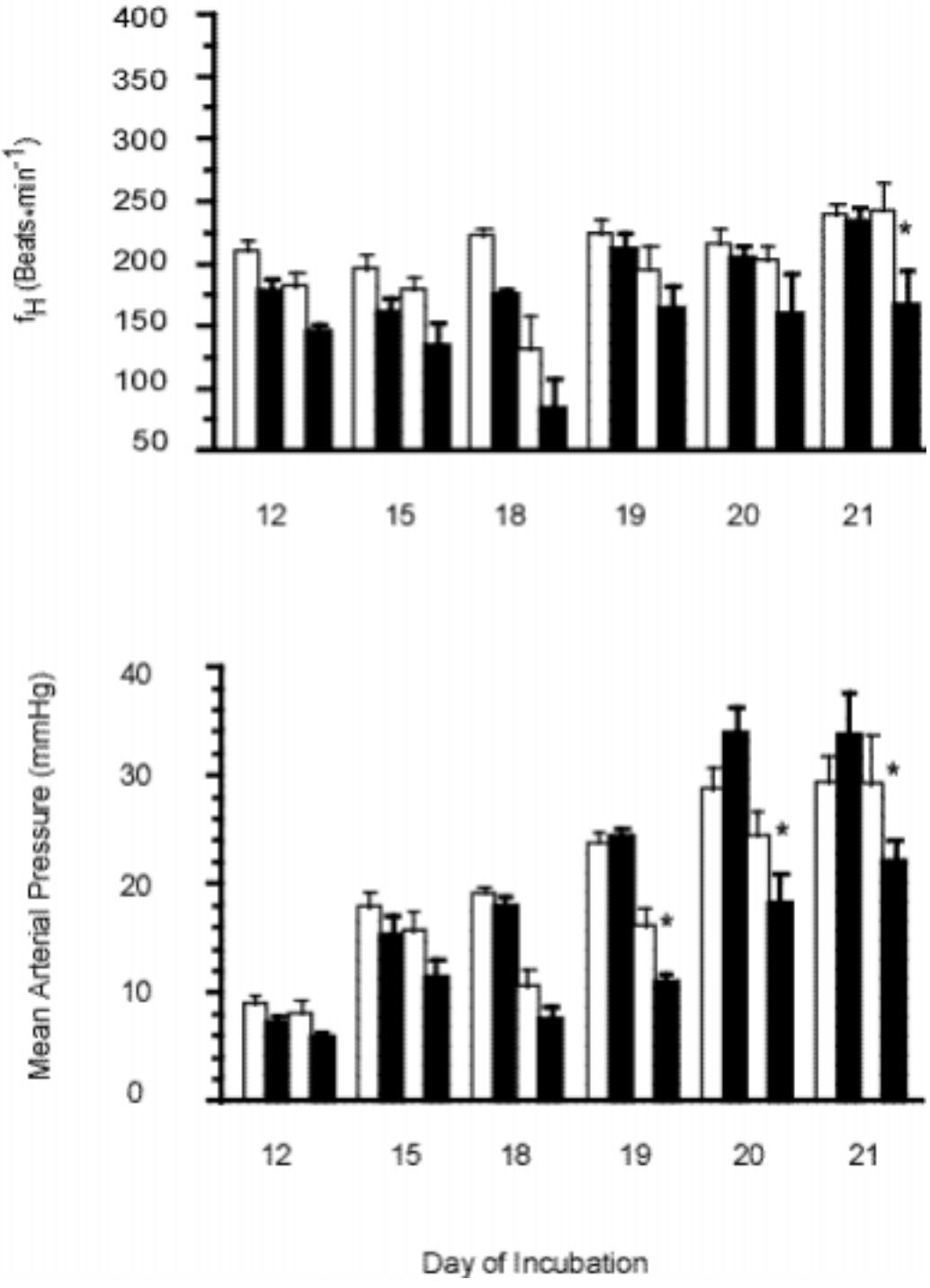
0
Mean Arterial Pressure (mmHg)
Day of Incubation

Figure 6.4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f_H (Beats·min^{-1})
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40
30
20
10
0
Mean Arterial Pressure (mmHg)
12
15
18
19
20
21
*
*
*
Day of Incubation

CHAPTER VII

CONTRIBUTION OF THE AUTONOMIC NERVOUS SYSTEM TO CARDIOVASCULAR ADJUSTMENT DURING ACUTE HYPOXIA IN CHICKEN EMBRYOS (*Gallus gallus*): THE EFFECT OF CHEMICAL SYMPATHECTOMY AND GANGLIONIC BLOCKADE.

Introduction

Chicken embryos demonstrate dramatic alterations of normal cardiovascular function during periods of reduction in ambient oxygen levels throughout development. These changes include a clear hypotensive bradycardia over the majority of incubation, with a loss of pressure sensitivity late in development (Tazawa 1981, Girard 1973, Van Golde et al 1998, Mulder et al 1997). Adult respond to hypoxia in a markedly different manner than embryonic chickens, with a pronounced tachycardia coupled with a decrease in arterial pressure developing during hypoxic exposure (Butler 1967, Ray and Fedde 1969, Dufree and Stuckie 1963). Adult reactions to low O_2 have been attributed to an increase in vagal activity as well as β-adrenergic reactions resulting in an increase of heart rate, with the fall in pressure attributed to the direct action of O_2 (Butler 1967). While adult cardiovascular regulation during hypoxia is well characterized, maturation of these regulatory mechanisms during development is poorly understood.

In Chapter 6 it was determined that embryonic chickens acquire some cardiovascular regulatory ability during the final portion of incubation (Ch 6 this thesis). This regulatory mechanism is composed of adrenergic components which, in the final 4 days of incubation, act on peripheral vessels resulting in significant changes in arterial pressure during exposure to hypoxia. However, the origin of these catecholamines was previously undetermined in embryonic chickens. Fetal sheep are known to release catecholamines from sympathetic terminals as well as the adrenal medulla during bouts of hypoxic stress, with both direct action of low O_2 and central stimulation responsible for catecholamine release (Lewis et al. 1984). Thus, the origin of adrenergic-induced cardiovascular response to hypoxia in chicken embryos may be due to both central as well as directly induced release of catecholamines.

The present investigation in chicken embryos sought to clarify the origin of previously determined adrenergic regulation of cardiovascular function during bouts of hypoxic stress. Utilizing selective chemical sympathectomy, the role and maturation of centrally mediated changes in cardiovascular function during hypoxia was determined. In addition, ganglionic blockade was used to specify the efferent paths which chicken embryos utilize to induce adrenergic augmentation of cardiovascular function.

Materials and Methods

Eggs of the domestic chicken, *Gallus gallus*, of the White Leghorn strain were purchased from Texas A&M and shipped overnight to the University of

North Texas Department of Biological Sciences. On arrival, eggs were placed in incubation at 38 ± 0.5 °C, 60-70% relative humidity and turned automatically every 3 hrs. For the purpose of the study, experimental manipulation was conducted on days 9, 12, 15 and18-21 of a 21-day incubation period.

Surgical Procedures

On the day of study, eggs were removed from the incubator, candled to locate a chorioallantoic artery and placed in a temperature-controlled chamber. A portion of the eggshell was removed exposing the previously located artery. The artery was temporarily occluded up stream with a 6 zero silk ligature and a second ligature was placed downstream to eliminate retrograde flow following cannulation. A small cut was then made in the artery and a heat-pulled, saline-filled catheter was then used to occlusively cannulate the vessel. All the procedures were carried out under a dissection microscope (Wild M3Z). Once cannulation was completed the catheter was fixed to the shell with cyanoacrylic glue and the egg was placed in the experimental chamber (described in Chapter 4). Four embryos were studied simultaneously.

Signal recording and calibration

Each catheter was attached to a pressure transducer (WPI, type BLPR), which in turn was connected to a bridge amplifier (CB Sciences, model ETH-400) and the pressure trace stored in a computer using PowerLab data acquisitions software. Heart rate was continuously calculated from the pressure signal via an acquisition tachograph. In all cases, zero was initially set at the top of the

experimental bath, and all values were corrected after the experiment as described in Chapter 2.

Experimental Protocol

Sympathectomy

During each of the study periods, eggs were allowed 30 min to reach control values, defined as stable readings for 5 min. Following the control period, eggs were exposed to a 5-min of 10% O_2 then returned to normoxia for 30 min. Upon completion of initial exposures eggs where allowed an addition 10 min to recover, after which 1 $mg_{*}kg^{-1}$ of the sympathetomizing agent 6-hydroxydopamine (6-OH) was injected as described in Ch 4. Blood pressure and heart rate were allowed to stabilized prior to the initiation of a 30-min control period. This control period was followed by a second dosage of 20 $mg_{*}kg^{-1}$ 6-OH as explained in Ch 4. A recovery period of 20 min after return to control levels was allowed between each dosages of 6-OH. Upon completion of sympathectomy, embryos were again allowed to recovery for 20 min. In each case changes in arterial pressure and heart rate were monitored. A second 5-min exposure to 10% O_2 was conducted upon completion of 6-OH treatment with responses recorded as described above. Following the completion of the study, eggs were euathanized with an overdose of xylocaine and KCl then quickly frozen for later analysis.

Ganglionic blockade

On each of the study periods eggs were allowed 30 min to reach control values followed by a bout of 10% hypoxia as described for the sympathectomy design. Upon completion of initial exposure eggs were allowed an addition 10 min to recover, after which 25 $mg_{*}kg^{-1}$ ganglionic blocker hexamethonium was injected as described in Ch 4. Blood pressure and heart rate were allowed to stabilized prior to the initiation of a 30-min control period. This control period was followed by a second 5-min exposure to 10% O_2 with responses recorded as described for sympathectomy. Following the completion of the study, eggs were euthanized with an overdose of xylocaine and KCl then quickly frozen for later analysis

Statistical Analysis

A paired Student t-test was used to access significant differences between control and hypoxic exposure for all levels. A one-way ANOVA was conducted on the arcsine transformed percent difference between control and hypoxic exposure levels in all variables to determine significant changes between days of incubation. A similar analysis was conducted within a day of incubation to determine differences in reactions to changing levels of hypoxia. Fisher's LSD post-hoc comparison was used to isolate significant differences between days of incubation as well as to determine differences in hypoxia. The fiduciary level of significance for all tests was taken at $p<0.05$. All data are presented as mean± s.e.m.. For all experimental procedures, a sample number of 5 eggs was used unless otherwise specified.

Results

Initial exposure to 10% O_2 produced similar cardiovascular responses to those previously described in Ch 5 (Table 7.3) for both experimental treatments, with the exceptions of days 20 and 21. In addition, 6-OH treatments produced similar pressure and heart rate responses from days 19 to 21 to those described in Ch 4 (Table 7.3). For all treatments systolic and diastolic pressure changes mirrored the changes in mean arterial pressure (Map), therefore these data are presented alone with no specific description in Tables 7.1, 7.2, 7.4 and 7.6.

Sympathectomy

Initial injections of 20 $mg_{*}kg^{-1}$ 6-OH resulted in a transient increase in all pressure parameters (Fig 7.1). Heart rate was increased following treatment, similar to that described in Chapter 4 on days 19 and 21 of incubation. All responses dissipated over time and control values returned within an average of 29 min after treatment. In addition, treatment with 1.0 $mg_{*}kg^{-1}$ altered pressure values significantly on days 12 and 19 with heart rate altered on days 15 and 19 (Fig 7.1).

Hypoxic response

Control hypoxia resulted in characteristic changes in cardiovascular function on all days of incubation, as discussed in Ch 5. Briefly, from days 12 to 19 hypoxia resulted in a reduction in both arterial pressure and heart rate (Fig 7.2), This pattern differed on day 20 of incubation during which hypoxia was ineffective in altering any pressure parameters (Fig 7.2). Negative chronotropic

actions were present on day 20 of incubation as shown in previous chapters (Fig 7.2). Day 21 of incubation exhibited a significant negative pressure response to hypoxia as previously demonstrated (Fig 7.2). In addition, embryonic heart rate was constant during control hypoxic treatment (Fig 7.2) in eggs at 21 days of incubation. Each of these responses differed from previously reported data in Ch 5.

Post 6-OH Response

Sympathectomy treatment in eggs from day 12 to 18 of incubation was ineffective at altering cardiovascular reactions to acute 10% O_2 (Fig 7.2). This pattern was altered on day 19 which exhibited an accentuated negative chronotropic response to hypoxia ($p<0.05$), from 37 to 48 beats$*$min^{-1}, following 6-OH treatment (Fig 7.2). Map changes during hypoxia post-6-OH treatment were significantly different ($p<0,05$) on day 20 of incubation (Fig 7.2). Heart rate response also differed significantly ($p<0.05$) on day 20 during post-6-OH hypoxia (Fig 7.4), falling an average of 23 beats$*$min^{-1} more (Fig 7.2). This trend was also exhibited on day 21 of incubation with heart rate falling an average of 30 beats$*$min^{-1} more during post-6-OH hypoxia (Fig 7.2).

Ganglionic blockade

Treatment with 25 beats$*$min^{-1} hexamethonium produced little change in cardiovascular function throughout the period of chicken incubation studied (Fig. 7.3). A positive chronotropic response to hexamethonium treatment was evident on day 12 of incubation resulting in a rise of 26 beats$*$min^{-1} above control levels

(Fig 7.3). Hexamethonium also altered heart rate on day 19 of incubation, but the response was dampened in comparison to that established on day 12, rising only 4 beats$_*$min^{-1} (Fig 7.3). Pressure parameters were altered only on day 20 of incubation with a significant decrease in mean arterial pressure of 1.4 mmHg (Fig 7.3).

Between Stage comparison

Treatment with hexamethonium on all days of incubation produced varying effects as indicated in Table 7.5. Elevation in heart rate that followed treatment on day 12 was significantly different from the reactions on all other days of incubation (Table 7.5). In addition a significant difference in Map reaction was present between day 21, which responded with a slight elevation, and days 18 to 20 which decreased in response (Table 7.5).

Post-Treatment Hypoxia

Cardiovascular response to 10% O_2 were unaffected by pre-treatment with 25 mg$_*$kg^{-1} of hexamethonium over the first 85% of incubation (Fig 7.5). This trend changed on days 20 and 21 with significant differences ($p<0.05$) in pre- and post- treatment responses to hypoxia (Fig 7.5). During hypoxia, Map fell an average of 1.2 mmHg on day 20 of incubation following ganglionic blockade (Fig 7.5) which differed from control responses. Hypoxic induced alterations in f_H following ganglionic blockade also differed on day 20, changing from an initial average fall of 20 beats$_*$min^{-1} to 4 beats$_*$min^{-1} post blockade (Fig 7.5). Pressure response to hypoxia was also altered on day 21 as indicated in Figure 7.5. Mean

arterial pressure during hypoxic exposure prior to ganglionic blockade fell an average of 5 mmHg. However, following hexamethonium treatment Map was relatively unaffected falling 1.4 mmHg on average (Fig 7.5).

Discussion

Acute hypoxic cardiovascular responses have been well characterized over the duration of embryonic development in chickens (Tazawa 1981a, Tazawa 1985, Girard 1973, Van Golde et al. 1998, Mulder et al. 1997). In general, hypoxia induces a hypotensive bradycardia that is present throughout the majority of development with pressure response lost in the final day's incubation. While these patterns have been thoroughly assessed in developing chickens, the mechanisms that mediate these changes were previously left undetermined. This thesis has established that hypoxia induced changes in cardiovascular function during the final four days of chicken incubation are primarily due to adrenergic systems, with cholinergic mechanisms remaining. However, the origin of this adrenergic response was unknown with the possibility of both sympathetic and adrenal influences contributing to cardiovascular responses. This study has established that a portion of these adrenergically induced hypoxic changes in cardiovascular function is due to sympathetic activity in the final 3 days of incubation. In addition, during the final 2 days of chicken development adrenal secretion during hypoxia comes partially under central regulation. During chicken development treatment with the sympathectomizing agent 6-hydroxydopamine produced similar results to those reported previously in Ch 4 of

this thesis during chicken development. Further, embryonic cardiovascular responses to acute hypoxic exposure were within the values previously reported for chickens with the exception of day 21 reactions (Tazawa 1981 Tazawa 1985, Van Golde et al. 1998, Mulder et al. 1997). Given that experimental protocols were conducted under identical conditions in this chapter as well as Ch 4-6, a rationale for these differences is difficult to construct. A possible cause could be attributed to the difficulty of establishing the exact age of each externally piped chick. However, with the limited knowledge of the final days of development in birds, further research is needed to ascertain whether this is a viable explanation.

Sympathetic Hypoxic Action

Sympathetically mediated central regulation of cardiovascular responses to hypoxia becomes operational over the final three days of chicken development (Fig 7.3). This sympatheticly originating adrenergic stimulation acts primarily on the heart and is important for limiting the negative chronotropic actions that occur during hypoxia in chicken embryos. Previously in Ch 6 of this thesis selective adrenergic receptor blockade suggested that the primary function of adrenergic systems during hypoxia was to alter arterial pressure with no chronotropic affects. This apparent discrepancy may be ascribed to a predominant adrenergic action on peripheral vessels. Following β-blockade the overall elevation of arterial pressure during hypoxia may mask any changes in f_H that would accompany blockade. This masking could be the result of an increase in venous return that would decrease heart-filling time and possibly accommodate for any

chronotropic changes potentially caused by β-blockade. The findings presented here strongly suggest that this occurs during hypoxia in late stage embryonic chickens. In addition, since adult chickens possess a strong β-adrenergic mediated hypoxic tachycardia, this late embryonic sympathetic cardiovascular action may represent the onset of an adult regulatory mechanism (Butler 1967). It is acknowledged that, due to the possibility of incomplete removal, the developmental window for sympathetic importance in the hypoxic response may be extended. However, given the findings in this work, sympathetic action during hypoxic is important for heart rate responses during the final 3 days of chicken development.

Arterial pressure responses to hypoxia on day 20 of incubation were strongly affected by sympathetic removal. While the transient nature of this response is difficult to ascribe to a specific phenomenon, it may indicate sympathetic action on peripheral vessels. Day 20 embryos thus rely on sympathetic adrenergic systems to maintain perfusion pressure during hypoxic stress. The pronounced nature of this reaction may be buffered on day 21 of incubation due to the maturation of other regulatory mechanisms. Due to the transient nature of this response as well as the absence of blood flow determinations during hypoxia, statements on central control of peripheral vascular beds should be limited. Further study is needed to determine whether differential changes in vascular resistance during hypoxia are mediated via sympathetic action on day 20 of chicken incubation.

Ganglionic blockade

Treatment with hexamethonium produced little cardiovascular response throughout the period of chicken incubation studied. These results were similar to those previously reported in chicken embryos from days 13 to 16 (Tazawa et al. 1992). Thus, resting embryonic cardiovascular function is maintained without centrally mediated release of adrenal catecholamines during chicken development.

Adrenergic systems are an important regulator of cardiovascular responses to acute hypoxia during the final 4 days of chicken incubation (Ch 6). Sympathetically mediated catecholamine secretion from the adrenal glands may comprise a portion of this response on the final 2 days of incubation (Fig 4). The transient pattern evident on day 20 and 21 may reflect the onset of neural control of adrenal catecholamine output during hypoxic stress. The differences in hypoxic cardiovascular responses between days 20 and 21 following ganglionic blockade are difficult to explain. However, the maturation of multiple regulatory systems during this short window of chicken development limits the information that can be drawn from the data. Further study is needed to establish the role catecholamines play in the final days of incubation and possible transition of their action on the cardiovascular system.

Conclusions

Hypoxia produces clear changes in cardiovascular function during the development of chickens. As demonstrated in Ch 6 catecholamines play an

important role in hypoxic induced cardiovascular changes during the final 4 days of chicken incubation. The origin of these catecholamines and their actions on the embryonic cardiovascular system differs during this final period. Chapter 6 illustrated an important hypoxic vascular response that was the result of the combined actions β-adrenergic vasodilatation and α–adrenergic constriction with a dominating beta driven dilation. The current chapter has shown a sympathetically mediated increase in heart rate during hypoxia on days 19 to 21, with vascular reactions on day 20 only. This suggests that the majority of vascular pressure changes are mediated via an increase in plasma catecholamines during these final days of development. Ganglionic blockade has revealed that these catecholamines are released via direct action of hypoxia on the adrenal tissue without sympathetic mediation. Thus circulating catecholamines initially results in an alteration of vascular pressure with an augmentation of heart rate attributed to central regulation systems during embryonic hypoxia.

The onset of sympathetically mediated changes in heart rate may represent an adult hypoxic cardiovascular response. As previously shown, adults exhibit a marked tachycardia during acute bouts of hypoxic stress. While this response was absent in late stage embryos their ability to maintain heart rate close to control levels may represent the maturation of an adult reaction. Further study is needed to fully address this issue, but there is a clear sympathetic role in hypoxic heart rate adjustment during the final three days of chicken development.

Table 7.1. Systolic (Sys) and diastolic (Dia) pressures responses to 1 $mg_{*}kg^{-1}$ (1) and 20 $mg_{*}kg^{-1}$ (20) 6-OH treatment. Asterisk indicates significant ($p< 0.05$) as determined by a paired Student t-test. Data are presented as mean ± s.e.m.. In all cases, five embryos were used for analysis unless noted.

		1 $mg_{*}kg^{-1}$ 6-OH		20 $mg_{*}kg^{-1}$ 6-OH	
Day		Sys	Dia	Sys	Dia
12	C	7.5±0.9	4.7±0.5	7.3±0.6	5.1±0.5
	T	7.8±0.8	5±0.5	7.5±0.7	5±0.5
15	C	16±1.1	9.1±0.8	16±0.8	8.6±0.7
	T	16.3±1.1	9.2±0.8	17.8±1.4	10±1
18	C	23.4±1.8	14.2±0.6	22.8±2.2	14.4±1.1
	T	24.3±1.6	14.9±0.8	27.3±2.3 *	17.9±1.4 *
19	C	25.7±1.3	14.4±0.9	25.5±1.2	15.1±1.1
	T	27.6±1.7	16.5±1.1 *	28.9±1.8 *	17.4±2.1
20	C	27.7±2	19.1±1.4	29.8±2.1	21.1±1.7
	T	31±2.1	21±1.1	33.4±2.1 *	23.5±1.9
21	C	36.5±4.1	23.2±2.9	34.7±2.9	21.5±2.1
	T	38.2±4	24.1±3.2	42.6±3.4 *	27.1±3.1 *

Table 7.2. Systolic (Sys) and diastolic (Dia) pressures responses to hypoxia (10) pre (C) and post (T) completion of sympathectomy. Asterisk indicate significant ($p<0.05$) difference from control values for each parameter as determined by the paired Student t-test. Data are presented as mean±s.e.m.. In all cases, 5 embryos were used for analysis unless noted.

		C				T			
Day		Sys		Dia		Sys		Dia	
12	N	8.6±1.2		5.1±0.6		7.4±0.7		4.9±0.5	
	10	6.6±0.7	*	4.1±0.6	*	5.6±0.6	*	4.1±0.5	*
15	N	17±0.7		9.2±0.4		16.4±1.1		8.9±0.8	
	10	14±0.7	*	6.8±0.4	*	13.9±1	*	7.1±0.7	*
18	N	23.6±1.8		14±1.4		21.8±2.7		13.9±1.6	
	10	19.6±1.6	*	11.2±0.8		19±2.5	*	11.9±1.3	*
19	N	25.8±1.1		15.3±0.7		27.5±1.2		17.6±0.8	
	10	22.8±1.3	*	12±0.8	*	24.8±1.3	*	13.6±1.2	*
20	N	26.9±1.7		16.3±0.7		30.1±2.3		20.4±1.2	
	10	27.5±1.5		16±1.2		27.1±2.1	*	15.3±0.8	*
21	N	31.7±2.4		20±1.7		35.1±2.4		21.7±2.2	
	10	27.7±1.7	*	16.2±1.3	*	29.4±1.3	*	17±1.1	*

Table 7.3. Differences in mean arterial (Map), systolic (Sys) diastolic (Dia) pressures and heart rate (f_H) changes in response to10% O_2 treatment. Values are the differences between control and hypoxia, pre- (1) and post- (2) 6-OH injection. Asterisk indicates significant differences ($p<0.05$) in responses between pre- and post- values as determined by a one way ANOVA conducted on the arcsine transformed percent changes. Data are presented as mean±s.e.m. In all cases, 5 embryos were used for analysis unless noted.

Day		Map		Sys		Dia		f_H	
12	1	-1.4±0.3		-2±0.6		-1±0.2		-61±17	
	2	-1.2±0.1		-1.8±0.3		-0.9±0.2		-82±19	
15	1	-2.9±0.3		-3±0.3		-2.5±0.6		-42±10	
	2	-2.3±0.3		-2.5±0.6		-1.8±0.3		-44±8	
18	1	-3.5±0.9		-4±1.1		-2.8±1.1		-30±10	
	2	-2.2±0.5		-2.8±0.6		-2±0.7		-51±6	
19	1	-3.2±0.5		-3±0.7		-3.3±0.4		-38±5	
	2	-3.3±0.6		-2.7±0.6		-4±0.7		-47±4	*
20	1	0±1.1		0.6±1.6		-0.3±0.8		-39±9	
	2	-4±0.4	*	-3.1±0.5		-5±0.8	*	-62±13	*
21	1	-3.7±0.8		-4±1.1		-3.8±0.8		-7±12	
	2	-5.1±1.7		-5.7±2		-4.7±1.6		-30±10	*

Table 7.4. Cardiovascular reaction to treatment with 25 $mg_{*}kg^{-1}$ hexamethonium (H) compared to control (N) values. Asterisk indicate significant difference ($p<0.05$) result of paired Student t-test. Data are presented as mean±s.e.m. In all cases, 5 embryos were used for analysis unless noted.

Day		Sys	Dia	
12	N	8.4±0.5	5.6±0.3	
	H	8.5±0.5	5.6±0.4	
15	N	18.4±1.8	10.4±1.3	
	H	18.3±1.6	10.4±1.1	
18	N	21.7±1.5	12.2±0.7	
	H	21±1.3	11.6±0.6	
19	N	23.2±1	15±0.9	
	H	23.5±0.8	13.9±0.5	
20	N	29±1	19.9±0.6	
	H	27.3±0.8	18.3±0.5	*
21	N	30.7±1	16.6±1.2	
	H	31.4±0.8	17.8±1.3	

Table 7.5. A comparison of the degree of change in cardiovascular function induced by 25 $mg_{*}kg^{-1}$ hexamethonium. Days of incubation which reacted in similar ways are noted with like lettering as dictated by the significant ($p<0.05$) difference determined via an ANOVA.

Day		Map		Sys	Dia		f_H	
12	H	0.2±0.1	A	0.2±0.1	0±0.2	AB	26±6	A
15	H	0±0.2	A	-0.1±0.2	0±0.3	A	-2±1	B
18	H	-0.7±0.3	B	-0.8±0.4	-0.6±0.2	BC	0±3	B
19	H	-0.3±0.3	AB	0.3±0.4	-1.1±0.5	C	4±1	B
20	H	-1.5±0.5	B	-1.7±0.7	-1.6±0.5	C	-3±8	B
21	H	0.6±0.7	A	0.6±1.4	1.2±0.6	A	2±4	B

Table 7.6. Systolic (Sys) and diastolic (Dia) pressures responses to hypoxia pre- (C) and post- (H) 25 $mg*kg^{-1}$ hexamethonium treatment. Asterisk indicates significant ($p<0.05$) difference from control values for each parameter as determined by the paired Student t-test. Data are presented as mean ± sem. In all cases, five embryos were used for analysis unless noted.

	C		T			
Day	Sys	Dia	Sys		Dia	
12	-1.1±0.1	-0.5±0.1	-1.5±0.1		-0.5±0.2	
15	-3.8±1.4	-1.9±0.6	-3.2±0.4		-2.2±0.4	
18	-2.2±1.2	-3.1±0.8	-2.9±0.7		-3.4±0.9	
19	-2.3±0.7	-2.7±0.9	-4.6±0.8		-3.8±0.5	
20	0.1±0.8	1.3±0.3	-1.6±0.4	*	-1±0.4	*
21	-5.5±0.4	-4.9±1.1	-0.4±1.7	*	-2.8±1	

Figure legend

Figure 7.1

Collective responses of heart rate (A) and mean arterial pressure (B) to treatment with 1 and 20 $mg_{*}kg^{-1}$ 6-hydroxydopamine. Significant difference between controls (open column) and treatments (filled column) are indicated by asterisk.

Figure 7.2

Heart rate (f_H) and mean arterial pressure (Map) comparison between control (open column) and response to 10% O_2 (filled column) before (first column set) and following (second column set) sympathectomy. Asterisk indicates significantly different responses to hypoxia following drug treatment as determined by the ANOVA conducted on the arcsine transformed percent change.

Figure 7.3

Heart rate (f_H) and mean arterial pressure (Map) responses to 25 $mg_{*}kg^{-1}$ hexamethonium injection. Significant difference between controls (open column) and treatments (filled column) are indicated by asterisk.

Figure 7.4

Heart rate (f_H) and mean arterial pressure (Map) comparison between control (open column) and response to 10% O_2 (filled column) before (first column set) and following (second column set) ganglionic blockade. Asterisk indicates significantly different responses to hypoxia following drug treatment as determined by the ANOVA conducted on the arcsine transformed % change.

Figure 7.1

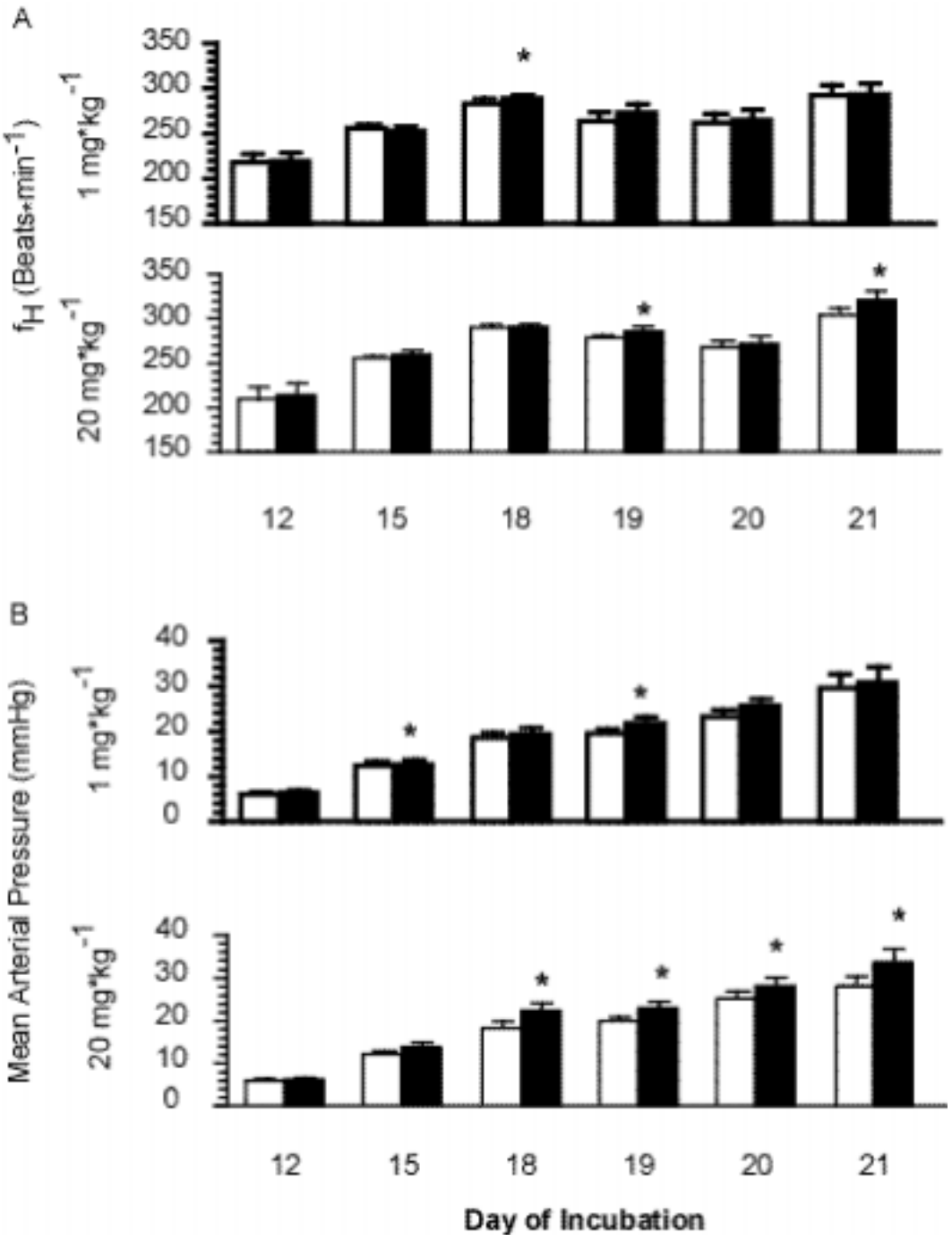
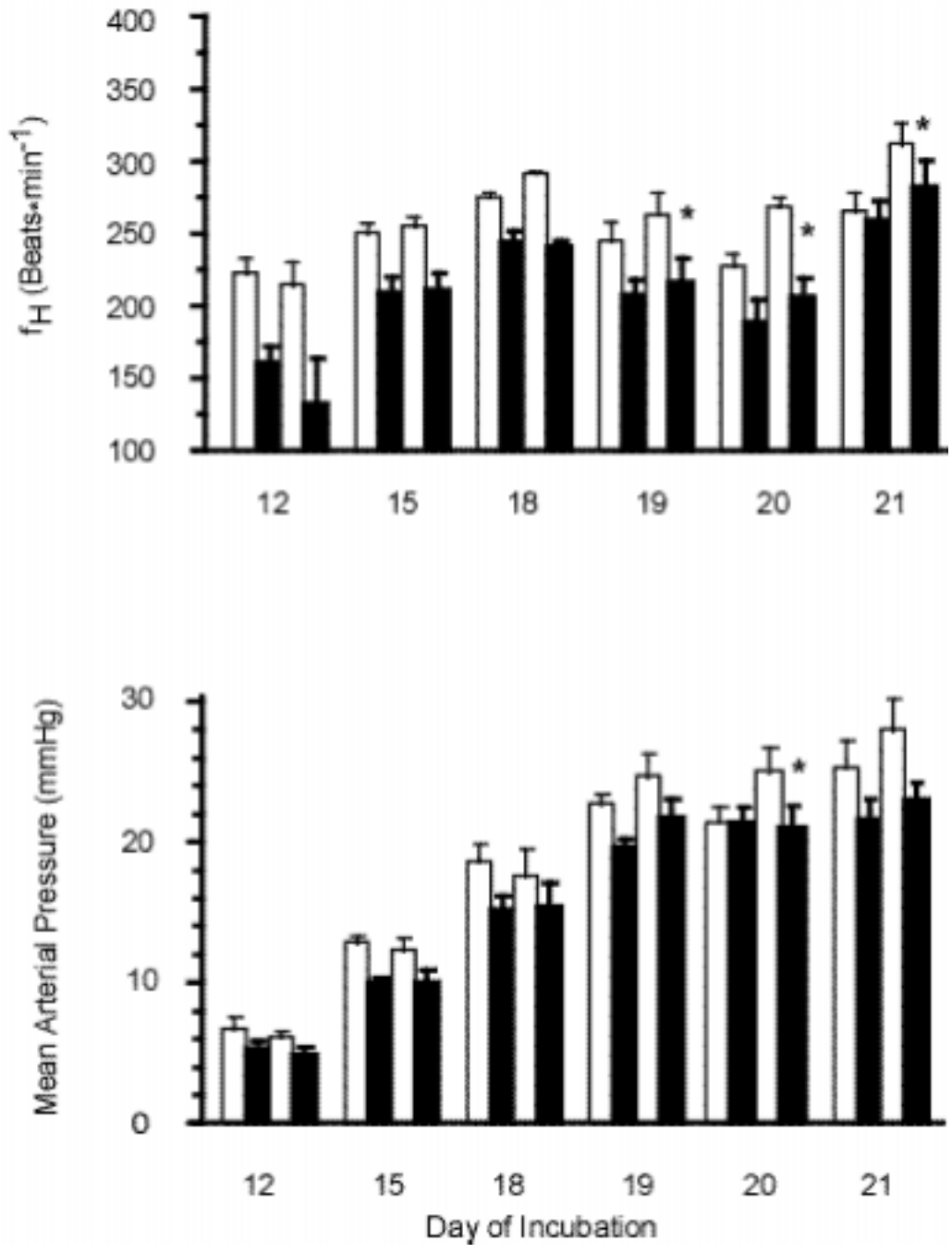

A
f_H (Beats*min^{-1})
1 mg*kg^{-1}
20 mg*kg^{-1}
350
300
250
200
150
12
15
18
19
20
21
B
Mean Arterial Pressure (mmHg)
1 mg*kg^{-1}
20 mg*kg^{-1}
40
30
20
10
0
Day of Incubation

Figure 7.2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Mean Arterial Pressure (mmHg)
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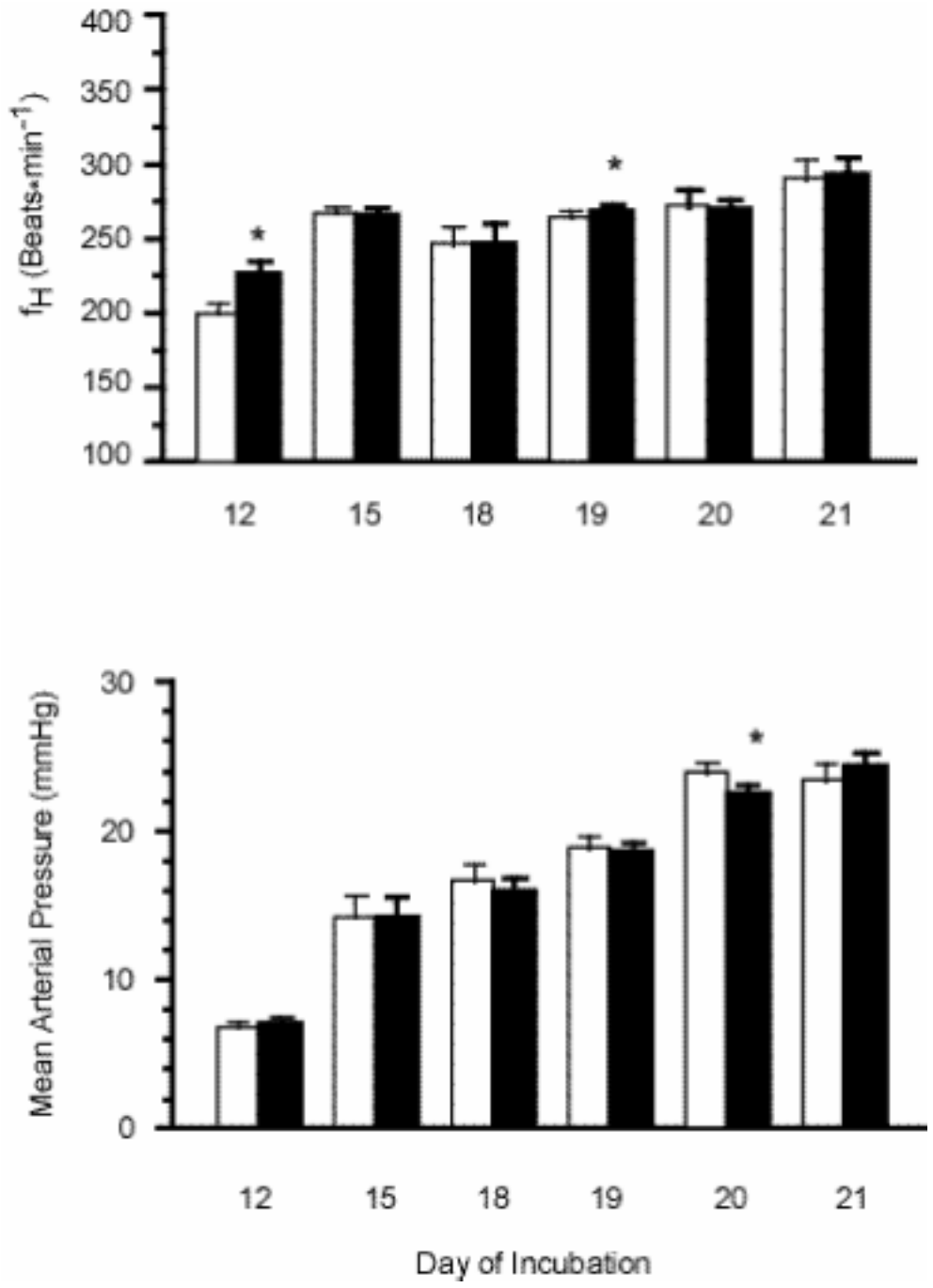
Day of Incubation

Figure 7.3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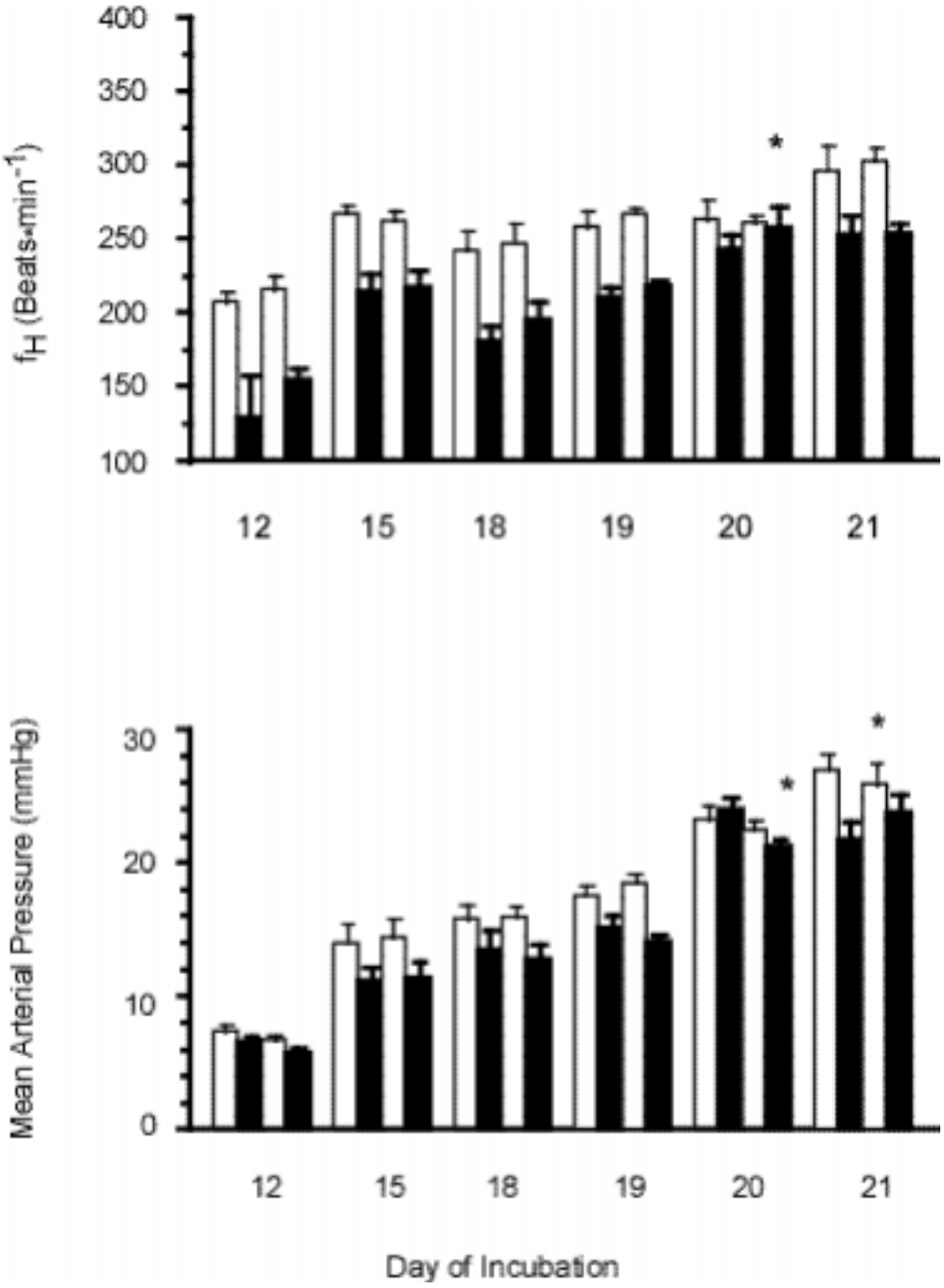
Day of Incubation

Figure 7.4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Mean Arterial Pressure (mmHg)
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21
Day of Incubation

CHAPTER VIII

MATURATION OF CARDIOVASCULAR FUNCTION IN EMBRYOS OF THE DESERT TORTOISE *Gopherus agassizii*

Introduction

The embryonic cardiovascular (CV) system is the first functional organ system in developing vertebrates, providing essential O_2 as well as nutrients to developing tissues. These functions are accomplished while the CV system itself undergoes a series of morphological and phyiological changes to reach the adult form. The majority of what is know of CV development in vertebrates has been based on two models, fetal sheep and the embryonic chicken (Pappano 1977, Lewis et al. 1984, Lewis and Sisho 1982, Tazawa 1981a, Tazawa et al 1985, Hu and Clark 1989, Girard 1973). Chicken embryos specifically have been used extensively to determine developmental changes in hemodynamics, gas transport properties and cardiovascular regulation. However, the avian embryo may not be the ideal model for understanding developmental changes in vertebrates which utilize *in-ovo* development. Reptilian eggs, specifically, are potentially subject to developmental challenges that are absent in birds, possibly necessitating differential development between the two groups.

Reptilian embryos experience different environmental and parental conditions that may require the onset of regulatory mechanisms prior to those

found in the avian embryo. Typically, with few exceptions, reptiles place their eggs in a given location and provide no further parental care. The developing animal must compensate for any alterations in the nest microenvironment without parental assistance. Given the established diurnal variability found within the reptilian nests environment the potential for acute environmental challenge is tremendous (Packard et al 1985). Therefore, environmental stress could adversely affect the cardiovascular system of reptilian embryos, necessitating unique ontogenic patterns.

The goal of this study was to characterize developmental changes in cardiovascular function in the desert tortoise *Gopherus agasizzii*, a representative reptilian species. Further, I sought to establish periods of cardiovascular sensitivity to autonomic blockade in a reptilian embryo. Finally, it was hypothesized that autonomic regulation of the cardiovascular system will develop prior to that known in avian embryos.

Materials and Methods

Donated eggs of the desert tortoise (*Gopherus agassizii*) from captive females were collected following oviposition and returned to the Department of Biological Sciences at the University of Nevada, Las Vegas. In the lab 110 eggs were numbered, weighed, randomly placed into plastic boxes and set in incubator (Lyon electronics ProfHi) at 29±0.5 °C. At experimental intervals of 10% incubation, five eggs where randomly selected for acute cardiovascular study beginning at 30% of incubation time.

Catheterization Procedure

Prior to experimentation, eggs were candled to locate a major chorioallontoic membrane (CAM) artery. Once located, eggs were placed in a water-jacketed temperature control chamber to maintain an environment close to incubation conditions. A small hole was then made in the shell with a 21-gauge needle, allowing the removal of a 1-cm square section of shell. A branch of an underling artery was then encircled with three 6-00 silk ligatures to act as catheter anchors. The distal ligature was then tightened, occluding blood flow, followed by proximal ligature closure limiting flow. Micro-fine scissors were then used to cut the vessel to allow anterograde insertion of a saline filled polyethylene catheter. Catherters were constructed of PE-90 (1.27 mm OD / 0.86 mm ID, Clay-Adams) heat pulled to a tip outer diameter smaller than 0.5 mm OD. Following successful cannulation, the intermediate ligature was tightened followed by the removal of the proximal ligature. The catheter was then fixed to the shell with cyanoacrylic glue and plugged with a pin occluder.

Signal recording and calibration

Each catheter was attached to a pressure transducer (WPI, type BLPR), which, in turn, was connected to a bridge amplifier (CB Sciences, model ETH-400), and the pressure trace was stored in a computer using PowerLab data acquisitions software. Heart rate was continuously calculated from the pressure signal via an acquisition tachograph. In all cases, zero was initially set at the top

of the temperature-controlled chamber set at 29±0.5 °C, and all values were corrected after the experiment as described in chapter 2.

Acute Study Procedures

Following surgery, each embryo was allowed to recover for 30 min to establish control conditions. After the control period, a 20 μl infusion of 0.9 % saline for egg ranging from 30-50% of incubation and 50 μl for eggs ranging from 60 to pre-hatch was used to determine the effects of volume loading. Volume loading tests were followed by the serial infusions of sodium nitroprusside, phenylephrine, atropine, and propranolol. Concentrations of 10^{-5} M and 10^{-4} M were used to establish maximal responses to each drug at a given period of incubation. Following completion of the study, arterial blood samples were collected for determination of hematocrit. Temperature of each egg was then taken, followed by an infusion of 1-percentage xylocaine and separation of the egg components to determine wet and dry masses.

Statistical Analysis

A paired Student t-test was used to access significant differences between pre- and post- drug infusion for all variables measured on each day of development. A one-way ANOVA was conducted on each cardiovascular parameter with incubation to determine developmental changes. Fisher's LSD post-hoc comparison was used to isolate significant differences between days of incubation. The fiduciary level of significance for all tests was taken at $p<0.05$.

All data are presented as mean ± 1 sem. For all days of study n = 5 eggs, were used to determine cardiovacular responses.

Results

Hematocrit values are presented in Table 8.1 with the exception that in the first series of samples all levels were similar during embryonic development. Mean arterial pressure (Map) changes during embryonic development were indicative of all pressure parameter changes (Table 8.2). Control Map in 40% embryos was 4.1 mmHg while heart rate (f_H) was 89 beats$*$min^{-1} (Fig 8.2). Mean arterial pressure remained constant over the next 40% of incubation rising significantly ($p<0.05$) during the last 20% of embryonic development (Fig 8.2). Heart rate was initially elevated at 40% of incubation, averaging 89 beats$*$min^{-1}, then fell over the remainder of development reaching 67 beats$*$min^{-1} at 90 % of incubation, a level that was significantly different ($p<0.05$) than the first 30% of the study period (Fig 8.2).

Cholinergic tone

Treatment with 50 μl of 10^{-5} M atropine produced no significant change in any cardiovascular parameter measured (Table 8.2). Cholinergic receptor presence and function on the embryonic cardiovascular system was tested in pilot studies. These studies established that acetylcholine 10^{-4} M injection stopped the embryonic heart (Fig 8.1A). This response was eliminated by treatment with atropine indicating receptors were present and atropine was effective at blocking responses.

Intra-arterial injections of atropine 10^{-4} M concentration produced significant reductions in Map from 70% to 95% of total incubation (Fig 8.3). These reductions ranged from 0.1 to 1.1 mmHg over the interval of action. Heart rate was constant over all intervals tested with the exception of the 60% interval during which injections produced a slight fall (Fig 8.3).

β-Adrenergic

β-Blockade with 10^{-5} M propranolol produced an increase in Map that became significant during the 70% incubation interval (Table 8.4). This change ranged from 0.6 to 2.7 mmHg over the period of significant response ($p<0.05$). Heart rate was consistently reduced following treatment with 10^{-5} M propranolol over the period of study (Table 8.4). This reaction in f_H was transiently significant from 60 to 90% of incubation with a reduction of 4 beats$_*$min^{-1} on average over this period (Table 8.4). Pilot studies with isoproterenol injections verified this level of b-blockade was sufficient to eliminating cardiovascular responses.

Mean arterial pressure responses to 10^{-4} M propranolol injection was significantly ($p<0.05$) elevated from 80% to 95% of incubation (Fig 8.4). Over this incubation interval, pressure increases ranged from 1.6 to 4.3 mmHg following drug injections. Heart rate was altered over the same interval as noted for the previous concentration of propranolol with an average fall of 7 beats$_*$min^{-1} (Fig 8.4).

Vascular Responses

Mean arterial pressure fell following injection of 10^{-5} M sodium nitroprusside (SNP) during all incubation intervals tested (Table 8.7). These pressure changes were significant ($p<0.05$) over the final 50% of tortoise development with an average change ranging from 0.1 to 4.3 mmHg. While treatment altered pressure, heart rate was relatively unaffected by this level of sodium nitroprusside treatment (Table 8.7). Injection of 10^{-4} M sodium nitroprusside accentuated the decreased in Map demonstrated for the initial dosage. Pressure reduction ranged from 0.4 to 4.1 mmHg following administration of 10^{-4} M sodium nitroprusside and the period of significant response was extended to include 40% of incubation (Fig 8.5). Average heart rate response was minimal following drug injection, with the exception of a significant increase ($p<0.05$) at 70% of tortoise incubation (Fig 8.5).

Treatment with 10^{-5} M concentration of the α–agonist phenylephrine resulted in a general increase in Map over the period of study. This increase ranged from 0.5 to 1.8 mmHg and was significant ($p<0.05$) at 40,50,70 and 80% of tortoise incubation (Table 8.9). Following injection of the second concentration of phenylephrine Map was elevated significantly ($p<0.05$) during all incubation intervals tested, with increases ranging from 0.06 to 1.8 mmHg (Fig 8.6). Heart rate was relatively unaltered by drug treatment throughout the incubation interval tested with the exception of a slight reduction at the 90% interval of incubation (Fig 8.6).

Discussion

Cardiovascular development has been intensively investigated in mammalian models, in an effort to characterize and understand potential pathologies that may occur during human gestation. While this has been essential work, the understanding of CV development in other vertebrate groups has remained poorly characterized. Given the clear differences between ex-utero and in-utero development there are compelling reasons to believe this process may differ between vertebrate groups. This study has characterized changes in cardiovascular function during the development of a reptilian species, a vertebrate group that is poorly understood. In addition, fundamental cardiovascular regulatory mechanisms were assessed to establish the potential for embryonic response to environmental stress. Utilizing pharmacology, this work determined that arterial pressure responses to selective agonists and antagonist were similar to those demonstrated in chicken embryos. All responses were simply accentuated by the increase in drug concentration. Further, β-adrenergic tone on the cardiovascular system of embryonic reptiles was in place at 60% on development, while cholinergic tone was absent throughout incubation. As established in Ch 2 of this thesis, cholinergic tone must become functional very late in tortoise development or sometime in the beginning of hatchling life.

Cardiovascular Patterns

General cardiovascular function exhibited a unique developmental pattern during the incubation of the desert tortoise. Mechanical cardiovascular

development in the desert tortoise mimicked that of embryonic chickens, with arterial pressures starting low and rising to a maximum at hatch. However, several differences were apparent when a stage by stage analysis of pressure change was conducted. In the desert tortoise, Map remained constant until the final 20% of incubation (Fig 8.2). This pattern was very different from that exhibited by chicken embryos which, during a comparable period, show a 10-fold increase in mean pressure (Girard 1973). In addition, average maximal pressure at each given percentage of tortoise incubation was consistently lower than pressure values demonstrated by chicken embryos at similar times (Girard 1973). Therefore, there are fundamental mechanical differences in cardiovascular function between the two species during their development.

Developmental heart rate patterns further illustrated the differences between the two species as gestation progressed. Embryonic chickens exhibit an increase in heart rate over the first 50% of incubation which then remains constant until the animal hatches (Girard 1973). The pattern exhibited by desert tortoise embryos differs, with an initial rise from 30 to 40% of incubation followed by a fall over the remainder of development (Fig 2). This trend has also been shown in embryos of the african brown house snake (*Lamprophis fuliginosis*) and the common snapping turtle (*Chelydra serpentina*), suggesting a bell pattern in heart rate change may be common among reptiles (Crossley unpublished, Birchard and Reiber 1996). Thus, there are basic developmental differences in cardiovascular function between chickens and reptiles during incubation. Further

analysis is needed to determine the functional basis behind this difference. However, changes in peripheral resistance may account for a portion of these findings.

Cholinergic tone

Cholinergic tone was absent throughout embryonic development in the desert tortoise (Fig 8.4) a characteristic that is identical to that found in chicken embryos. Fundamental anatomical and cellular developmental studies, which have been conducted on chicken embryos, are lacking in embryonic reptiles, thus limiting the interpretation of the present findings. If it is assumed that cholinergic receptors and nerve terminals reach the cardiovascular system prior to hatch, as occurs in chickens, then clearly cholinergic tone is nonfunctional during development in the desert tortoise. It could be argued that embryonic muscarinic receptors are insensitive to cholinergic blockade during tortoise development. This scenario could be a potential concern however, since the chronotropic effects of acetylcholine could be successfully eliminated via pre-treatment with atropine it is unlikely (Fig 8.1). Therefore, resting cardiovascular function in the embryonic tortoise is maintained without tonic cholinergic input as determined in chicken embryos. Collectively these data indicate that vagal tone is absent during the development of terrestrial ex-utero developing species.

β-Adrenergic tone

Unlike the CV responses to cholinergic blockade, a pronounced negative chronotropic action of β-blockade was evident over the latter 40% of tortoise

incubation (Fig 8.4). Upon initial comparison, this could be interpreted as a fundamental difference between chickens (which exhibit a strong β-tone at 40% of development) and desert tortoise embryos. However, due to the tremendous mortality evident following drug treatment at 40 and 50% of incubation, this interpretation could be inaccurate. In addition, because of experimental techniques utilized in this work, changes in sensitivity to antagonist during development were undetermined. However, the finding presented here clearly indicate β-adrenergic tone is important for the maintenance of resting chronotropic action in developing desert tortoise (Fig 8.4).

β-Adrenergic vascular tone was first evident at 80% of incubation in the desert tortoise (Fig 8.4). Thus, as in embryonic chickens, β-adrenoreceptors are an integral component which maintains a relative vasodilated state during the final 20% of development. A portion of the pressure increase demonstrated following propranolol treatment might be directly attributed to chronotropic changes resulting in an increase in output pressure. Due to the general administration techniques used in this work, a proper distinction between cardiac and vascular changes is difficult. However, it is clear that embryos of the desert tortoise, as with chicken embryos, respond to β-blockade in a way that is similar to that of most other vertebrates. While the cardiac β-adrenergic tone seems to be almost universal in vertebrates, the degree of hemodynamic changes due to β-adrenoreceptor vasodilation might be accentuated in embryos, especially if the chorioallantoic vasculature has an active population of β-adrenergic receptors.

This has been suggested to be the case in the placenta of the fetal lamb during the latter third of gestation (Carter 1993). Therefore, β-adrenergic receptors may become important regulators of vascular tone during the latter 20% of desert tortoise development. Preliminary study of the isolated chorioallantoic membrane vasculature from chicken embryos suggests that a significant vasodilation can be induced via β-adrenergic stimulation (Crossley, Altimiras and Burggren in prep). These finding further suggest that a resting β-adrenergic tone on the vasculature is possible during the later 20% of desert tortoise incubation.

α–Adrenergic response

A predominant pressure increase following α–adrenoreceptor stimulation was evident over the final 50% of tortoise incubation, with limited chronotropic action (Fig 8.6). This response was similar to those shown in chicken embryos over a similar period of development (Tazawa et al. 1992). It is important to recognize that while this study indicated the cardiovascular system of the desert tortoise is responsive to α–stimulation, the question of tonic control was unanswered. Given the pronounced α–tone found in embryonic chickens and fetal sheep, each with a hypertensive reaction to α–stimulation, the desert tortoise may also rely on an α–tone during incubation (Tazawa et al. 1992, Assali et al. 1978). In addition, possible changes in α–adrenergic sensitivity were undetermined due to the constant volume technique used in this study. Further

study is needed to determine if the cardiovascular system of embryonic desert tortoise relies on an α–adrenoreceptor tone during development.

Nitric Oxide effects

In the present study, injections of sodium nitroprusside, a nitric oxide donor, decreased arterial pressure throughout the development of the desert tortoise (Fig 8.5). Cardiovascular responses to nitric oxide (NO) treatment were primarily manifested as reductions in arterial pressure with little change in heart rate (Fig 8.5). These effects were similar to those shown in various adult reptiles and embryonic chickens (Millard and Moalli 1980, Stephens et al. 1983, Crossley et al. in review). Thus, the nitric oxide pathway is present at 50% of tortoise development as previously shown in embryonic chickens. While the role of nitric oxide tone during tortoise development was undetermined, this work established that vascular smooth muscle is responsive to nitric oxide. Prior study has shown that multiple cellular components are required for the transudation of the nitric oxide signal from the endothelium to vascular smooth muscle (Moncada and Higgins 1993). Thus, the hypotension exhibited by desert tortoise embryos following sodium nitroprusside injection suggests an important cardiovascular function during development. An in depth study is needed to determine whether desert tortoise embryos rely on an NO tone during development or if this system is activated by selective cardiovascular challenges.

Conclusion

Our understanding of cardiovascular maturation during reptilian development has previously been based on limited morphological descriptive studies with few attempts to quantify physiological changes. The current study has characterized general hemodynamic changes during the final 60% of incubation in a representative reptilian species. Embryos of the desert tortoise exhibit a constant arterial pressure over the majority of development. This pattern differs dramatically from that known to occur in embryos of the domestic chicken, suggesting fundamental mechanical differences between the two species. While these species may differ mechanically during development, regulatory components are similar.

As previously shown in chickens, cholinergic tone on the cardiovascular system of desert tortoise embryos is absent during development. Thus, basal cardiovascular performance and maturation is achieved without tonic vagal input during tortoise incubation. This finding contrasts the tonic vagal control of cardiovascular function that has been established in adult turtles (Burggren 1976). Thus, vagal function must become operational sometime during the hatchling life period. As indicated in chapter 2, parasympathetic tone may therefore be nonessential for normal cardiovascular development of terrestrial egg laying species. However, to definitively determine this, extensive multi-species comparisons must be conducted.

While vagal tone was absent throughout incubation of the desert tortoise, a clear β-adrenergic tone on cardiovascular function was operational. In

addition, α–adrenoreceptor stimulation suggested that an important α–tone might also be present. The interpretation of these findings should be limited given that critical information on anatomical integrity is needed. Collectively, these findings suggest that adrenergic components, either neural or humoral in origin, are important for cardiovascular development in the desert tortoise. Further study is needed to determine the role that adrenergic systems play in cardiovascular development of the desert tortoise as well as other egg laying terrestrial vertebrates.

Table 8.1. The change in hematocrit (Hct) as incubation progressed. Number (N) of eggs sampled on each interval.

%	N	Hct
40	2	21.7±7.9
50	3	34.5±1.5
60	5	27.3±2.9
70	8	28.0±1.5
80	11	25.1±2.0
90	9	28.7±1.8
95	15	25.3±6.2

Table 8.2. Change in mean arterial pressure (Map) systolic pressure (Sys), diastolic pressures (Dia) and heart rate (f_H) during the percentage of incubation noted. Number (N) of eggs used at each portion of development is noted. Data are presented as mean ± sem.

%	N	Map	Sys	Dia	f_H
40	8	4.1±0.3	4.9±0.4	3.3±0.4	89±6
50	5	4.1±0.6	4.9±0.6	3.4±0.5	84±6
60	6	4.5±0.3	5.2±0.4	3.7±0.2	79±4
70	9	4.6±0.4	5.7±0.4	3.5±0.5	80±3
80	6	5.7±0.8	8.3±1.0	3.6±0.6	77±3
90	7	9.6±0.8	13.7±1.2	4.9±1.0	67±2
95	14	11.3±0.6	14.8±0.8	8.2±0.6	72±2

Table 8.3. The change in mean arterial pressures (Map) systolic pressures (Sys), diastolic pressures (Dia) and heart rate (f_H) following a 50 µl injection of 10^{-5} M atropine into embryos at the given percentage (%) of incubation. The paired Student t-test between control (C) and drug treatment (T) revealed drug produced no response. Egg numbers (N) are presented. Data are presented as mean ± sem.

%		N	Map	Sys	Dia	f_H
40	C	4	3.0±0.5	3.7±0.6	2.4±0.4	94±5
	T		3.1±3.8	3.8±0.5	2.5±0.2	84±9
50	C	3	3.4±4.1	4.1±0.6	2.7±0.3	86±8
	T		4.0±4.9	4.9±1.1	3.2±0.8	83±8
60	C	5	5.4±6.4	6.5±0.6	4.5±0.4	77±6
	T		5.2±6.4	6.4±0.3	4.3±0.3	75±7
70	C	9	5.5±0.4	6.7±0.6	4.3±0.4	80±3
	T		5.4±0.4	6.7±0.5	4.3±0.4	80±3
80	C	11	6.9±0.7	9.9±0.9	4.5±0.5	74±2
	T		7.1±0.7	10.2±1	4.5±0.5	73±1
90	C	11	12.2±1.2	16.9±1.5	8.0±1.0	68±2
	T		12.0±1.2	16.7±1.5	8.0±0.9	68±2
95	C	3	19.7±6.1	24.7±8.1	14.7±4.5	61±5
	T		19.8±5.8	24.8±7.6	15.1±4.5	65±5

Table 8.4. The change in mean arterial (Map) systolic (Sys), diastolic (Dia) pressures and heart rate (f_H) following a 50 µl injection of 10^{-4} M atropine into embryos at the given percentage (%) of incubation. Asterisk indicates significant result ($p<0.05$) the paired Student t-test between control (C) and drug treatment (T). Egg number (N) is presented. Data are presented as mean ± sem.

%		N	Map		Sys		Dia		f_H	
40	C	6	3.5±0.5		4.2±0.6		3.1±0.4		72±8	
	T		3.5±0.5		4±0.7		3.1±0.5		61±14	
50	C	4	4.1±0.7		4.8±0.8		3.5±0.7		83±4	
	T		4±0.6		4.8±0.7		3.4±0.6		83±6	
60	C	5	5±0.4		5.9±0.5		4.2±0.3		77±6	
	T		4.8±0.4		5.8±0.5		4.1±0.4		76±6	*
70	C	9	5.4±0.4		6.8±0.5		4.2±0.4		79±3	
	T		5.3±0.4	*	6.6±0.5		4.1±0.4		78±3	
80	C	10	7.3±0.7		10.6±0.9		4.7±0.5		73±1	
	T		7.1±0.6	*	10.3±0.8	*	4.5±0.5	*	72±1	
90	C	12	11.6±1.2		16.1±1.5		7.6±1		67±2	
	T		11±1.2	*	15.2±1.5		7.2±1	*	67±2	
95	C	11	15.8±1.7		20.7±2		11.4±1.5		64±2	
	T		14.5±1.5	*	19.5±1.8	*	10.1±1.2	*	64±2	

Table 8.5. The change in mean arterial pressures (Map) systolic pressures (Sys), diastolic pressures (Dia) and heart rate (f_H) following a 50µl injection of 10^{-5} M propranolol into embryos at the given percentage (%) of incubation. Asterisk indicates significant result ($p<0.05$) the paired Student t-test between control (C) and drug treatment (T). Egg number (N) is presented. Data are presented as mean ± sem.

%		N	Map		Sys		Dia		f_H	
40	C	2	2.8±0.1		3.5±0.1		2.3±0.2		95±13	
	T	2	2.9±0.3		3.5±0.4		2.5±0.3		90±12	
50	C	2	3.5±0.4		4.4±0.8		2.8±0		87±14	
	T	2	3.3±1.1		4±1.7		2.6±0.5		68±3	
60	C	5	5.1±0.4		6.1±0.5		4.2±0.3		74±7	
	T	5	5.6±0.4		6.6±0.6		4.7±0.3		70±6	*
70	C	9	5.1±0.5		6.5±0.5		4±0.4		78±3	
	T	9	5.7±0.5	*	7.1±0.6	*	4.4±0.5	*	75±3	*
80	C	8	7.5±0.7		10.6±1		4.9±0.6		73±2	
	T	8	8.5±0.9	*	12.1±1.2	*	5.5±0.6	*	71±2	*
90	C	10	10.6±1.2		15.1±1.3		6.8±1		68±2	
	T	10	13.4±1.2	*	18.4±1.6	*	9±1	*	65±2	*
95	C	4	17.4±3.5		21.6±4.8		12.9±2.1		64±4	
	T	4	20.1±4.5		24.6±5.7		15.6±3		61±6	

Table 8.6. The change in mean arterial pressures (Map) systolic pressures (Sys), diastolic pressures (Dia) and heart rate (f_H) following a 50 µl injection of 10^{-4} M propranolol into embryos at the given percentage (%) of incubation. Asterisk indicates significant result ($p<0.05$) of the paired Student t-test between control (C) and drug treatment (T). Egg number (N) is presented. Data are presented as mean ± sem.

%		N	Map		Sys		Dia		f_H	
40	C	5	4±0.5		4.7±0.6		3.4±0.5		84±11	
	T	5	4.2±0.5		4.9±0.7		3.6±0.5		56±18	
50	C	4	4.8±0.5		5.7±0.6		4±0.4		78±2	
	T	4	4.7±0.8		5.6±1		3.9±0.6		51±17	
60	C	6	5.1±0.4		6±0.5		4.3±0.3		71±5	
	T	6	5±0.3		5.9±0.5		4.3±0.2		65±5	*
70	C	8	5.5±0.6		6.9±0.7		4.3±0.6		77±3	
	T	8	5.4±0.6		6.8±0.7		4.3±0.6		71±3	*
80	C	13	7.1±0.8		10.2±1.1		4.5±0.6		73±1	
	T	13	8.7±0.9	*	12.6±1.2	*	5.5±0.6	*	66±1	*
90	C	12	12.6±1.3		17.3±1.6		8.3±1		64±2	
	T	12	14.6±1.4	*	20.2±1.9	*	9.9±1.1	*	58±2	*
95	C	12	15.3±1.9		19.2±2.1		11.3±1.6		67±3	
	T	12	19.6±1.9	*	24.9±2.3	*	14.7±1.5	*	60±2	*

Table 8.7. The change in mean arterial pressures (Map) systolic pressures (Sys), diastolic pressures (Dia) and heart rate (f_H) following a 50 μl injection of 10^{-5} M sodium nitroprusside. Asterisk indicates significant result ($p<0.05$) of the paired Student t-test between control (C) and drug treatment (T). Egg number (N) is presented. Data are presented as mean ± sem.

%		N	Map		Sys		Dia		f_H	
40	C	6	3.9±0.5		4.6±0.4		3.3±0.5		93±7	
	T		3.7±0.5		4.3±0.5		3.2±0.5		90±7	
50	C	5	4.4±0.5		5.2±0.6		3.6±0.5		87±6	
	T		3.6±0.5	*	4.3±0.5	*	3.1±0.5	*	86±6	
60	C	5	4.8±0.1		5.6±0.1		4.1±0.2		77±6	
	T		4.1±0.2	*	4.8±0.2	*	3.6±0.3	*	77±7	
70	C	9	4.5±0.4		5.7±0.4		3.5±0.4		81±4	
	T		3.7±0.5	*	4.7±0.5	*	2.9±0.5	*	82±3	
80	C	12	5.6±0.5		8.3±0.7		3.5±0.3		74±2	
	T		4.5±0.3	*	6.7±0.5	*	2.8±0.2	*	74±2	*
90	C	12	9±0.5		13±0.8		5.4±0.4		67±2	
	T		7.1±0.4	*	10.6±0.6	*	4.2±0.3	*	67±2	
95	C	6	12.8±2.3		16.9±3.1		8.9±1.6		64±3	
	T		9.6±1.4	*	13.1±2.1	*	6.6±1	*	64±4	

Table 8.8. The change in mean arterial pressures (Map) systolic pressures (Sys), diastolic pressures (Dia) and heart rate (f_H) following a 50 μl injection of 10^{-4} M sodium nitroprusside. Asterisk indicate significant result ($p<0.05$) of the paired Student t-test between control (C) and drug treatment (T). Egg number (N) is presented. Data are presented as mean ± sem.

%		N	Map		Sys		Dia		f_H	
40	C	6	4.1±0.4		4.9±0.4		3.5±0.4		77±5	
	T		3.6±0.4	*	4.2±0.4	*	3.2±0.4		77±5	
50	C	4	4±0.6		4.8±0.6		3.3±0.6		87±8	
	T		2.9±0.5	*	3.5±0.6	*	2.5±0.6	*	86±9	
60	C	7	4.4±0.4		5.2±0.4		3.7±0.4		77±5	
	T		3.3±0.3	*	3.8±0.3	*	2.9±0.4	*	79±5	
70	C	16	5.3±0.4		7.3±0.6		3.6±0.3		77±2	
	T		3.7±0.4	*	5.1±0.5	*	2.7±0.3	*	78±2	*
80	C	5	5.5±0.6		8.3±1		3.3±0.4		74±2	
	T		3.9±0.7	*	5.9±1.2	*	2.4±0.4	*	76±3	
90	C	13	8.8±0.5		12.8±0.8		5.3±0.3		66±2	
	T		6±0.3	*	8.9±0.5	*	3.7±0.2	*	67±2	
95	C	18	11.6±0.8		15.6±1.1		8±0.6		68±2	
	T		7.7±0.6	*	10.6±0.9	*	5.3±0.3	*	70±2	

Table 8.9. The change in mean arterial pressures (Map) systolic pressures (Sys), diastolic pressures (Dia) and heart rate (f_H) following a 50 µl injection of 10^{-5} M phenylephrine. Asterisk indicates significant result ($p<0.05$) of the paired Student t-test between control (C) and drug treatment (T). Egg number (N) is presented. Data are presented as mean ± sem.

%		N	Map		Sys		Dia		f_H
40	C	6	3.5±0.4		4.1±0.4		3±0.4		78±6
	T		4±0.3	*	4.7±0.4	*	3.5±0.4	*	88±9
50	C	5	3.5±0.4		4.1±0.5		2.9±0.4		86±7
	T		4.4±0.4	*	5.4±0.4	*	3.6±0.4	*	89±8
60	C	4	4.1±0.2		4.9±0.3		3.4±0.1		76±8
	T		5.1±0.5		6.2±0.8		4.2±0.4		74±8
70	C	9	5±0.4		6.3±0.4		3.9±0.4		79±3
	T		6.8±0.5	*	8.8±0.7	*	5.1±0.5	*	78±3
80	C	12	5.7±0.6		8.3±0.8		3.5±0.4		75±2
	T		6.6±0.7	*	9.8±0.9	*	3.9±0.5	*	73±2
90	C	11	9.2±0.6		13.4±0.9		5.6±0.5		64±2
	T		10±0.7		14.4±0.9		5.9±0.5		64±2
95	C	10	13.2±1.4		17.3±2.2		9.5±1		66±3
	T		14.9±1.6		19.2±2		11±1.5		66±3

Table 8.10. The change in mean arterial pressures (Map) systolic pressures (Sys), diastolic pressures (Dia) and heart rate (f_H) following a 50 µl injection of 10^{-4} M phenylephrine. Asterisk indicates significant result ($p<0.05$) the paired Student t-test between control (C) and drug treatment (T). Egg number (N) is presented. Data are presented as mean ± sem.

%		N	Map		Sys		Dia		f_H	
40	C	6	3.1±0.4		3.8±0.4		2.4±0.5		93±9	
	T	6	3.7±0.3	*	4.5±0.4		2.9±0.4		96±9	
50	C	5	4.4±0.6		5.3±0.6		3.7±0.6		81±6	
	T	5	5±0.6	*	6.1±0.7	*	4.1±0.5		87±6	
60	C	5	3.8±0.5		4.6±0.6		3.2±0.5		72±4	
	T	5	4.6±0.6	*	5.7±0.7	*	3.6±0.6	*	73±4	
70	C	6	5.4±0.6		6.6±0.7		4.3±0.6		79±3	
	T	6	7±0.7	*	8.8±0.8	*	5.5±0.7	*	79±3	
80	C	11	6.5±0.6		9.5±0.8		4±0.5		74±2	
	T	11	8.1±0.8	*	12±1.1	*	4.9±0.6	*	72±1	
90	C	13	9.4±0.7		13.5±0.7		5.7±0.4		65±2	*
	T	13	11.2±0.7	*	16.1±0.7	*	6.9±0.4	*	64±2	
95	C	10	13.3±2		18.5±2		8.6±0.8		63±2	
	T	10	15.1±2.1	*	20.6±2.1	*	9.6±1		63±2	

Figure legend

Figure 8.1.

Representative traces demonstrating arterial pressure (P) and heart rate (f_H) responses to various drug treatments in an 80% desert tortoise embryo. Trace A illustrates P and f_H response to acetylcholine (1) atropine (2) and repeated acetylcholine (3). Trace B shows that cardiovascular response to atropine treatment of 10^{-5} M (1) and 10^{-4} M (2). Trace C illustrates cardiovascular responses to propranolol treatment of 10^{-5} M (1) and 10^{-4} M (2).

Figure 8.2.

Change in control f_H and Map during tortoise incubation. Letters indicate the result of an ANOVA with differing letters indicating significant ($p<0.05$) difference between percentages of incubation. Data are presented as mean ± s.e.m.

Figure 8.3.

Response of embryonic f_H and Map to injection of 10^{-4} M atropine during desert tortoise incubation. Each percentage of development responses are presented as control (open bar) and treatment (closed bar) with differences indicated by asterisk. Data are presented as mean ± s.e.m.

Figure 8.4.

Response of embryonic f_H and Map to injection of 10^{-4} M propranolol during desert tortoise incubation. Each percentage of development responses are presented as control (open bar) and treatment (closed bar) with differences indicated by asterisk. Data are presented as mean ± s.e.m.

Figure 8.5.

Response of embryonic f_H and Map to injection of 10^{-4} M soduim nitroprusside during desert tortoise incubation. Each percentage of development responses are presented as control (open bar) and treatment (closed bar) with differences indicated by asterisk. Data are presented as mean ± s.e.m.

Figure 8.6.

Response of embryonic f_H and Map to injection of 10^{-4} M phentolamine during desert tortoise incubation. Each percentage of development responses are presented as control (open bar) and treatment (closed bar) with differences indicated by asterisk. Data are presented as mean ± s.e.m.

Figure 8.1

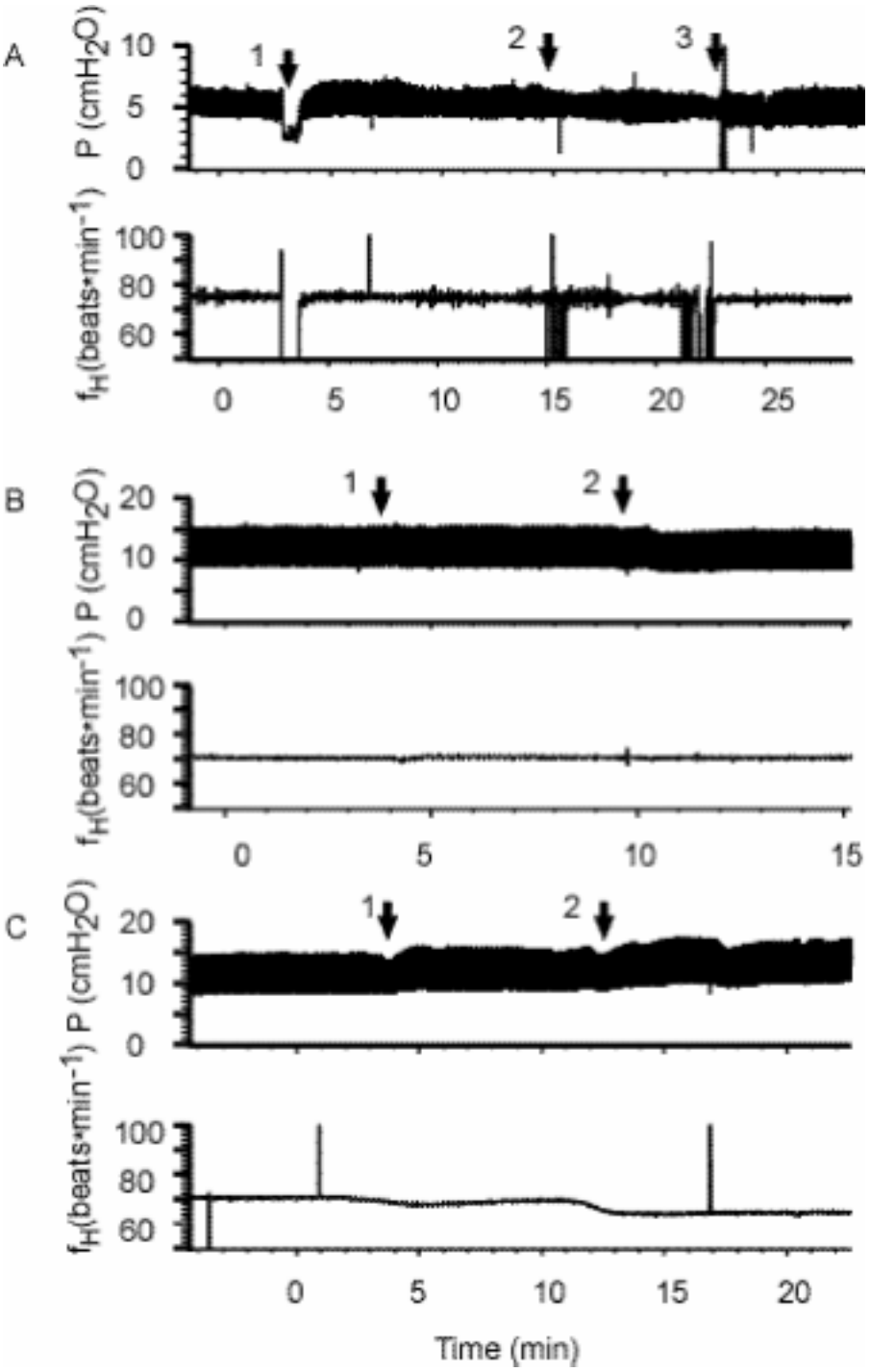

A
B
C
1
2
3
P (cmH2O)
fH(beats•min-1)
10
5
0
20
100
80
60
0
5
10
15
20
25
Time (min)

Figure 8.2

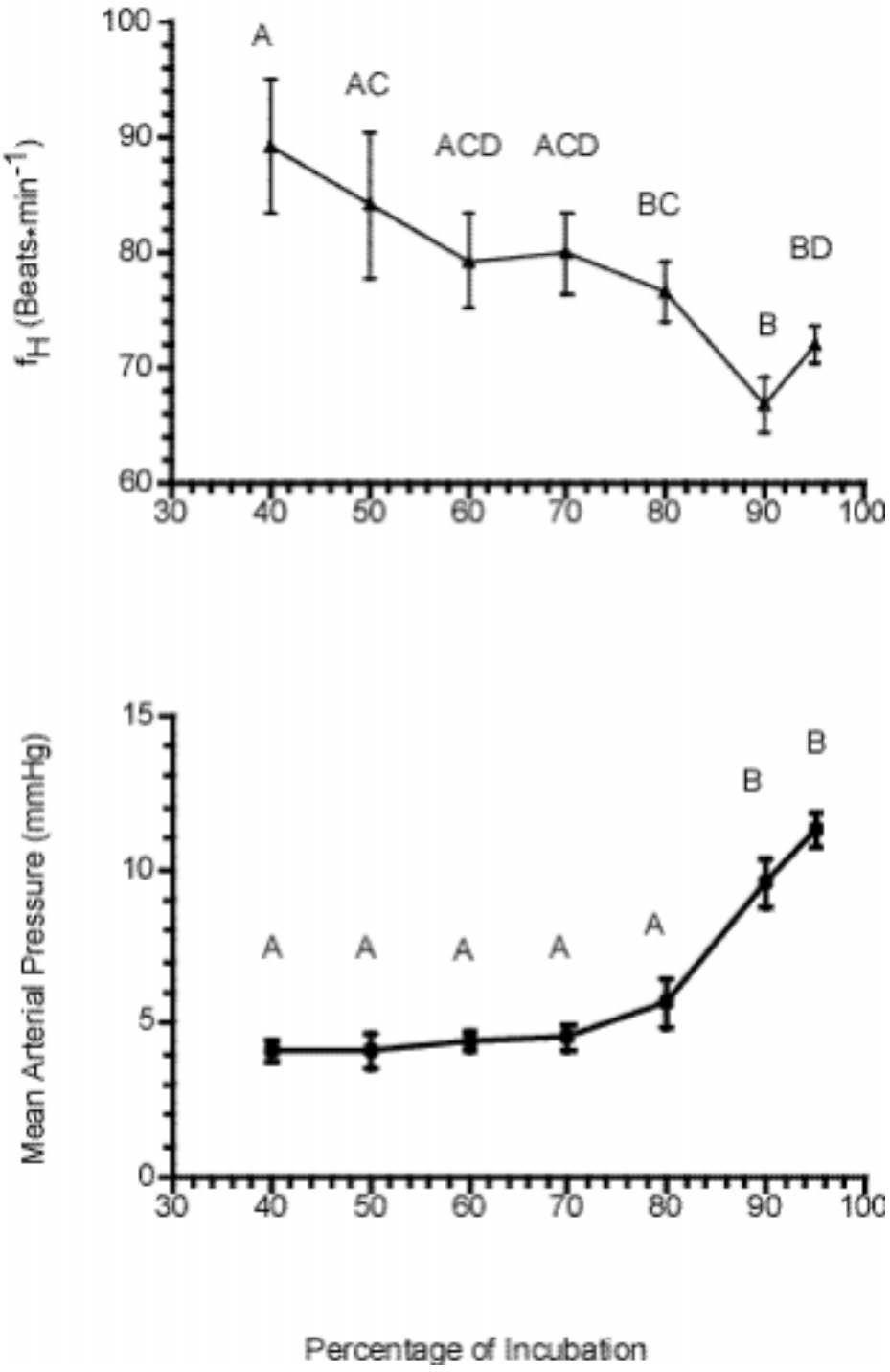

100
90
80
70
60
f_H (Beats·min^{-1})
A
AC
ACD
ACD
BC
BD
B
30
40
50
60
70
80
90
100
15
10
5
0
Mean Arterial Pressure (mmHg)
A
A
A
A
A
B
B
Percentage of Incubation

Figure 8.3

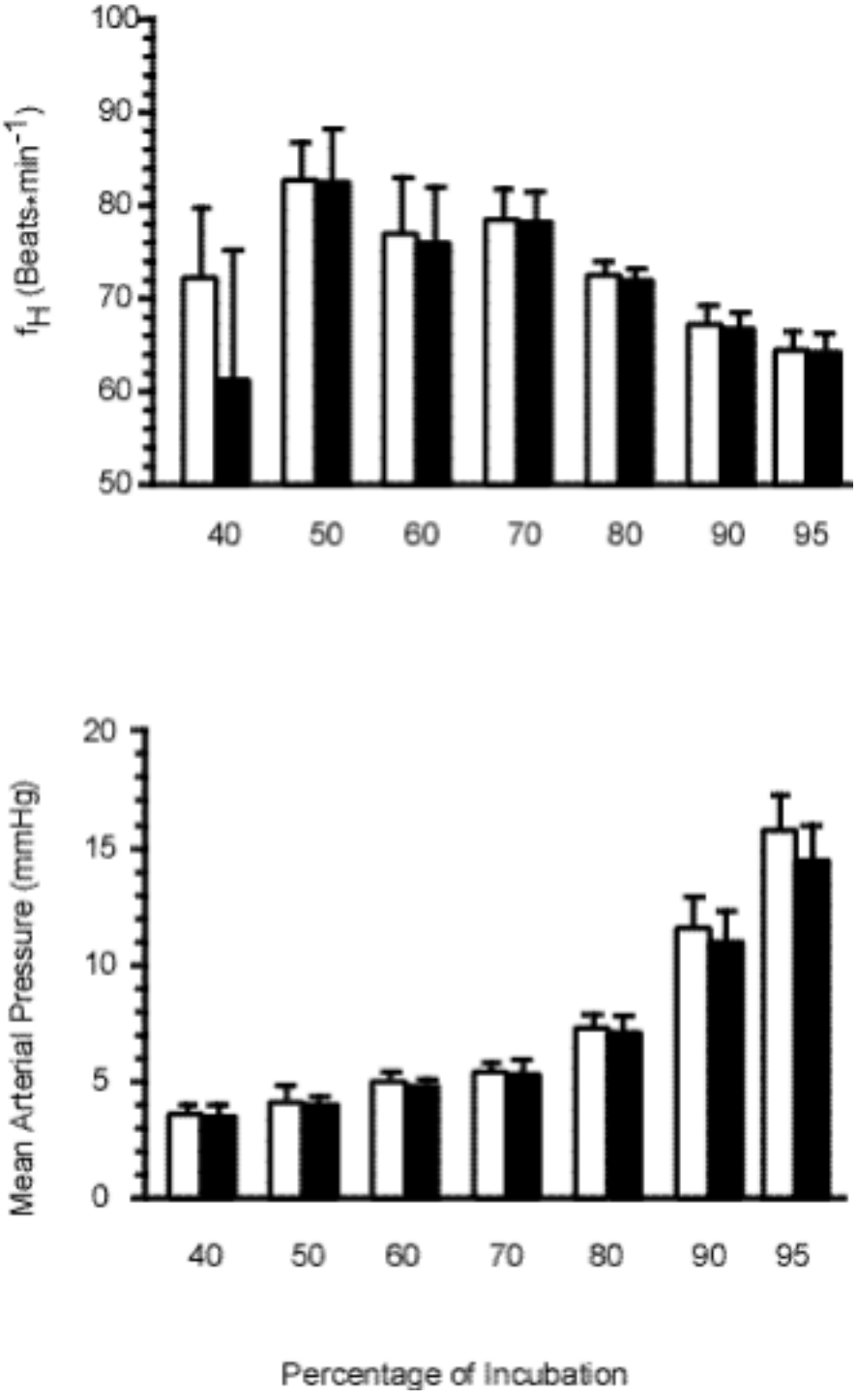

100
90
80
70
60
50
f_H (Beats·min^{-1})
40
50
60
70
80
90
95
20
15
10
5
0
Mean Arterial Pressure (mmHg)
40
50
60
70
80
90
95
Percentage of Incubation

Figure 8.4

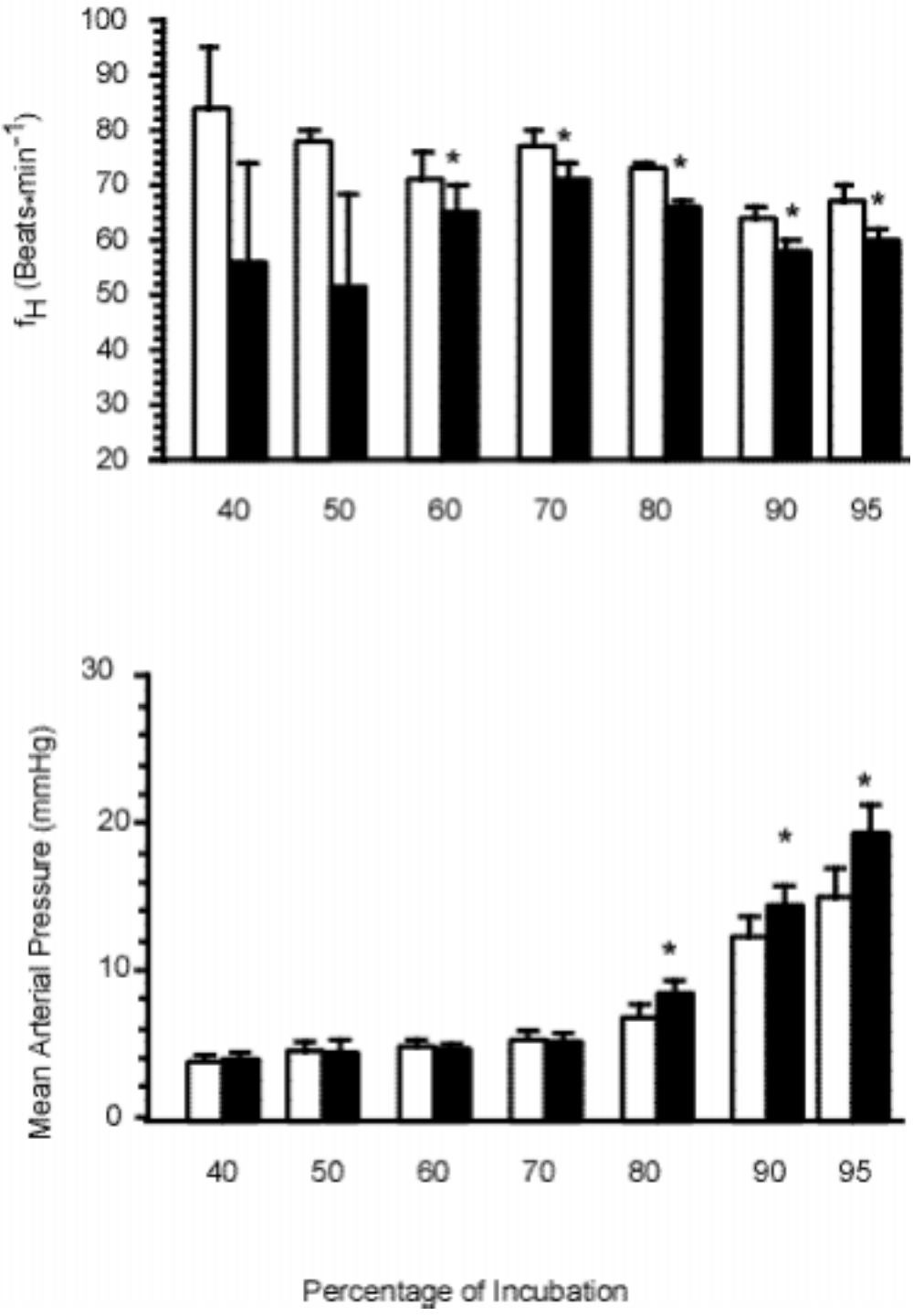

100
90
80
70
60
50
40
30
20
f_H (Beats•min^{-1})
40
50
60
70
80
90
95
30
20
10
0
Mean Arterial Pressure (mmHg)
40
50
60
70
80
90
95
Percentage of Incubation

Figure 8.5

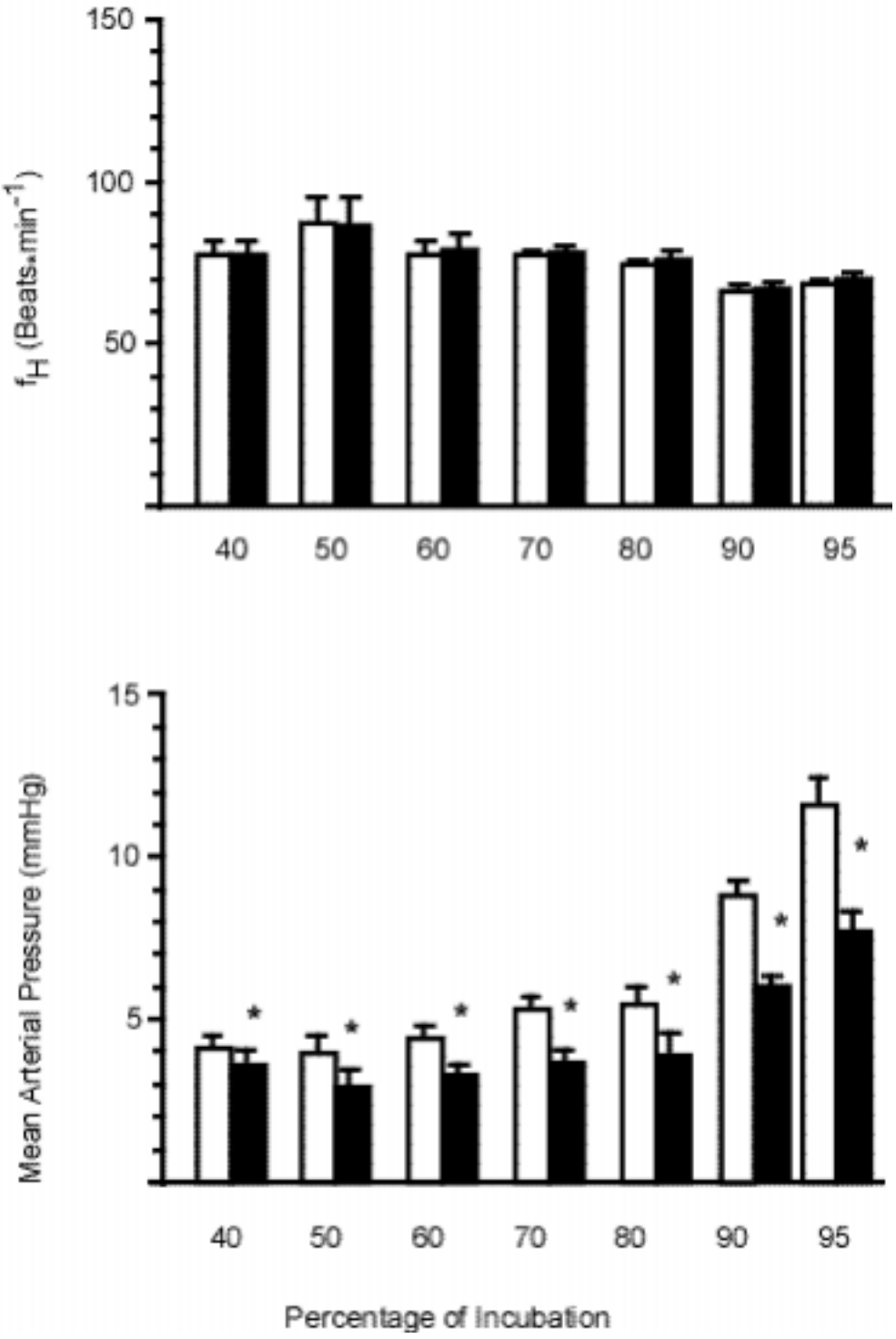
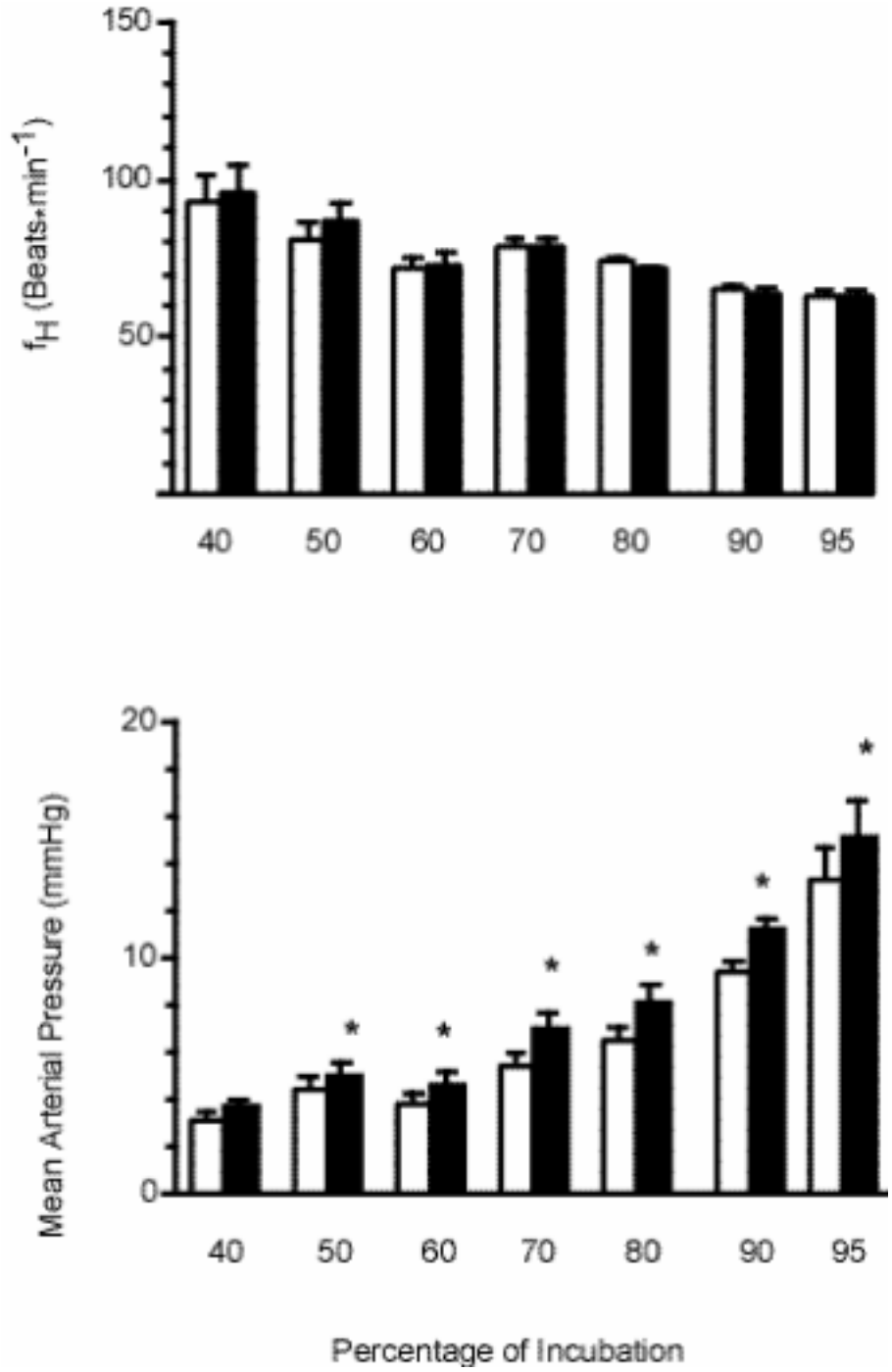

150
100
50
f_H (Beats.min^{-1})
40
50
60
70
80
90
95
15
10
5
Mean Arterial Pressure (mmHg)
*
40
50
60
70
80
90
95
Percentage of Incubation

Figure 8.6


150
100
50
f_H (Beats·min^{-1})
40
50
60
70
80
90
95
20
10
0
Mean Arterial Pressure (mmHg)
*
Percentage of Incubation

CHAPTER IX

CARDIOVASCULAR REGULATORY SYSTEMS IN AN AVIAN EMBRYO

Summary

Collectively, the findings compiled in these studies have characterized cardiovascular control mechanisms that are available to developing embryonic chickens. In addition, windows during which adult cardiovascular regulatory systems become operational were established. Embryonic chickens rely on circulating catecholamines to maintain cardiovascular function during intermediate development. Acute bouts of selective cardiovascular stress produce a general hemodynamic compromise, to which intermediate embryonic chickens are unable to respond. While the potential for regulatory contributions from other humoral or local mechanisms during development stress remains, it was clear that autonomic regulation is absent prior to day 18 of chicken incubation.

Sympathetically mediated changes in heart rate and arterial pressure were evident on day 18 of incubation, while the parasympathetic system remain inactive. This was surprising, given the pronounced role the parasympathetic system plays in basal cardiovascular function in adults. The predominance of adrenergic systems during development has been established in fetal sheep, as well as embryos of the desert tortoise suggesting that, in vertebrates,

cardiovascular maturation occurs without vagal tone. Hypoxic stress induced a pronounced depression of cardiovascular function, with changes mediated in part by sympathetic action as well as an augmented humoral catecholamine action on the cardiovascular system. Further, the baroreflex response begins to operate during late development of embryonic chickens. Therefore, a critical period exists between days 18 and 21, during which adult regulatory systems begin to function. Given some of the differences in hypoxic response exhibited by chickens in late embryonic development, there is a potential for regulatory mechanisms that are uniquely embryonic which are lacking in adult animals. These general patterns were also demonstrated in embryonic desert tortoise during development.

A comparative study of desert tortoise embryos illustrated similarities, as well as differences, to chicken maturation of cardiovascular function and regulation during development. Mechanically, embryos of the desert tortoise and domestic chicken differ dramatically during development. However, from the perspective of embryonic cardiovascular regulation the desert tortoise has a pronounced adrenergic tone, with the notable absence of vagal tone during embryonic development that is evident in chickens. While further cross taxa comparisons are needed to fully delineate the extent of similarities between these species, the lack of a vagal tone might represent a universal pattern for terrestrial egg laying vertebrates.

Future Studies

Studies presented in Chapters 2-8 have provided a necessary foundation for the development of a general model of embryonic cardiovascular regulation. They have also established a timeline during which adult systems become functional, as well as their patterns of maturation. The following investigations should provide the needed information to solidify the developmental model of cardiovascular regulation in terrestrial egg laying vertebrates.

Experiment 1.

Changes in peripheral resistance which accompanies documented cardiovascular responses to alteration of ambient gas composition must be determined in embryonic chickens. Several of the findings suggest that during hypoxic stress embryos utilize selective alteration of vascular tone within different organs to maintain oxygen delivery to vital systems. Simultaneous measurement of "umbilical" and systemic blood flow changes during hypoxia will provide important information on mechanisms that allow possible changes in cardiac output distribution.

Experiment 2.

Determination of plasma catecholamine levels pre- and post- exposure to a hemodynamic stress, will further delineate periods when catecholamine mobilization is the primary embryonic response mechanism. The current studies have provided evidence that a transition occurs between days 15 and 18 during which an adrenergic response to hypoxia becomes operational. Determination of plasma catecholamine levels would provide further support for this hypothesis.

Experiment 3.

Direct neural recording and stimulation will provide the data necessary to determine the role of centrally mediated regulation of cardiovascular function during chicken incubation. Prior work, including the studies presented here, utilized agonist and antagonist pharmacology to deduce potential periods when autonomic regulation of cardiovascular function becomes active. Vagal as well as sympathetic stimulation and recording will provide important data on the operation of these autonomic divisions during development.

Experiment 4.

These proposed investigations, coupled with previous work, will contribute substantially to the general model of neural control of embryonic cardiovascular function. Research utilizing selective blockade of other humoral and locally released vasoactive substances is needed to further characterize embryonic mechanisms of altering cardiovascular function. In fetal sheep, components such as angiotensin, vasopressin and prostaglandin's are important for fetal cardiovascular adjustments to hypoxia. Therefore, these substances could be important mechanisms utilized by embryonic chickens to maintain gas transport during bouts of altered ambient gas concentrations.

Experiment 5.

These, as well as completed studies should be repeated during the final 4 days of chicken incubation, in a time dependent manner. An intensive study of time windows within each of the final four days of chicken incubation will allow

the isolation of periods when adult systems become operational. Environmental manipulation studies during these windows will demonstrate the degree to which adult function is altered by challenge during these critical periods.

Experiment 6.

Completion of these studies will further our understanding of cardiovascular regulation in embryonic chickens. However, due to selective breeding and domestication of *Gallus gallus* findings might be limited in their application to other egg laying vertebrates. Thus, additional bird species with different degrees of out of egg development should be tested to determine species which exhibit different developmental patterns and the possible causes which underlie them.

Closing

This collection of studies has filled an important gap in our understanding of cardiovascular regulation in developing vertebrates other than mammals. Further, essential information has been produced that will allow future study to established the contributions of specific cardiovascular regulatory systems during embryonic development. One basic assumption, while not specifically addressed by individual investigations, was collectively addressed in this text. That assumption was, embryos will respond to stress conditions by altering cardiovascular function, as well as other actions, in an effort to tolarate the period of stress. This appears to be an incorrect assumption during early chicken development. Embryonic chickens, prior to day 18, either tolarate periods of

environmental challenge or the embryo dies. In either case an embryo is unable to alter function of the cardiovascular system via central or adrenergic mechanisms to improve chances of survival. Further, study is needed to determine if some, as yet undetermined, system is activated during periods of stress which provides some benefit to developing embryonic chickens.

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