UNIVERSITY OF CALIFORNIA,
IRVINE

Plasticity Along the Cardiac-Gastrointestinal Axis in Carnivorous Reptiles

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

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2015

DEDICATION

To Mom

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ACKNOWLEDGMENTS

I would be foolish not to begin by thanking the Boss Man, Jim Hicks, who supervised this work. Thank you, Jim, for the many hours you spent demonstrating that being a successful scientist requires, in addition to scientific expertise, a robust network of collaborators and friends, involvement in the right academic societies, political savvy, frugality, and the ability to avoid awkward conversations with those much smarter than you by talking about sports (I just wish I knew something about basketball). I'm thankful for the opportunities you gave me to work with your collaborators and friends, and I appreciate the money you earmarked over the years to support my projects. Thank you for teaching me an incomprehensible amount of comparative cardiorespiratory physiology, a handful of surgical techniques, and that drinking akvavit after a dissertation defense will result in falling asleep in a bathroom sink.

In addition to Jim, I owe many thanks to the current and former members of the Hicks Lab with whom I was fortunate enough to interact: Jason Blank, Tomasz Owerkowicz, John Eme, June Gwalthney, Ashley Ossher, Alex Jackson, and Corey Jew. Thank you for, in addition to the general exchange of ideas, maintaining institutional memory, specifically as it relates to using rarely-utilized equipment and finding stuff I'd lost.

Thank you to my graduate advisory committee members, Dr. Timothy Bradley and Dr. Matthew McHenry, who, like all faculty in UCI's Center for Organismal Biology, consistently provided feedback with the appropriate mixture of candor and encouragement. Thank you to the remainder of my advancement committee, Dr. Donovan German, who has always provided, without hesitation, access to his ideas and his lab (sorry about the hole I burned in your floor), and Dr. Sabee Molloi, who spent considerable time and resources encouraging non-invasive imaging of non-model organisms.

Thanks to my fellow graduate students in Eco Evo for too many priceless exchanges to recall. Specifically, to the members of my cohort, Drs. Batmalle, Cabral, Gilbreath, Jackson, Kivlin, Stewart, and Zimmerman: I appreciate your friendship and admire your accomplishments.

I am tremendously thankful for an army of Danes who were critically-important in facilitating Chapter 1 of this dissertation. Many thanks to Dr. Tobias Wang, for opening his lab, his home, and many bottles of booze for me, and for putting me in contact with Sanne Enok, an intense coauthor and friend who was supposed to "show [me] where things are" in Tobias's lab and then spent several hours a day for 2 months helping me finish experiments. I appreciate the technical expertise and bravery of Heidi Jensen and Rasmus Buchanan, and assistance from Kasper Hansen and Henrik Lauridsen.

I'd like to thank Dr. Dane Crossley who allowed me to work with him in the summer of 2011, and provided valuable training and experience in developmental physiology, embryology and pharmacology. Thanks as well to his former graduate students, Dr. Kevin Tate and Dr. Zac Kohl, colleagues with whom I look forward to sharing a career worth of scientific discoveries,

and with whom I've probably already shared a (shortened) lifetime's worth of terrible beer, Meatloaf, etc.

Thank you to Dr. Ruth Elsey of the Louisiana Department of Wildlife and Fisheries, who, despite promising me it was impossible, delivered an abundance of yearling alligators (facilitating Chapter 2 of this dissertation), and provided impeccable hospitality and kindness during visits to Rockefeller Wildlife Refuge.

Speaking of animals, I acknowledge the contributions of all the animals that were sacrificed in these studies. Their lives benefitted our knowledge of comparative physiology and my own professional development. All animals were treated in accordance with Danish Federal Regulations and UCI Institutional Animal Care and Use Committee protocols 1999-2123, 2009-2876, and 2010-2921.

Research was supported by the National Science Foundation [grants IOB 0445680 and IOS 0922756 to James W. Hicks, a Graduate STEM Fellowship in K-12 Education (DGE-0638751), and a Graduate Research Fellowship to me] and the Danish Research Council (to Tobias Wang). Additional travel funds (supporting travel to Denmark for completion of Chapter 1) were provided by the Society for Integrative and Comparative Biology's Fellowship for Graduate Student Travel. I received financial support from the Department of Ecology and Evolutionary Biology at UCI.

The text of Chapter 1 of this dissertation is a reprint of the material as it appears in *The Journal of Experimental Biology* and is reproduced with permission from The Company of Biologists, Ltd. Two coauthors, Dr. James W. Hicks and Dr. Tobias Wang directed, supervised, and supported this research and we again acknowledge the many contributions of co-author Sanne Enok to the research. This research forms the basis for the first chapter of this dissertation.

* * *

On a personal note, I want to thank my wife, Holly, who is among the most compassionate and supportive people on Earth. Her excellence in her career as a neonatal intensive care unit nurse has left her well-suited to deal with the eccentricities of a frazzled, early-career academic. I'm thankful for her love and support.

I'm also unbelievably lucky to have been raised by two immensely-supportive parents who have motivated my success. My late mother, to whom this dissertation is dedicated, quite literally taught me to read and write (as my kindergarten teacher), and my father who, in addition to fetching all the baseballs I threw over his head, taught me the art of revision (bring it on, referees!). I'm grateful for the support of my brother, Matthew, who from the moment he could speak wanted to be an organismal biologist – a goal I only adopted during my undergraduate career.

I hope I've made all of these individuals proud.

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ABSTRACT OF THE DISSERTATION

Plasticity along the cardiac-gastrointestinal axis in carnivorous reptiles

By

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Doctor of Philosophy in Ecology and Evolutionary Biology

University of California, Irvine, 2015

Professor James W. Hicks, Chair

Digestion of meals and assimilation of nutrients are energetically-costly endeavors for all animals, and especially for intermittently-feeding ectothermic predators. The costs of digestion are attributable to a suite of aerobically-demanding processes, and interactions of digestive organs with the functionally and anatomically diverse hearts of non-avian reptiles have been hypothesized to support these processes. Specifically, postprandial cardiac hypertrophy is thought to increase convective oxygen transport to meet the aerobic demands of digestion and assimilation, and the right-to-left shunt (R-L) of crocodilians is thought to aid in acid secretion and provision of metabolic substrates.

In Chapter 1, I examined the renowned rapid postprandial cardiac hypertrophy of Burmese pythons, predicting that because it likely exists to augment oxygen delivery during digestion, it should be triggered by an oxygen supply-demand mismatch. To test this prediction, I experimentally manipulated the magnitude of the O_2 supply-demand mismatch by rendering animals anemic prior to feeding. I argued, because we found cardiac hypertrophy only in this group of manipulated animals, that an autonomically-mediated response to a "low O_2 signal" resulted in altered postprandial hemodynamics and subsequent hypertrophy.

In Chapter 2, I utilized a similar experimental protocol on alligators to determine whether they normally exhibited postprandial cardiac hypertrophy, and if not, whether it could be artificially induced by rendering animals anemic prior to feeding. I found only modest postprandial increases in cardiovascular function, enlargement of only stomach and liver in fed animals, and no other differences in visceral organ mass. I argued that there are neither down-regulations following fasting nor up-regulations following feeding of most visceral organs in alligators.

In Chapter 3, I tested the hypothesis that the R-L shunt of crocodilians facilitates rate of net energy gain in crocodilians. Despite significant changes in ventricular hemodynamics and a near doubling of ventricular mass in animals with surgically-occluded left aortae, I found no consequences of elimination of the shunt. This and other findings support the idea that the R-L shunt is an evolutionary remnant which has never been selected against.

Together these results illustrate the complex and plastic interactions between the digestive and circulatory systems.

INTRODUCTION

All organisms must obtain energy from the environment to facilitate physiological processes related to growth, homeostasis, behavior, and reproduction. For animals specifically, acquisition of energy is accomplished through the sequential processes of feeding, digestion, and assimilation of nutrients. This suite of processes is, itself, energetically demanding, and animals employ a diversity of strategies dependent on natural history, ecological niche, and thermal biology to acquire and assimilate food while maximizing net energy gain. Among these strategies, the approach utilized by ectothermic sit-and-wait predators is particularly dramatic.

These predators, most famously snakes, can endure prolonged fasts upwards of months at a time during periods of low prey availability ("starvation"; e.g. Benedict, 1932; Wang et al., 2006; McCue, 2007). During this fasting period, adaptive down-regulation of digestive and accessory digestive organs occurs, serving primarily to reduce energy consumption (e.g. Secor, 2000). When prey becomes available, however, animals may feed voraciously, consuming meals equivalent to 25% of their body mass or more (e.g. Greene, 1983; Secor and Diamond, 1997), and when considering the extremely low standard metabolic rates (SMR) of these animals, the energy content of large meals may represent several hundred times an animal's daily energetic requirement (Benedict, 1932). Digestion of such a sizeable meal, both in terms of mass and energy content, is a costly endeavor.

Upon feeding in previously-fasted snakes, metabolic rate can increase by more than 40-fold (though more modest responses are more typical), and remain elevated for several days (Secor and Diamond, 1997; Andrade et al., 2005; McCue, 2006; Secor, 2009). This increase in metabolic rate is attributable to energetic costs associated with preabsorptive (e.g. swallowing

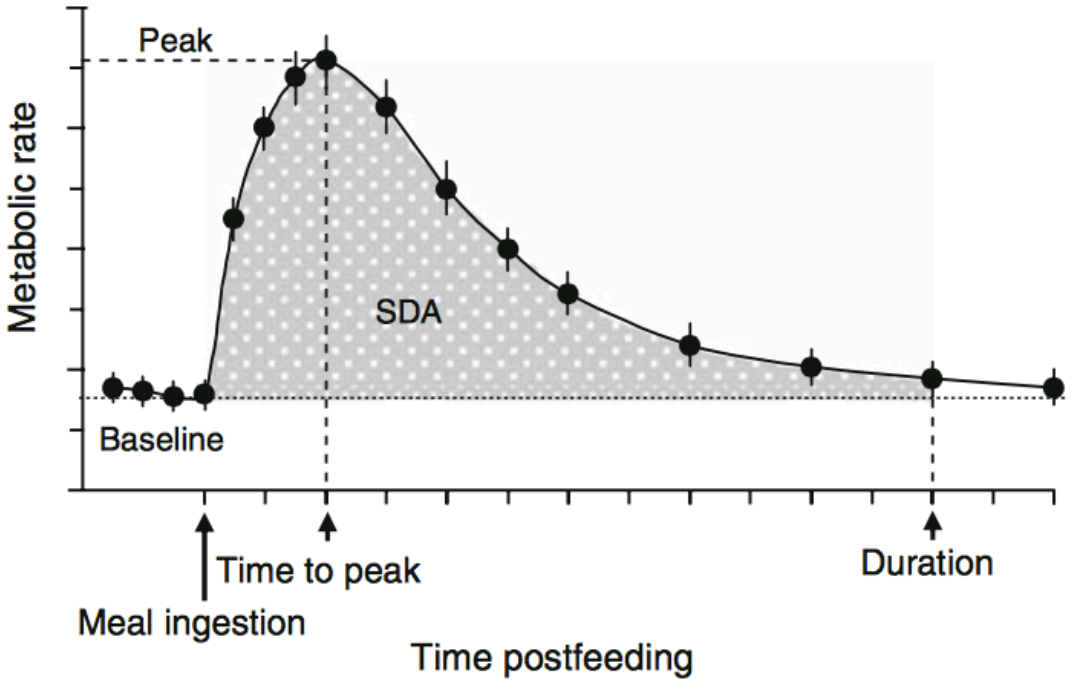

Figure I.1. Typical specific dynamic action (SDA) curve. This plot of metabolic rate vs. time illustrates the stereotypical shape of the metabolic rate vs. time curve of a digesting animal. While the general shape of the curve is similar among vertebrates, the "scope" or ratio between "peak" and "baseline" values, differs widely between vertebrates, with ectothermic sit-and-wait predators often exhibiting scopes an order of magnitude greater than birds and mammals. This is a consequence of the higher "baseline" of endothermic animals and of the relative meal size consumed by carnivorous reptiles. From Secor (2009).

and secretion of acid), absorptive (e.g. transepithelial transport of nutrients) and postaborptive (e.g. assembly of new biomolecules) processes (Andrade et al., 2005; McCue, 2006; Secor, 2009). While the relative energetic demands of individual digestive processes remain subject to debate (e.g. Overgaard et al., 2002; Secor, 2003; Enok et al., 2013), it is clear that this energy demand is met aerobically (Overgaard et al., 1999; Busk et al., 2000), and the postprandial period is therefore also characterized by an immediate and prolonged increase in oxygen consumption ($\dot{V}O_2$). The increase in postprandial metabolic rate and $\dot{V}O_2$ over resting values is termed specific dynamic action (SDA), and follows a similar pattern in all animals (Andrade et

al., 2005; McCue, 2006; Secor, 2009, Figure I.1), though the duration and scope are dependent largely on SMR, feeding frequency, and meal size. In Burmese pythons (*Python bivittatus*), which have been termed a "model of extreme physiologic regulation" due to the physiologic responses to their prolonged fasts and voracious feeding, peak postprandial $\dot{V}O_2$ can meet or exceed peak $\dot{V}O_2$ during exercise and may therefore represent the maximal rate of oxygen delivery and consumption, $\dot{V}O_{2max}$ (Secor and Diamond, 1998; Secor et al., 2000; Figure I.2.). Clearly, digestion in intermittent, sit-and-wait predators represents a perturbation to oxygen homeostasis. This occurs in addition to perturbations in acid-base balance (see Wang et al., 2005).

Anatomical features or physiological mechanisms which aid in resolving these perturbations or otherwise increasing the net rate of energy gain should be considered adaptive responses if they ultimately increase an animal's fitness (Lauder 1996; Secor, 2001). In addition to adaptive responses of digestive and accessory digestive organs (Secor, 2001; Ott and Secor, 2007), a number of cardiorespiratory mechanisms are hypothesized to facilitate or enhance digestion and assimilation by affecting the quantity or "quality" (i.e. content) of blood flow to the gut. Metabolically-active digestive organs require blood both for acquisition of oxygen and transport of nutrients and substrates around the splanchnic bed and beyond. The response to digestion in vertebrates is therefore unsurprisingly characterized by a gastrointestinal hyperemia, and this response is particularly pronounced in reptiles. Estuarine crocodiles (*Crocodylus porosus*), for example, experience a near doubling of gastrointestinal blood flow during digestion, and Burmese pythons exhibit a greater than 10-fold increase in flow through both the mesenteric artery and hepatic portal vein (Axelsson et al., 1991; Secor and White, 2010). In addition to a general redistribution of flows as directed by autonomic and nonadrenergic,

noncholinergic factors (Wang et al., 2001), increased cardiac output can serve the gastrointestinal hyperemia.

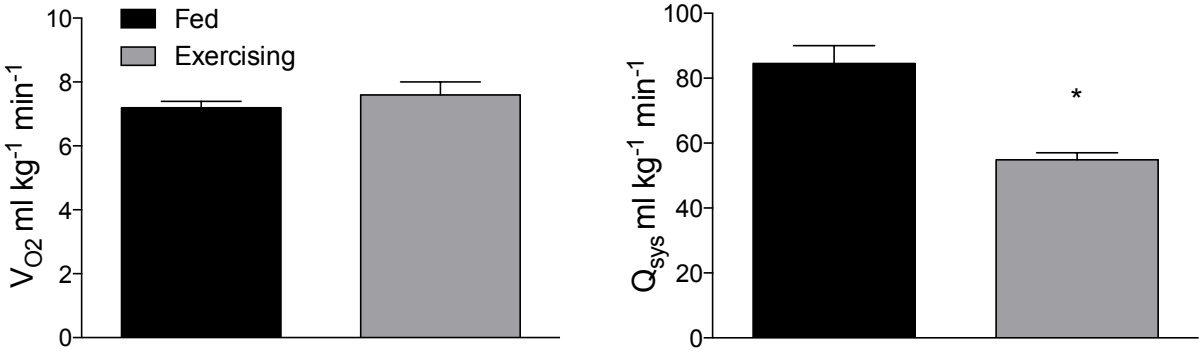

Figure I.2. Metabolic and cardiovascular variables in fed and exercising Burmese pythons (*Python bivittatus*). Peak metabolic rate (oxygen consumption as determined via respirometry, left) is similar for pythons during digestion (black bars) and activity (grey bars). Peak cardiac output (Q_{sys}, right) is higher in digesting animals than active animals. This indicates that the metabolic demands of exercise and digestion are similar in Burmese pythons, and cardiac output is presumably elevated to meet both the aerobic demands of digestion as well as the demand for nutrient transport (gastrointestinal hyperemia). Redrawn from Secor et al. (2000).

The increase in postprandial cardiac output has been well-described in Burmese pythons (Secor et al., 2000; Secor and White, 2010) with a nearly-immediate increase in flow which persists for several days. Augmenting this elevated flow is a pronounced cardiac hypertrophy, which can occur astonishingly quickly (within 48 h), is of impressive magnitude (a ~40% increase in heart mass), and is reversible (atrophies within 28 days) (Andersen et al., 2005). This hypertrophy, despite its rapid onset and impressive magnitude, is functionally and molecularly similar to physiologic cardiac hypertrophy in mammals, which is healthy, "adaptive," reversible, and serves to augment convective oxygen transport and $\dot{V}O_{2max}$ (Hill and Olson, 2011; Riquelme et al., 2011; Wagner, 1996). Postprandial cardiac hypertrophy in Burmese pythons is likely an adaptive mechanism to facilitate gastrointestinal hyperemia, but due to its sporadic occurrence, has been labeled a facultative rather than obligatory response to digestion (Jensen et al., 2011).

While circulating free fatty acid concentration has been shown to induce postprandial cardiac hypertrophy (Riquelme et al., 2011), it remains unclear which other "triggers" and in which other animals postprandial cardiac hypertrophy might occur.

In addition to postprandial cardiac hypertrophy, which should be considered a plastic response, the unique cardiovascular morphology of reptiles may also inherently affect the quantity or quality of flow to the gut (Fig. I.3.). While the mammalian and avian cardiovascular arrangements result in fully-divided pulmonary and systemic circulations, due to varying degrees of functional and anatomical separation of the ventricle and the presence of the dual aortic arch system, the hearts of non-avian reptiles are capable of differentially distributing blood between the pulmonary and systemic circuits (see Hicks, 1998). During shunting, O_2-rich blood returning from the lungs can be immediately returned back to the lungs, bypassing the systemic circulation (a left-to-right "systemic bypass" shunt), or O_2-poor blood returning from the systemic circulation can be immediately returned back to the systemic circulation, bypassing the lungs (a right-to-left "pulmonary bypass" shunt). Consequently, shunting can result in altered blood composition (specifically arterial O_2, CO_2, H^+, and HCO_3^-) and altered distribution of flow (see Hicks, 1998; Hicks, 2002; Hicks and Wang, 2012)

Despite a century's worth of hypotheses proposing a functional advantage of L-R and/or R-L shunting, almost no convincing experimental evidence exists to support these hypotheses (e.g. Hicks and Wang, 2012). One intuitively-appealing hypothesis with limited experimental support is the idea that blood shunted past the lungs is relatively high in CO_2, which can be used to augment secretion of stomach acid, and is high in HCO_3^-, which is an important substrate for anabolic processes (Jones and Shelton, 1993; Farmer et al., 2008; Farmer et al., 2011). Additionally, this relatively hypercapnic blood may be preferentially delivered to the digestive

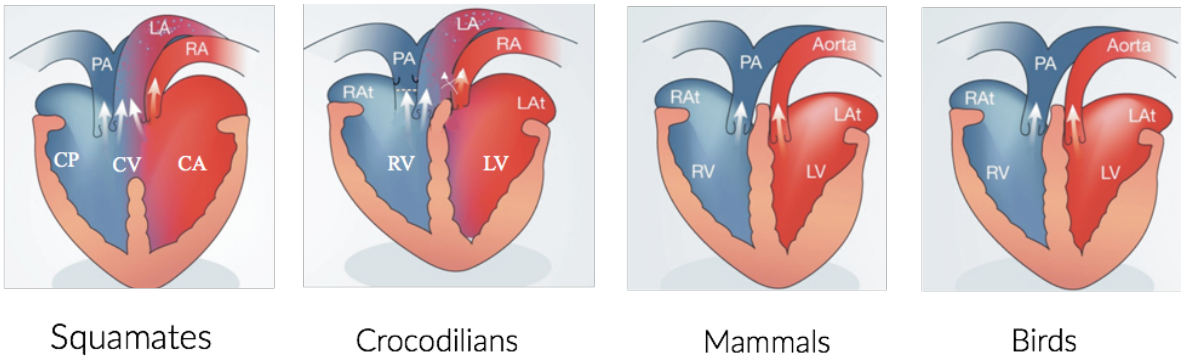

Figure I.3. Diversity of cardiac morphology among vertebrates. While the mammals and birds have completely divided ventricles, permitting complete separation of pressures and flows between the pulmonary and systemic circulations, all non-avian reptiles, including squamates (lizards and snakes) and crocodilians, can differentially distribute pulmonary and systemic flows, or "shunt." Shunting occurs due to the presence of the dual aortic arch system (both left aorta, "LA," and right aorta, "RA," above) in all non-avian reptiles, and due to varying degrees of anatomical and functional septation in squamates. Because crocodilians have a completely divided ventricle, they are a useful model for determining the functional advantage of the R-L shunt, because the left aorta (LA/LAo) can be surgically occluded, abolishing the shunt. Modified from Summers (2005).

tract due to specialized vascular anatomy. This idea is appealing, considering the massive complex meals that carnivorous reptiles are capable of consuming, and is, fortunately, testable due to the complex cardiovascular morphology of the crocodilians. Because crocodilians possess a fully-divided ventricular septum, the R-L shunt can be removed via occlusion of the left aorta, the major outflow tract returning desaturated blood to the systemic circulation. Using this approach, Farmer et al. (2008) have shown that rates of bone demineralization are faster in animals that possess the capacity to shunt as compared to those that do not. These results conflict, somewhat, with the reports of Eme et al. (2010) and Jones and Gardner (2010), who report that neither rates of growth nor food consumption are affected by abolition of the R-L shunt. It is unclear, then, whether the R-L shunt is critically important to the rate of energy gain in reptiles.

It is clear, however, that the cardiovascular response to digestion in intermittently-feeding reptiles exists as a complex and adaptive interaction between evolutionarily-derived morphology, active regulation of hemodynamic parameters, and phenotypic plasticity. While many questions regarding adaptive interactions along the cardiac gastrointestinal axis have been addressed in recent years by a (surprisingly small) number of research groups, the unanswered questions are among the most significant in the field of comparative cardiorespiratory physiology (Burggren et al., 2014). This dissertation seeks to address what triggers postprandial cardiac hypertrophy in Burmese pythons (Chapter 1) and other intermittently-feeding reptiles (Chapter 2), to what extent visceral organ size and function are affected by prolonged fasts (Chapter 2), and whether the R-L shunt of reptiles is an adaptive trait, specifically as it relates to the rate of energy acquisition (Chapter 3). These studies shed light on the remarkable complexity of the reptilian cardiovascular system.

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CHAPTER 1:

Reduction of blood oxygen levels enhances postprandial cardiac hypertrophy in Burmese python (*Python bivittatus*)

INTRODUCTION

Burmese pythons (*Python bivittatus*) (Kuhl, 1820), like many large snakes, utilize an intermittent 'sit-and-wait' feeding strategy, where prolonged fasts are punctuated by brief and voracious feeding bouts when prey is available. Digestion of these large meals (up to 25–100% of their body mass) is associated with pronounced upregulation of a suite of digestive functions and a large postprandial increase in oxygen uptake ($\dot{V}O_2$), termed specific dynamic action (SDA), where $\dot{V}O_2$ may exceed that during aerobic activity and last for several days (Benedict, 1932; Secor and Diamond, 1995; Secor and Diamond, 1997; Secor and Diamond, 1998; Secor, 2008; Cox and Secor, 2008; Secor et al.., 2000b; Wang et al.., 2001b). To support the high $\dot{V}O_2$ during digestion cardiac output increases drastically above resting values through a combination of increased stroke volume and heart rate (fH) (Secor et al.., 2000a; Secor and White, 2010). This hemodynamic response is mitigated largely by a reduction in cholinergic tone and positive chronotropic effects of non-adrenergic, non-cholinergic (NANC) factors, including an increased histaminergic tone (Wang et al.., 2001a; Skovgaard et al.., 2009; Enok et al.., 2012; Enok et al.., 2013; Burggren et al.., 2014).

The rise in stroke volume has been linked with a 40% increase in ventricular mass within 48 h of eating (Andersen et al.., 2005) that Riquelme et al.. described as being 'physiological' in nature and triggered by humoral factors, including increased levels of circulating free fatty acids (Riquelme et al.., 2011). The universality and stimulus of the postprandial cardiac hypertrophy, however, remain unclear as Jensen et al.. found no postprandial cardiac hypertrophy in Burmese

or Ball pythons (Python regius) under a similar experimental protocol (Jensen et al.., 2011). They argued, therefore, that postprandial cardiac hypertrophy should be considered a 'facultative' rather than 'obligatory' response to feeding (Jensen et al.., 2011), and the lack of postprandial cardiac hypertrophy was recently reported in two additional studies (Hansen et al.., 2013; Enok et al.., 2013).

The correlation between the magnitudes of SDA and postprandial cardiac hypertrophy is not well understood (Jensen et al.., 2011). Thus, while $\dot{V}O_2$ consistently increases following feeding, the postprandial cardiac hypertrophy is inconsistent. We hypothesize that postprandial cardiac hypertrophy is triggered when systemic metabolic demand outpaces systemic oxygen delivery. To investigate this hypothesis, we established an oxygen supply–demand mismatch in postprandial pythons by rendering specimens anemic prior to feeding, with the prediction that anemic pythons would exhibit greater postprandial cardiac hypertrophy than fasted pythons with normal blood oxygen levels.

MATERIALS AND METHODS

Animal acquisition and husbandry

Burmese pythons (*Python bivittatus*; N=31) of both sexes were acquired from commercial vendors and housed for several months prior to experimentation at the vivarium facilities of Aarhus University or the University of California, Irvine. Animals ranged from 0.24 kg to 11.5 kg with a mean body mass of 1.83±0.52 kg. Snakes were kept in individual vivaria at 27–30°C, and had access to heated surfaces that reached 32°C. A 12 h light:12 h dark photoperiod was maintained. All animals always had access to water, vigorously consumed rodent meals every 1–2 weeks, and gained mass during captivity. All snakes were fasted for a

minimum of 28 days prior to experimentation. Animals were housed and treated according to Danish Federal Regulations and UCI IACUC protocol 2009-2821.

Surgical procedures

Snakes (27 of the 31) were instrumented with arterial catheters for measurement of MAP and f_H, as well as for withdrawal of arterial blood samples to determine blood C_{O_2} and blood pH. To induce anesthesia, individual snakes were placed in a sealed container containing gauze soaked in isoflurane (Baxter, Allerød, Denmark) until they lost muscle tone and could be intubated for artificial ventilation with 2% isoflurane at 5 breaths min^{-1} and 50 ml kg^{-1} tidal volume, using a vaporizer (EZ-155, EZ Systems, Bethlehem, PA, USA) and an HI 665 Harvard Apparatus respirator (Holliston, MA, USA). A 5 cm incision close to the cloaca enabled the dorsal aorta to be accessed by blunt dissection, so a catheter (PE-50) containing heparinized saline (50 IU ml−1) could be inserted and externalized via a small cutaneous puncture and secured to the skin with 2-0 braided silk suture. Approximately 0.15 ml of whole blood was then withdrawn from the catheter to determine Hct by spinning the blood in glass capillaries for 3 min at 12,000 rpm.

A subset of 14 randomly selected snakes was rendered ‘anemic’ (see discussion of experimental groups, below) by withdrawing blood while the snakes were still anesthetized. Aliquots of 10% of the estimated blood volume (6–7% of body mass) (Lillywhite and Smits, 1984) were placed in sterile 1.5 ml Eppendorf tubes and centrifuged at 6000 rpm for 5 min. The supernatant plasma was returned via the arterial catheter. Hct was remeasured 15 min after reinjection of plasma and the process was repeated until Hct was reduced to ~10% (mean 10.1±0.3%).

The snakes were ventilated with room air until they regained muscle tone and resumed spontaneous ventilation. They were then returned to their enclosures, given access to water, and placed in a 30°C temperature controlled chamber. Animals were allowed to recover from surgery undisturbed in their enclosures for 24 h to ensure low plasma catecholamine levels (Olesen et al.., 2008).

Experimental and feeding protocols

Following the 24 h recovery period, we measured MAP and f_H, from each snake while they remained minimally disturbed in the climactic chamber. The catheters were connected to pressure transducers (PX600, Baxter Edwards, Irvine, CA, USA) calibrated with a vertical water column and connected to an in-house built amplifier sampling at 200 Hz (MP100 BioPac Systems, Inc., Goleta, CA, USA). MAP and f_H were analyzed over 5–10 min intervals.

Each animal was randomly assigned to one of four treatments: fasted control (N=8), fasted anemia (N=6), fed control (N=9) or fed anemia (N=8). Following the measurements of MAP and f_H, the 'fasted' animals remained undisturbed at 30°C, whereas 'fed' animals consumed rodent meals equivalent to 25±0% body mass. Contingent upon catheter patency, 48 h after recovery (72 h after surgery), MAP and f_H were obtained and the RPP was calculated (f_H x MAP) from each animal. Cardiac output and thus work was not measured, but we used the RPP as a proxy for myocardial work. From each animal, an arterial blood sample of ~0.5 ml was withdrawn to determine C_{O_2} (Tucker, 1967), blood pH (glass electrode maintained at 30°C and connected to a PHM 73; Radiometer, Copenhagen, Denmark) and Hct. Immediately after blood sampling, adrenergic and cholinergic tone were assessed by sequential infusion of atropine and

propranolol (see Enok et al.., 2012) and calculated from the standard equations, modified for use of f_H rather than R–R interval (e.g. Altimiras et al.., 1997):

$$\text{Chol}\ (\%) = \frac{\frac{1}{f_{H_{cont}}} - \frac{1}{f_{H_{atr}}}}{\frac{1}{f_{H_{dbl}}}} * 100 \qquad \text{(Eq. 1.1)}$$

and

$$\text{Adr}\ (\%) = \frac{\frac{1}{f_{H_{dbl}}} - \frac{1}{f_{H_{atr}}}}{\frac{1}{f_{H_{dbl}}}} * 100 \qquad \text{(Eq. 1.2)}$$

where $f_{H,cont}$ is the control heart rate, $f_{H,atr}$ is the heart rate following administration of atropine, and $f_{H,dbl}$ is the double-blocked heart rate (i.e. following administration of atropine and propranolol).

Tissue harvest

Immediately following assessment of autonomic tone, animals were killed via intraperitoneal injection of sodium pentobarbital (>100 mg kg−1) whereupon a long ventral incision allowed for the heart, liver, stomach, small intestine, large intestine and kidneys to be removed. All organs were rinsed with isotonic saline and blotted dry with gauze to remove blood and chyme before determining wet mass. A small representative sample was removed from each organ and weighed before and after it had been dried in an oven at 60°C for 72 h to determine the M_d:M_w ratio.

Statistical analyses

Mass-specific organ mass (g tissue per kg body mass), C_{O_2}, f_H and MAP data were compared using two-way ANOVA and post hoc Tukey's HSD in JMP statistical software

(Version 7, SAS Institute, Inc., Cary, NC, USA) following assurance of homogeneity of variance and normal distribution of data. Post hoc tests were performed only when the ANOVA yielded significance ($P \leq 0.05$), and were considered significant when $P \leq 0.05$. Hematocrit, adrenergic tone, cholinergic tone and $M_d:M_w$ were arcsin square-root transformed and compared using a two-way ANOVA in JMP. Effects, where reported, are the results of the effect tests conducted as part of the ANOVA model and are distinguished by the single degree of freedom. Regression plots were generated using GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA, USA) and slopes were analyzed using the software's linear regression analysis. Slopes of the regression lines were considered significantly different from 0 at the level of $P \leq 0.05$. All values are reported as means ± s.e.m.

RESULTS

Hematological parameters and blood gases

Our experimental procedure for rendering animals anemic resulted in significantly reduced blood oxygen carrying capacity (Table 1). At the time of sacrifice, anemic animals (both fed and fasted) exhibited 61% lower hematocrit (Hct) than controls ($F_{1,28} = 103.0$, $P < 0.0001$) and 53% lower arterial blood oxygen concentration (C_{O_2}) than control animals ($F_{1,19} = 27.8$, $P < 0.0001$). Among fed animals, C_{O_2} was significantly reduced in anemic animals as compared to control animals ($F_{3,19} = 9.4$, $P < 0.0001$), which was critical for testing the hypothesis. Arterial pH did not differ between anemic and control snakes and was not affected by digestion ($F_{3,21} = 1.15$, NS).

Table 1.1. Blood parameters in fasting and fed pythons.

	Fasted		Fed	
	Control	Anemic	Control	Anemic
C_{O_2} (mM)	3.24±0.84[a]	1.76±0.47[b]	3.94±0.24[a]	1.69±0.26[b]
Hct (%)	24.0±1.7[a]	7.4±0.5[b]	21.1±1.7[a]	9.9±1.0[b]
pH	7.49±0.08[a]	7.65±0.05[a]	7.60±0.09[a]	7.66±0.04[a]

C_{O_2}, arterial blood oxygen concentration.
Values with same superscript letters are not significantly different from one another.

Cardiovascular parameters

While manipulation of Hct alone did not significantly elevate the heart rate (f_H) of fasting snakes, feeding elicited significant increases in f_H among both anemic (50% increase) and control (78% increase) snakes. Coupling anemia with feeding, however, resulted in a 126% difference between fasted controls and fed anemic snakes (Figure 1A; $F_{3,20} = 9.2$, $P < 0.001$)

Mean arterial pressure (MAP) was 85% higher in fed controls than in fasted controls and 71% higher in fed anemic animals than in fasted controls (Figure 1B; $F_{3,20} = 5.9$, $P < 0.05$). As a consequence of the markedly elevated f_H and MAP, particularly in fed anemic snakes, the rate pressure product (RPP) was 2.9-fold higher in fed anemic snakes than in fasted controls (Figure 1C; $F_{3,20} = 6.2$, $P < 0.05$).

The changes in f_H were attended by changes in autonomic tone on the heart (Figure 2). Feeding alone elicited a 42% reduction in adrenergic tone among control animals, but the response was blunted in anemic animals, resulting in more modest 27% reduction. The greatest reduction in adrenergic tone was the 47% difference between fasted anemic snakes and fed control snakes (Figure 2A; $F_{3,16} = 10.3$, $P = 0.001$).

There were significant effects of digestive status ($F_{1,16} = 10.4$, $P < 0.05$) and Hct ($F_{1,16} = 5.0$, $P < 0.05$) on cholinergic tone (Figure 2B), with a modest difference existing between fasted

controls and fed controls (47%), and a more impressive difference between fasted controls and fed anemic animals (73%)(Figure 2B; $F_{3,16} = 8.1$, $P < 0.005$).

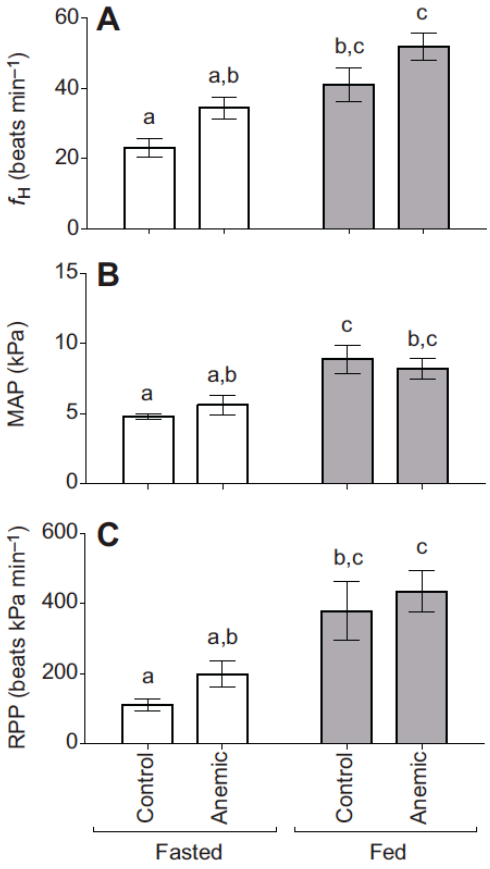

Fig. 1.1. Hemodynamic parameters in fasting and fed (48 h into digestion) Burmese pythons (*Python bivittatus*). Heart rate (A; f_H) was significantly higher in digesting snakes than in fasted controls, with a greater difference between fed anemic snakes and fasted controls. (B) Mean arterial blood pressure (MAP) was significantly higher in digesting snakes than in fasting control snakes, while anemia alone did not influence MAP. (C) The rate–pressure product (RPP, a proxy for cardiac work) was also significantly higher in digesting snakes than in fasted controls with a greater difference between fed anemic snakes and fasted controls. Groups with the same lowercase letter do not differ significantly. Data are presented as means ± s.e.m. Fasted controls, N=4; fasted anemic, N=5; fed controls, N=6; fed anemic, N=7.

The effect of feeding alone was significant in determining double-blocked f_H ($F_{1,17}$ = 16.9, $p < 0.005$) whereas Hct did not have a significant effect ($F_{1,17} = 2.0$, NS), but fed anemic animals had higher f_H than either group of fasted animals ($F_{3,17} = 6.9$, $p < 0.005$) (Figure 2C).

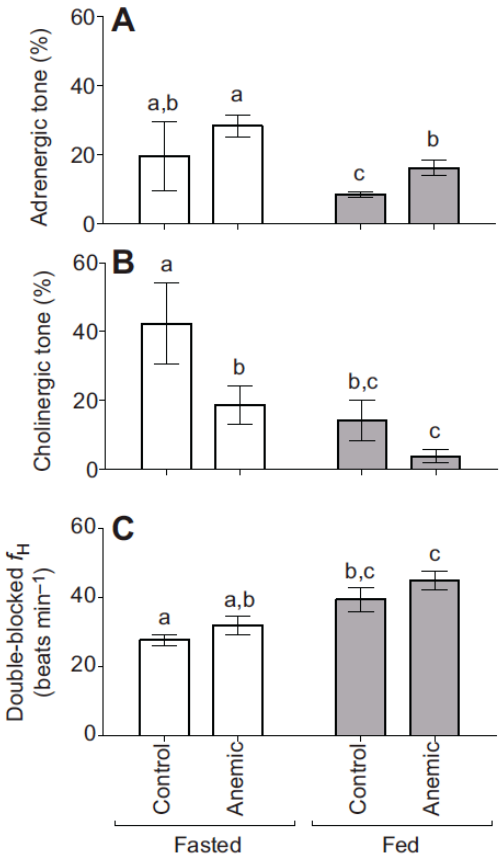

Fig. 1.2. Adrenergic and cholinergic cardiac tone in fasting and postprandial (48 h into digestion) Burmese pythons (P. bivittatus). Both cholinergic (A) and adrenergic (B) tone were lower in digesting snakes, and cholinergic tone was reduced during anemia in both fasting and digesting snakes. Double blocked f_H (C) was significantly higher in fed animals than in fasted controls, with no significant effect of anemia alone. Groups with the same lowercase letter do not differ significantly. Data are presented as means ± s.e.m. Fasted controls, N=3; fed controls, N=4; fasted anemic, N=4; fed anemic, N=7.

Cardiac hypertrophy

Heart mass of snakes with normal Hct did not increase during digestion, and anemia did not elicit cardiac growth in fasting snakes (Fig. 3A). However, the anemic snakes, 48 h into

digestion, had a ventricular mass of 1.8 g kg^{-1}, which is 39% larger than the ventricle of fasting snakes with normal Hct and 28% larger than the ventricle of fed animals with normal Hct. Thus, there was a significant difference ($F_{3,30}$=3.0, P<0.05) in ventricular wet mass between treatments. The effects of Hct on ventricular mass ($F_{1,30}$=5.3, P<0.05) were greater than the effects of digestion ($F_{1,30}$=2.6, NS). There was no significant difference in the dry mass:wet mass ratio (M_d:M_w) between treatments (Fig. 3B; $F_{3,30}$=0.7, NS). Total wet heart mass, i.e. combined

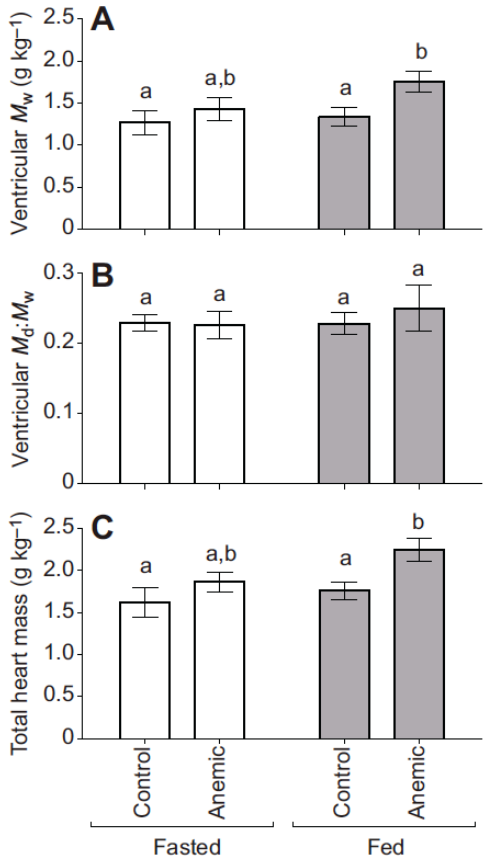

Fig. 1.3. Ventricular wet mass, dry to wet mass ratio and total heart mass in fasting and postprandial (48 h into digestion) Burmese pythons (*P. bivittatus*). Ventricular wet mass (A; M_w), was significantly higher in fed anemic snakes than both fasting control and fed control snakes, while there were no differences in dry to wet mass ratio (M_d:M_w; B). Total heart mass (C) was significantly higher in fed anemic snakes than in fasted and fed controls. Groups with the same lowercase letter do not differ significantly. Data are presented as means ± s.e.m. Fasted control, N=8; fasted anemic, N=6; fed control, N=9; fed anemic, N=8.

ventricular and atrial wet with a modest difference existing between fasted controls and fed controls (47%), and a greater difference between fasted controls and fed anemic animals (73%) (Fig. 3B; $F_{3,16}$=8.1, P<0.005).

Total wet heart mass, i.e. combined ventricular and atrial wet masses, also differed between treatments (Fig. 3C) ($F_{3,30}$=3.9, P<0.05), with fed anemic animals again having the largest hearts (38% larger than hearts of fasted controls and 22% larger than hearts of fed controls), but not significantly larger than hearts of fasted anemic snakes. Hct exerted a greater effect on total heart mass ($F_{1,29}$=7.3, P<0.05) than digestion ($F_{1,29}$=2.9, NS). Atrial wet mass was also 36% greater in fed anemic animals than in fasted controls ($F_{3,30}$=4.3, P<0.05), again, with a significant effect of Hct ($F_{1,29}$=8.8, P<0.05) but not feeding status ($F_{1,29}$=3.5, NS). There was no significant difference in atrial M_d:M_w between groups of animals ($F_{1,29}$=0.3, NS).

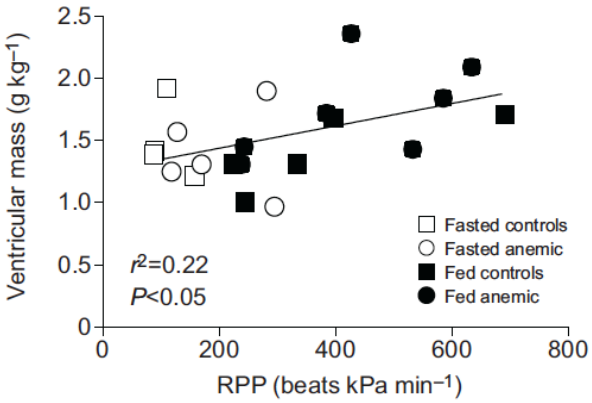

Fig. 1.4. Correlation between ventricular wet mass and RPP across all experimental groups (fasted control, fasted anemic, fed control and fed anemic) of Burmese pythons (Python bivittatus). The RPP is equal to the product of MAP and fH, and is a proxy for cardiac work.

We correlated ventricular mass with RPP, where this value estimates myocardial oxygen consumption and thus provides a proxy for cardiac work (Fig. 4). Ventricular mass was positively and linearly correlated with RPP ($P<0.05$, $R^2=0.22$).

Plasticity of the digestive organs

Stomach wet mass was significantly greater in fed control than in fasted control animals (Table 2; $F_{3,30}=5.2$, $P<0.05$), but there was no difference in stomach dry mass between groups ($F_{3,30}=2.4$, NS). Wet mass of the small intestine was also significantly larger in digesting snakes ($F_{3,30}=10.0$, $P<0.0001$), with similar trends for dry mass (albeit with no statistical difference between fasted anemic and fed control intestines; $F_{3,30}=16.0$, $P<0.005$). There were no significant differences in large intestine mass (wet $F_{3,30}=1.5$, NS; dry $F_{3,29}=2.3$, NS) or liver wet mass ($F_{3,30}=2.4$, NS), whereas liver dry mass differed significantly between groups ($F_{3,30}=16.5$, $P<0.005$). Fed anemic animals exhibited higher kidney wet mass than fasted controls (84% enlargement; $F_{3,30}=5.3$, $P<0.05$), but there were no significant changes in kidney dry mass ($F_{3,27}=1.7$, NS). While growth of the small intestine was due only to digestion ($F_{1,30}=27.1$, $P<0.0001$) and not Hct ($F_{1,30}=1.7$, NS), both digestion ($F_{1,30}=7.8$, $P<0.05$) and Hct ($F_{1,30}=7.1$, $P<0.05$) had significant effects on kidney wet mass.

DISCUSSION

Our study confirms that feeding alone does not elicit postprandial cardiac hypertrophy. Animals confronted with the simultaneous challenges of increased O2 demand (digestion) and reduced O2 supply (anemia) do, however, exhibit postprandial cardiac hypertrophy when compared with fasted, un-manipulated controls. This suggests that cardiac hypertrophy is

Table 1.2. Visceral organ masses

	Wet mass (g kg^{-1})				Dry mass (g kg^{-1})			
	Fasted		Fed		Fasted		Fed	
	Control	Anemic	Control	Anemic	Control	Anemic	Control	Anemic
Stomach	12.9±0.8^{a}	15.2±1.1^{ab}	17.7±2.2^{b}	17.0±1.2^{ab}	2.7±0.2^{A}	2.7±0.1^{A}	3.6±0.4^{A}	3.6±0.3^{A}
Small Intestine	14.5 ±1.4^{a}	17.1±0.8^{a}	25.4±3.2^{b}	29.6±1.7^{b}	2.8±0.3^{A}	3.0±0.1^{AB}	4.6±0.9^{BC}	5.9±0.4^{C}
Large Intestine	8.7±0.7^{a}	10.7±1.0^{a}	10.2±0.8^{a}	13.2±2.6^{a}	2.2±0.5^{A}	1.45±0.1^{A}	1.4±0.2^{A}	1.7±0.2^{A}
Liver	17.5±1.4^{a}	18.7±1.9^{a}	22.1±2.6^{a}	25.5±2.8^{a}	5.1±0.4^{A}	4.7±0.5^{A}	6.1±0.9^{AB}	8.2±0.8^{B}
Kidney	4.5±0.4^{a}	5.9±0.4^{ab}	6.0±0.6^{ab}	7.7±0.8^{b}	0.9±0.1^{A}	1.1±0.1^{A}	1.4±0.3^{A}	1.4±0.2^{A}

Values which share the same superscript letters (lowercase for wet mass and uppercase for dry mass) are not significantly different from one another.

triggered when oxygen supply/delivery cannot meet the elevated metabolic demands of digestion. Interestingly, cardiac mass of several other ectothermic vertebrates also responds to oxygen supply and demand mismatches, such as alligators reared in hypoxia (Warburton et al.., 1995; Crossley and Altimiras, 2005; Owerkowicz et al.., 2009) and fish rendered anemic (e.g. Sun et al.., 2009; Simonot and Farrell, 2007).

Our findings conflict with the previous reports of an obligatory postprandial cardiac hypertrophy (e.g. Andersen et al.., 2005; Riquelme et al.., 2011), and support the proposal that postprandial cardiac hypertrophy is a facultative response in pythons (Jensen et al.., 2011; Hansen et al.., 2013; Enok et al.., 2013). In contrast, postprandial enlargement of the small intestine, liver and kidneys seems consistent amongst studies (Secor and Diamond, 1995; Secor and Diamond, 1998; Starck and Beese, 2001; Ott and Secor, 2007; Cox and Secor, 2008; Jensen et al.., 2011; Hansen et al.., 2013; Enok et al.., 2013). Supporting the idea that expansion of the intestine is stimulated by the presence of chyme (Secor et al.., 2000b), there was no effect of Hct reduction on the rise in intestinal mass during digestion, though it is impressive that significant intestinal hypertrophy occurs in animals with severe oxygen limitation. Enlargement of the stomach seems to be another facultative response to digestion, as it is noted in some studies (Secor and Diamond, 1995; Jensen et al.., 2011) but not others (Cox and Secor, 2008; Ott and Secor, 2007). As in other studies (Secor and Diamond, 1995; Jensen et al.., 2011), kidney wet mass increased with digestion, but we also note that snakes with reduced Hct had enlarged kidneys, which may result from a stimulation of erythropoietic functions, but dry kidney mass did not differ between groups.

As shown in earlier studies (Wang et al.., 2001a; Skovgaard et al.., 2009; Enok et al.., 2012; Enok et al.., 2013), the postprandial tachycardia is largely governed by a reduction of cholinergic tone on the heart, whereas the adrenergic tone actually decreases during digestion. In the double-blocked heart, there was also a rise in the postprandial fH resulting from circulating NANC factors (Skovgaard et al.., 2009), although the specific nature of the stimulus remains to be identified (Enok et al.., 2012). Given that the NANC factor is likely to be released in direct response to digestion, possibly as a peptide from the digestive organs, it is not surprising that anemia did not affect the double-blocked f_H. The rise in f_H of the anemic snakes was likely a barostatic response to vasodilation and the attendant lowering of total peripheral resistance in response to lowered blood CO2, but could also result from the stimulation of chemoreceptors (Wang et al.., 1994; Wang et al.., 1997; Andersen et al.., 2003). In contrast to previous studies on digesting snakes, the postprandial tachycardia in our study was associated with a significant rise in MAP. However, because MAP did not increase proportionally to the rise in f_H, and because stroke volume is likely to have been elevated, digestion was probably attended by a reduced total peripheral resistance as blood flow to the digestive organs increases during digestion (Secor et al.., 2000a; Starck and Wimmer, 2005; Secor and White, 2010). In addition, lowering of Hct is likely to have reduced blood viscosity and hence could have alleviated the workload on the heart. However, anemia did not influence MAP, and the anemic snakes therefore did have a higher RPP than animals with normal Hct.

The observation that the postprandial cardiac hypertrophy of pythons is facultative rather than obligatory indicates that factors other than circulating signal molecules are involved, and our results suggest that increased cardiac work or myocardial oxygen consumption stimulate the postprandial cardiac growth in pythons. Compared with resting animals, postprandial cardiac

growth was elicited in anemic snakes with significantly higher RPP, suggesting increased workload and greater mechanical stress on the ventricles. In mammals, the molecular pathways stimulating physiologic cardiac hypertrophy are stimulated by increased mechanical stress, such that increased workload stimulates myocytes to synthesize and release growth factors, including insulin-like growth factor I (IGFI) (Serneri et al.., 1999; Hill and Olson, 2008). These growth factors are then involved in paracrine and/or autocrine activation of the phosphatidylinositol 3′-kinase (PI3K)–Akt-mTOR pathway, which ultimately leads to synthesis of contractile elements (Dorn and Force, 2005; Shiojima and Walsh, 2006; Dorn, 2007; Hill and Olson, 2008). AMPK, Akt, GSK3β and mTOR, all signaling molecules in mammalian physiologic hypertrophy pathways mediated by mechanical stress, are known to be active in the python model (Riquelme et al.., 2011). This suggests that the cardiac hypertrophy in pythons occurs in response to elevated mechanical stress on ventricular myocytes. This obviously does not rule out the possibility that circulating factors, such as free fatty acids (Riquelme et al.., 2011), may contribute to the postprandial hypertrophy. Nevertheless, such humoral regulation does not appear adequate without a sufficient elevation of cardiac work and mechanical stress.

General conclusions

Despite the universal presence of gastrointestinal hypertrophies in fed pythons, our study supports the concept that postprandial cardiac hypertrophy is not an obligatory response to elevated oxygen demands associated with digestion in the python. We describe postprandial cardiac hypertrophy in fed anemic animals, whose hearts are operating at significantly elevated f_H (as mediated by reduced C_{O_2}, subsequently reduced cholinergic tone, and the presence of a significant NANC tone), and elevated cardiac work (as indicated by the RPP). We posit that

regardless of the potential for other humoral signals (Riquelme et al.., 2011), significantly elevated cardiac work is required to 'trigger' the postprandial hypertrophy via common physiological hypertrophy signaling pathways. However, the precise level of cardiac work needed to induce cardiac hypertrophy is difficult to assess from the current analysis, as the experimental paradigm depends on a group analysis. Experiments measuring systemic flow, f_H, MAP, $\dot{V}O_2$ and heart size/mass need to be correlated during fasting and digestion, within individual animals. Advanced imaging techniques, which are becoming increasingly accessible to comparative physiologists (e.g. Hansen et al.., 2013), in combination with classical physiological measurements would provide the information to determine the trigger level needed to induce postprandial cardiac hypertrophy in the Burmese python.

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CHAPTER 2:

Absence of visceral organ plasticity during prolonged fast and subsequent feeding in American alligator (*Alligator mississippiensis*)

INTRODUCTION

Digestion of food and acquisition of energy from the nutrients contained within are themselves energetically-expensive processes. In addition to the metabolic costs of capturing and handling food, breakdown of that food into absorbable components, absorption of nutrients, anabolic synthesis of new compounds, and excretion of waste – all of which are supported aerobically (Overgaard et al., 1999; Busk et al., 2000) – there is a cost associated with maintenance of an active digestive tract (i.e. tissues which do everything listed above) (Andrade et al., 2005; McCue, 2006; Secor, 2009). For intermittently-feeding "sit-and-wait" predators, down-regulation of gut tissue between meals can serve to significantly reduce standard metabolic rate (SMR) and/or liberate energy for other physiological processes (Secor, 2001).

The Burmese python is arguably the "champion" of digestive plasticity (a so-called "model of extreme physiological regulation"; Secor and Diamond, 1998). Following prolonged fasts and the associated down-regulation of gut function, the digestive process in pythons is notable not only for an up to 40-fold increase in metabolic rate (Secor and Diamond, 1997), which is attended by an immediate and prolonged increase in heart rate and cardiac output (Secor et al., 2000; Secor and White, 2010), but a regular doubling of intestinal mass (Secor and Diamond, 1995; Secor and Diamond, 1998; Starck and Beese, 2001; Ott and Secor, 2007; Cox and Secor, 2008; Jensen et al.., 2011; Hansen et al.., 2013; Enok et al.., 2013), and an occasional rapid and dramatic increase in ventricular mass (Anderson et al., 2005; Riquelme et al., 2011;

Slay et al., 2014; but not Jensen et al., 2011). This cardiac hypertrophic response likely serves to augment oxygen delivery during the postprandial period of peak oxygen demand, and is at least partly mediated by humoral factors including circulating free fatty acids (Riquelme et al., 2011). It is also likely a more direct response to increased oxygen demand, with a "low systemic O_2" signal transduced through the autonomic nervous system and subsequent adjustments in adrenergic, cholinergic, and non-adrenergic, non cholinergic signals (NANC; Enok et al., 2012; Slay et al., 2014). Slay et al. (2014; Chapter 1 of this dissertation) argue that this low O_2 threshold must be reached before hypertrophy occurs, and the ability to meet demand with existing supply is likely the reason for occasional postprandial cardiac constancy (Jensen et al., 2011). Slay et al. (2014) were able to stimulate postprandial cardiac hypertrophy by rendering animals anemic during digestion and thus theoretically artificially increasing the magnitude of the oxygen supply-demand mismatch.

Rapid and pronounced postprandial cardiac hypertrophy has not been noted outside of the pythons, nor, in fact, in other python species (Jensen et al., 2011). In this study, we search for cardiac hypertrophy in the American alligator (*Alligator mississippiensis*). While crocodilians are quite different from Burmese pythons, they share several key similarities in digestive performance, including the capacity to endure prolonged fasts when food is not available (including during hibernation; Hernandez and Coulson, 1952), establishment of a pronounced "alkaline tide" due to perturbations of acid-base balance during digestion (Coulson et al., 1950; Busk et al., 2000), and 1.4 to 4 fold increases in metabolic rate during digestion (Coulson and Hernandez, 1979; Busk et al., 2000; Gienger, et al., 2011). Given these similarities, we hypothesize that (1) American alligators (*Alligator mississippiensis*) will demonstrate organ plasticity depending on cycles of fasting and feeding, especially of the heart and digestive

organs; (2) if postprandial cardiac hypertrophy does not occur under normal conditions, we can stimulate cardiac hypertrophy by confronting animals with simultaneous oxygen deprivation (anemia) and elevated oxygen demand (digestion); and that (3) cardiovascular responses to digestion will be mitigated primarily by a release of cholinergic tone on the heart and NANC factors. In the present study, we have compared four experimental groups of animals (fasted control, fasted anemic, fed control, and fed anemic) against one another, and have also compared two groups of fasted animals, a "fasted control" group which was fasted for nearly 2 months and a "briefly fasted" group, which was fasted for just over a week. We anticipated down-regulation and atrophy of digestive organs following prolonged fasts, a massive up-regulation in size and function of visceral organs within 72 hours of feeding, and intermediate sizes and functions following a short fast.

MATERIALS AND METHODS

Animal acquisition and husbandry

American alligators (*Alligator mississippiensis*) of both sexes were obtained as yearlings from Rockefeller Wildlife Refugee (Grand Chenier, LA, USA). Between hatching and the second year of life, animals were housed at the Louisiana State University Aquaculture Research Station and used for digestibility studies (Reigh and Williams, 2013; see paper for housing, husbandry, and feeding details). Animals were then returned to Rockefeller Wildlife Refuge where they were held until transported to the University of California, Irvine (UCI). At UCI, animals (N=23) were held in large stainless steel or fiberglass holding tanks (1m x 2.5m x 1.5m) at a density of 3-7 animals per tank. These enclosures were tilted at an angle to permit both

diving and basking, and the basking platform was warmed with a ceramic heat lamp. Tanks were otherwise maintained at an ambient temperature of 30 °C on a 12h:12: light:dark photocycle. Animals, presumably due to participation in earlier feeding trials, willingly consumed only pelletized commercial alligator chow (50%; Texas Farm Products, Nacogdoches, TX, USA), which they were fed 1-3 times per week *ad libitum*. Animals gained mass during their time in captivity and at the time of experimentation ranged from 1.2-2.6 kg (mean 1.9kg±0.6kg). Husbandry and experimental procedures were approved by UCI's Institutional Animal Care and Use Committee (Protocol 2009-2921).

Surgical Procedures

Following fasts of 5 or 56 days (see details of experimental groups, below), all animals were implanted with femoral arterial catheters, primarily for monitoring MAP and f_H, injection of cardioactive pharmaceuticals, and subsequent determination of adrenergic and cholinergic tone. Anesthesia was induced by placing each animal's head in a sealed container containing gauze soaked in isoflurane (Isoflo, Abbott Laboratories, North Chicago, IL, US). When animals lost body tone and the vestibulo-ocular reflect, they were intubated and artificially ventilated with isoflurane in room air (initially 4% and reduced to 1.5-2% following loss of the "paw-pinch" reflex). Isoflurane was vaporized (Foregger Fluomatic, Smithtown, NY, USA) upstream of a ventilator (SAR-830; CWE, Ardmore, PA, USA) set to ventilate each animal at 5 breaths min^{-1} and 50 ml kg^{-1} tidal volume.

Following loss of the "paw pinch" reflex, the surgical site on the ventral surface of the proximal left lower limb was scrubbed with iodine scrub (Prepodyne iodine scrub; WestAgro, Kansas City, MO, USA) and a local anesthetic (Lidocaine 2%) was administered. A small

incision (1.5-2 cm) was made through which the femoral artery was revealed. A small ligation was made in the femoral artery, into which polyethylene catheter (PE 50, SAI Infusion Technologies, Chicago, IL, USA) containing heparinized saline (50 IU ml^{-1}) was inserted, occluding the femoral artery. This was secured internally with 4-0 silk suture (Ethicon, Somerville, NJ, USA), externalized via a small cutaneous puncture on the dorsal surface of the limb, and secured to the animal with 3-0 braided silk suture (Ethicon). The surgical incision was thereafter closed with 3-0 silk suture and veterinary adhesive (Vetbond Tissue Adhesive; 3M, Maplewood, MN, USA).

Immediately following cannulation of the femoral artery, a small (0.15ml) whole blood sample was withdrawn from the supraspinal vein (Zippel et al., 2003), and spun in glass capillary tubes for 3 min at 12,000 rpm to determine Hct. Ten animals were chosen at random and rendered anemic (see discussion of experimental groups, below) according to protocols established in Slay et al. (2014). This was accomplished by removing whole blood from the supraspinal vein (at no more than 10% estimated blood volume per aliquot; Lillywhite and Smits, 1984), centrifuging it for 5 min at 6,000 rpm, and reinjecting supernatant plasma into the supraspinal vein. This process was repeated until Hct was approximately halved in each "anemic" animal. Following manipulation of Hct in anemic animals and closure of the surgical site in all other animals, alligators were ventilated with room air until spontaneous breathing resumed. Animals were thereafter placed in opaque containers with access to water, and catheters were led through breathing holes in the lids of these containers and secured to Mylar balloons, permitting access to catheters with minimal disturbance of animals. These containers were placed in an environmental chamber held at 30 °C while animals and catecholamine levels

recovered from handling and surgical procedures (Olesen et al., 2008). Catheters were flushed with heparinized saline daily.

Experimental and feeding protocols

As mentioned above, animals were divided at random into 5 groups and fasted for 5 or 56 days, depending on the group. Those 5 groups were classified as follows: (1) "fed control" animals, which were fasted for 56 days, cannulated, fed *ad libitum* 24 hours after surgery, and euthanized 72 hours after feeding; (2) "fasted control" animals, which were fasted for 56 days, cannulated, fasted for 96 hours after surgery, and thereafter euthanized; (3) "fed anemic" animals, which were fasted for 56 days, cannulated, rendered anemic, fed *ad libitum* 24 hours after surgery, and euthanized 72 hours after feeding; (4) "fasted anemic" animals, which were fasted for 56 days, cannulated, rendered anemic, fasted for 96 hours after surgery, and thereafter euthanized; and (5) "briefly fasted control" animals, which were fasted for just 5 days, cannulated, fasted for 96 hours after surgery, and thereafter euthanized. To clarify, all but the "briefly fasted control" animals were fasted for 56 days to examine downregulation of digestive performance in response to prolonged fasting. These animals served as baselines against which fed animals were compared to determine to what extent physiological performance is up-regulated following feeding. Half of both "fasting" and "fed" groups were rendered anemic to introduce a hypoxic challenge, with "fed anemic" animals confronted with the simultaneous challenges of reduced oxygen supply and increased oxygen demand and, therefore, the highest expected degree of oxygen supply-demand mismatch. The animals which were fasted for just 5 days were added post-hoc to determine to what extent downregulation of physiological performance actually occurred during the prolonged fast.

Twenty-four hours after cannulation of the femoral artery, resting f_H and MAP were measured via the arterial catheter in all animals. To accomplish this, catheters were connected to pressure transducers (PX600, Baxter Edwards, Irvine, CA, USA) which had been calibrated against a saline-filled column. Pressure transducers were amplified (ADInstruments bridge amplifier, Colorado Springs, CO, USA) and sampled at 120 Hz on a BioPac MP100 (BioPac Systems, Inc., Goleta, CA, USA). These traces were recorded for 1-3 hours, with MAP and f_H calculated by sampling steady and representative 5-10 minute pressure waves (Acq*Knowledge* Version 3.8.1, BioPac Systems, Goleta, CA, USA).

Immediately following hemodynamic assessment, "fed" animals were provided 3 liters of water (at 30 °C) and 10% of their body mass in commercial gator chow. Alligators were allowed to feed for 1 hour, after which they were briefly removed from their containers while the remaining food and water were poured through 200 micron mesh strainers. Remaining food particles were dried to a constant mass at 60 °C (at least 72 hours), and the mass of remaining food was subtracted from the mass of food offered to each animal to estimate the amount of food an individual consumed. "Fed" animals consumed between 2.3 and 4.7% of their body mass (average 3.01±0.24%). At 21.02 kJ g^{-1} of food (measured calorimetrically), "fed" animals consumed 1309.0±112.4 kJ of energy on average. While 3% body mass was a relatively modest amount of food, using energy curves provided in Chapter 3 of this dissertation, we have calculated that these meals are equivalent energetically to rodent meals averaging 6.34±0.51% body mass. Per the design of this experiment, "fasted control," "fasted anemic," and "briefly fasted" animals were not offered food.

72 hours after "fed" animals were offered food (and 96 hours after all animals were cannulated), f_H and MAP values were acquired again for all animals (as above). We calculated

the product of f_H and MAP, the so-called rate pressure product (RPP), which provides an adequate proxy for cardiac work in absence of flow data. We then sequentially administered atropine and propranolol to determine adrenergic and cholinergic tone on the heart (see Enok et al., 2012; Slay et al., 2014) with these values calculated from the standard equations (modified from Altimiras et al., 1997 for use of f_H rather than R-R interval):

$$\text{Chol (\%)} = \frac{\frac{1}{f_{H_{cont}}} - \frac{1}{f_{H_{atr}}}}{\frac{1}{f_{H_{dbl}}}} * 100 \qquad \text{(Eq. 3.1)}$$

and

$$\text{Adr (\%)} = \frac{\frac{1}{f_{H_{dbl}}} - \frac{1}{f_{H_{atr}}}}{\frac{1}{f_{H_{dbl}}}} * 100 \qquad \text{(Eq. 3.2)}$$

where f_{Hcont} is heart rate prior to administration of cardioactive drugs, f_{Hatr} is heart rate following administration of atropine, and f_{Hdbl} is the double-blocked heart rate, i.e., heart rate following administration of atropine and propranolol.

Tissue harvest

Following determination of autonomic tones, a blood sample was removed for assessment of Hct and [Hb] (as per Rice, 1967), and animals were euthanized via removal of the ventricle while under anesthesia. Animals were anesthetized (as above) and once peripheral reflexes were lost, a large ventral incision was made to expose the heart, which was immediately removed. Thereafter, liver, stomach, small intestine, large intestine, and kidneys were removed from all animals, rinsed with saline, gently blotted dry with gauze, and weighed. A small representative biopsy was removed from each organ, weighed, dried to a constant weight at 60 °C (>72 h), and weighed again. The ratio of wet mass to dry mass was multiplied by total organ wet mass to acquire organ dry mass.

Statistical analysis

All organ masses were expressed as dry mass-specific values (g dry tissue per kg body mass). For statistical comparison between fasted control, fasted anemic, fed control, and fed anemic animals we utilized a two-way analysis of variance (ANOVA) and a post-hoc Tukey's HSD (only when the ANOVA model yielded significance) in JMP (Version 7, SAS Institiute, Inc., Cary, NC, USA). All variables met the assumption of homogeneity of variance, but values for adrenergic tone, cholinergic tone, and Hct were arcsine square root transformed prior to statistical analysis. Effects, where reported, are the results of effect tests produced by the ANOVA model and are distinguishable by the single degree of freedom. Statistical comparisons between briefly fasted and fasted control groups were made using a one-way ANOVA. The relationship between RPP and ventricular mass was plotted and analyzed in GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA, USA). For all tests, significance was accepted at the level of $P \leq 0.05$. All values reported are means ± s.e.m.

RESULTS

Effects of Anemia and Feeding on Hemodynamics and Visceral Organ Size

Hematocrit remained depressed through the 96 hour period following initial reduction ($F_{3,14}=37.3$, $P<0.001$; Table 2.1). This is notable not only because it provides evidence that we successfully reduced Hct by about 50%, but it suggests that animals either did not compensate for the loss of red blood cells or compensated with a relative increase in both red blood cell and blood volume. Similarly, [Hb] remained low in the anemic group following experimental manipulation ($F_{3,14}=33.6$, $P<0.001$).

Heart rate differed significantly between groups ($F_{3,12}$=4.2, P<0.05), surprisingly due primarily to an effect of feeding ($F_{1,12}$=9.5 P<0.05) but not Hct ($F_{1,12}$=1.6, *NS*) or an interaction of feeding and Hct ($F_{1,12}$=0.30, *NS*). Heart rate in fasted control animals was significantly lower than in fed controls (60% difference) and fed anemic animals (73% difference, but f_H did not differ between groups of fed animals (Fig. 2.1A). Anemia alone did not cause an elevation of f_H in fasting or digesting animals. MAP also differed significantly between groups ($F_{3,12}$=3.5, P<0.05) again because of a significant effect of feeding ($F_{1,12}$=5.8, P<0.05), but not Hct ($F_{1,12}$=1.6, *NS*) or an interaction between feeding and Hct ($F_{1,12}$=1.7, *NS*), with MAP being highest in fed control animals, and significantly larger than in fasted controls and fed anemic animals (Fig 2.1B). Together, these differences resulted in a RPP that differed between groups ($F_{3,12}$=3.5, P<0.05) due to an effect of feeding ($F_{1,12}$=9.3, P<0.03) but not Hct ($F_{1,12}$=0.1, *NS*) or an interaction of feeding and Hct ($F_{1,12}$=1.1, *NS*). RPP was highest in fed control animals, which differed significantly only from fasted controls, where RPP in fed controls was 2.3-fold higher than in fasted controls (Fig. 2.1C). Again, anemia alone did not cause an elevated RPP for fasted or digesting groups of animals.

In assessing autonomic tone on the heart, we found no differences in adrenergic tone ($F_{3,12}$=0.9, *NS*), but there were significant differences in cholinergic tone ($F_{3,10}$=4.9 P<0.05), again because of an effect of feeding ($F_{1,10}$=8.7, P<0.05) but not Hct ($F_{1,10}$=8.7, P<0.05) or an interaction of feeding and Hct ($F_{1,10}$=8.7, P<0.05) (Fig. 2.2). While not significantly different from each other, fed control and fed anemic animals exhibited approximately half the cholinergic tone found in fasted control animals. There were no differences in intrinsic f_H, i.e., heart rate under dual autonomic blockade ($F_{3,10}$=0.8, *NS*).

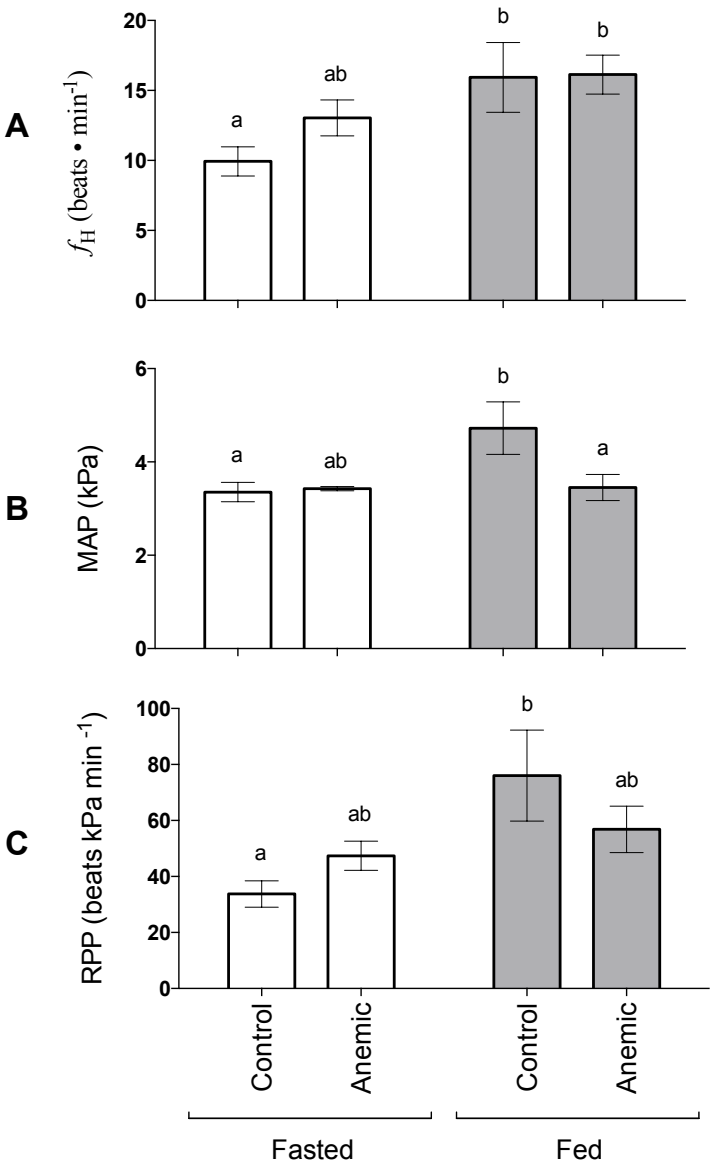

Figure 2.1 Hemodynamic parameters in alligators confronted with the simultaneous challenges of digestion and hypoxemia (anemia). Heart rate (f_H; A) differed significantly between groups, with fed controls and fed anemic animals exhibiting higher heart rates than fasted controls. There were effects of feeding on mean arterial pressure (MAP; B) and the rate-pressure product (RPP; C). Surprisingly, there were no effects of anemia (a 50% reduction in Hct) alone on hemodynamic values.

We did not find any predicted effect of feeding or anemia on the size of the heart (Fig. 2.3). Specifically, ventricular dry mass was quite similar in each of the four groups of animals ($F_{3,14}$=1.3, *NS*), as was atrial dry mass ($F_{3,14}$=0.5, *NS*). Indeed, there were no differences in the mass of any visceral organs except the stomach and kidneys (Table2.1). Predictably, differences in stomach mass ($F_{3,14}$=3.9, P<0.05), were due to an effect of feeding ($F_{1,14}$=13.9, P<0.05) and not Hct ($F_{3,14}$=1.2, *NS*) or an interaction effect ($F_{3,14}$=0.5, *NS*), and stomach mass was, on average, 34% higher in fed groups than in fasted groups of animals. Differences in kidney mass ($F_{3,14}$=7.3, P<0.005) were due to effects of feeding ($F_{1,14}$=6.9, P<0.05), Hct ($F_{1,14}$=3.9, P<0.05), and an interaction of feeding and Hct ($F_{1,14}$=4.7 P<0.05). Small intestine ($F_{3,14}$=2.0, *NS*), large intestine ($F_{3,14}$=1.3, *NS*), and liver ($F_{3,14}$=2.1, *NS*) did not differ between groups.

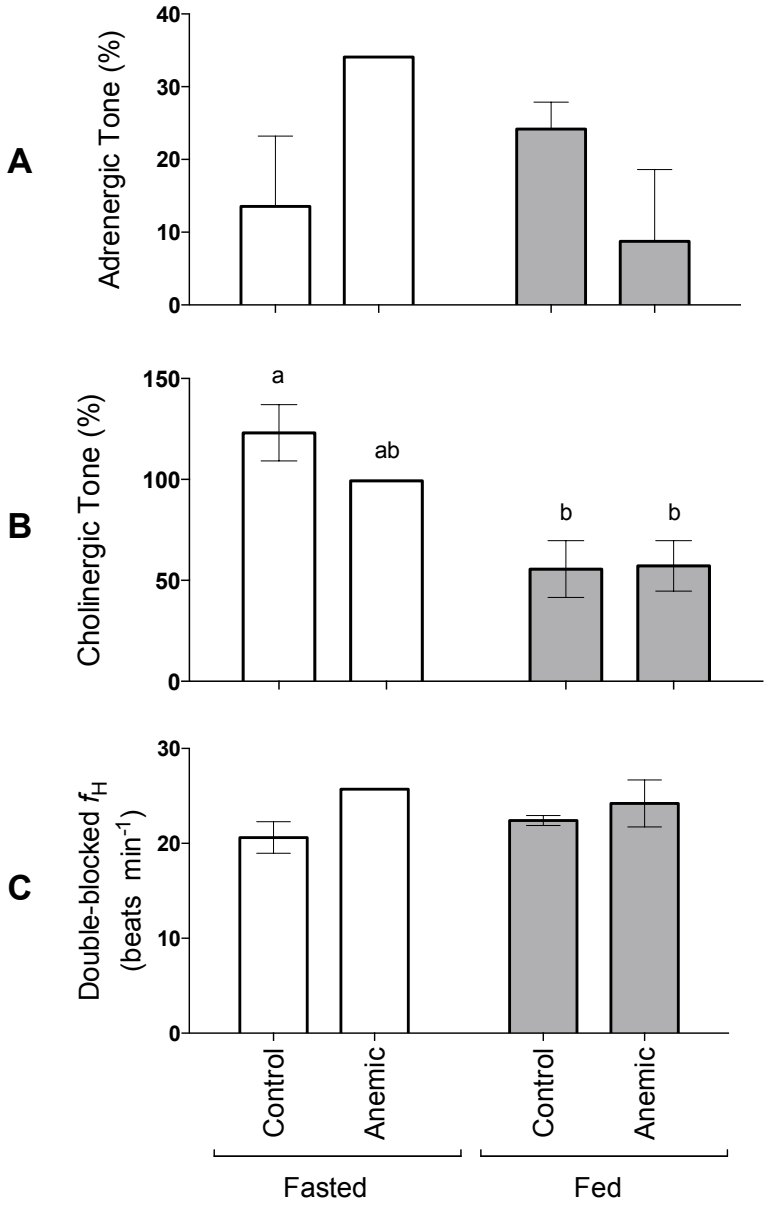

Figure 2.2. Control of heart rate in alligators confronted with simultaneous challenges of digestion and hypoxemia. There are no significant differences between groups in adrenergic tone on the heart (A), nor are there any apparent trends. There is, however, a significant effect of feeding on cholinergic tone (B), but no further reduction due to anemia. There are no differences in double-blocked heart rate (C) between animals, indicating that there is not likely a non-adrenergic, non cholinergic (NANC) factor acting on the hearts of digesting alligators.

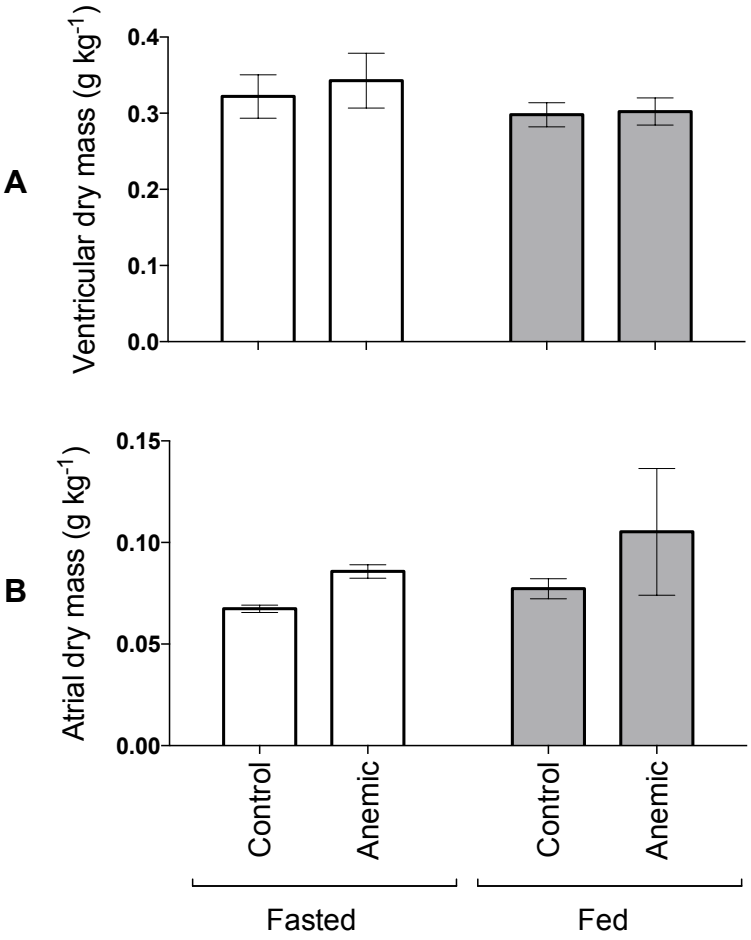

Figure 2.3. Comparisons of heart mass in alligators confronted with simultaneous challenges of anemia and digestion. Neither ventricular nor atrial dry mass was affected by feeding or reduced Hct.

Table 2.1. Group size, mass, hematocrit, hemoglobin, and organ mass.

Variable (unit)	Fasted control	Fasted anemic	Fed control	Fed anemic	Briefly fasted
n	4	3	4	7	5
Animals mass (kg)	2.10±0.16 [a]	1.84±0.08 [a]	2.09±0.04 [a]	2.04±0.06 [a]	1.5±0.09
Hct (%)	17.1±1.3 [a]	9.3±1.2 [b]	19.3±0.3 [a]	9.3±0.6 [b]	18.6±2.2
[Hb] (mM)	82.7±4.6 [a]	38.2±4.1 [b]	102.5±6.5 [a]	43.1±3.9 [b]	-
Stomach dry mass (g kg^{-1})	1.6±0.01 [a]	1.8±0.1 [ab]	2.2.1±0.1 [c]	1.9±0.0 [b]	1.3±0.3
Sm. Int. dry mass (g kg^{-1})	1.8±0.2 [a]	1.5±0.1 [a]	1.5±0.0 [a]	1.6±0.1 [a]	1.9±0.1
Lg. Int. dry mass (g kg^{-1})	0.71±0.10 [a]	0.72±0.09 [a]	0.67±0.02 [a]	0.80±0.09 [a]	0.62±0.02
Liver dry mass (g kg^{-1})	2.8±0.1 [a]	2.6±0.3 [a]	2.3±0.2 [a]	2.3±0.1 [a]	3.3±0.2*
Kidney dry mass (g kg^{-1})	0.44±0.01 [a]	0.58±0.02 [b]	0.56±0.04 [b]	0.55±0.02 [b]	0.53±0.02*

Values are means±s.e.m. Fasted control, fasted anemic, fed control, and fed anemic groups were compared with two-way ANOVA and if significance was determined, post-hoc Tukey's HSD was used for pairwise comparisons. Statistically similar groups share superscript letters. The briefly fasted group was compared only to fasted controls using a one-way ANOVA. Significance from that test (differences between the fasted control and briefly fasted groups) is indicated with an asterisk.

Effects of Fasting Duration on Hemodynamics and Visceral Organ Size

To identify potential anatomical or functional differences between animals subjected to a prolonged fast and animals fasted relatively briefly, we compared "fasted control" animals to "briefly fasted" animals and examined hemodynamics and visceral organ mass (Table 2.1). We found no differences in any hemodynamic variable between these two groups, as f_H ($F_{1,7}$= 0.1, *NS*) , MAP ($F_{1,7}$=1.2, *NS*), and RPP ($F_{1,7}$= 1.9, *NS*) were statistically similar between groups. Unsurprisingly, then, there were no differences in adrenergic ($F_{1,7}$= 1.9, *NS*) or cholinergic ($F_{1,7}$= 1.9, *NS*) tone, and no difference in double-blocked f_H ($F_{1,7}$= 2.1, *NS*). As might be expected given the lack of functional differences, there are no anatomical differences these hearts; neither ventricular mass ($F_{1,7}$= 1.0, *NS*) nor atrial mass ($F_{1,7}$= 1.3, *NS*) differed between groups.

Among the digestive organs, there were no differences in stomach ($F_{1,7}$= 0.8, *NS*), small intestine ($F_{1,7}$= 0.7, *NS*), or large intestine ($F_{1,7}$= 1.9, *NS*) mass between fasted controls and briefly fasted animals. There were, however, differences in liver ($F_{1,7}$= 5.7, P<0.05) and kidney mass, ($F_{1,7}$= 12.2, P<0.05), with the briefly fasted group having 22% and 23% larger livers and kidneys, respectively, than fasted control animals.

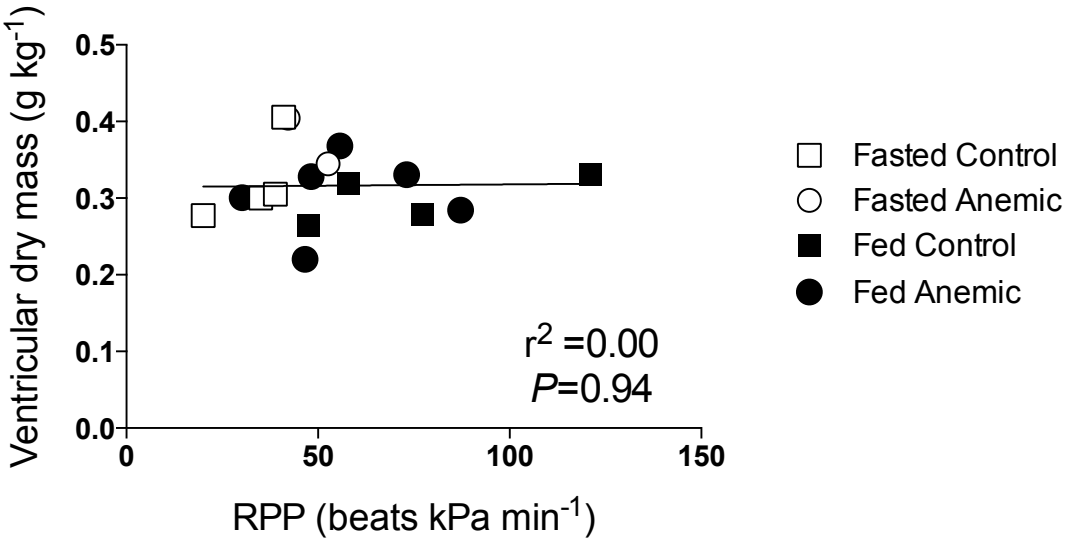

Figure 2.4. The RPP and ventricular mass relationship in American alligators is statistically insignificant. A positive relationship between RPP and ventricular dry mass in Burmese pythons (Slay et al., 2014, Chapter 1 of this dissertation) suggests that postprandial cardiac hypertrophy occurs when RPP (a proxy for cardiac work) is significantly elevated. The absence of that relationship here suggests cardiac hypertrophy does not occur in alligators because RPP never reaches the level required to stimulate ventricular enlargement, a probable consequence of the comparatively modest increases in oxygen demand and cardiovascular function in alligators.

DISCUSSION

We found only limited support for our hypotheses. While we report some differences in stomach, liver, and kidney masses, we found no evidence of postprandial cardiac hypertrophy, even when anemia was used to increase the magnitude of supply-demand mismatch. While there

were some modest hemodynamic differences between fasted and fed animals, feeding and anemia did not result in additive cardiovascular responses. There were, therefore, no changes in autonomic or NANC regulation of heart function due to a combination of both feeding and anemia. Still, however, we can draw interesting conclusions from this work. We report that generally the response to fasting in alligators is not characterized by significant gross anatomical changes (with the exception of some changes in stomach, liver, and kidneys). This is supported both by (1) the general lack of differences between fasted animals and fed animals and by (2) the general lack of differences between animals experiencing prolonged and brief fasts. In other words, not only is limited rapid up-regulation of visceral organ mass due to feeding in alligators, there is limited down-regulation of visceral organ mass during prolonged fasts. We also note the relatively small consequence of markedly reducing oxygen delivery capacity in alligators.

Prolonged fasts, feeding, and anemia

Anemia had been previously utilized as a tool to artificially increase the magnitude of oxygen supply-demand mismatch and stimulate postprandial cardiac hypertrophy in Burmese pythons (Slay et al., 2014). This was, the authors argued, a sequential consequence of detection of low blood oxygen levels, up-regulation of cardiac function through actions of the autonomic nervous system and NANC factors, elevated cardiac work (as measured experimentally using a proxy, the RPP), and physiologic cardiac hypertrophy as mediated through classical cellular pathways sensitive to stretch of the cardiomyocytes. This idea provides a framework we can utilize to determine why cardiac hypertrophy does not occur in pythons and other species. We begin, therefore, with a discussion of cardiac function.

Heart rate, MAP, and RPP were changed only through effects of feeding and not because of anemia. This is surprising, because Hct is typically closely regulated to permit adequate delivery of oxygen while minimizing the cost of transport associated with the positive relationship between hematocrit and viscosity of whole blood (Crowell and Smith, 1967). Exercise training, for example, results in elevated hematocrit in alligators (Eme et al., 2009). And yet there was no statistical consequence of reduced hematocrit (and consequently, reduced [Hb] and oxygen carrying capacity) on the heart rate of fasted or fed alligators, which indicates a blood oxygen carrying capacity that far exceeds the oxygen demands of resting alligators and, for a given increase in cardiac output, digesting alligators. There was, however, an influence of feeding on f_H, which presumably served to increase convective oxygen transport during a period when oxygen consumption can reach peak values 1.5- to 4-fold higher than standard metabolic rate. This difference in heart rate among digesting animals appeared to be supported exclusively through release of cholingergic tone, which is similar to what occurs in digesting *Boa constrictor* and Burmese pythons (Wang et al., 2001; Enok et al., 2012, Slay et al., 2014). There did not appear to be a NANC-mediated increase on f_H, which may indicate that circulating humoral factors are not as important (if they are at all) in regulation of postprandial f_H as they are in the Burmese python model (Skovgaard et al., 2009). MAP was elevated in digesting animals (which may be a consequence of increased peripheral resistance and shunting of blood to the splanchnic circulation), and this resulted in an elevated RPP. Overall, however, there was no significant relationship between RPP and ventricular mass (Fig. 2.4). Because we found only modest functional increments in the heart, this lack of relationship between RPP and ventricular mass supports the idea that elevated cardiac work and subsequent myocardial stretch stimulates postprandial hypertrophy. In other words, postprandial cardiac hypertrophy probably does not

occur in alligators because cardiac work (or RPP) is never elevated enough to induce such a change. We know that alligators experience significant ventricular enlargement which is at least to some degree physiologic hypertrophy (Eme et al., 2009; Eme et al., 2010), so the cellular "machinery" responsible for ventricular remodeling exists in reptiles. It is not activated, however, in this scenario.

The apparent lack of intestinal plasticity was a bit surprising because there are well-characterized gross anatomical and histological changes in the small intestine of caimans, alligators, and crocodiles (Starck et al., 2007; Tracy et al., 2015), but these may be due to hydrostatic inflation of villi (Starck and Beese, 2001) and not due to an absolute increase in intestinal dry mass. Increases in stomach mass are common among digesting reptiles (especially Burmese pythons; Secor and Diamond, 1995; Jensen et al.., 2011), though this appears to be a facultative response to digestion in snakes (see Cox and Secor, 2008; Ott and Secor, 2007; Slay et al., 2014 for stomach constancy) and the presence of this response in the current study suggests that the thick muscular wall of crocodilians is of significant importance during digestion. Kidneys of fasted control animals had reduced kidney size, and this might be a result of or result in reduced bicarbonate handling (Esbaugh et al., 2015).

Comments on the general lack of plasticity in alligators

Through comparisons between animals subjected to prolonged (56 day) and relatively brief (8 day) fasts, we argue that there is little anatomical (though not necessarily functional) down-regulation of the alligator visceral organs during extreme fasting events. Alligators do overwinter, generally without eating or at very least with reduced appetites (Hernandez and Coulson, 1950); do experience quite large increases in postprandial oxygen consumption; and do

experience acid-base perturbations due to consumption of large meals. However, crocodilians are not stereotypical sit-and-wait predators. Nile crocodiles (*Crocodylus niloticus*), for example, are initially insectivorous, then subside primarily on frogs and fish, and only consume large mammalian or avian prey after reaching 2 meters in length (Cott, 1961). The same species is reported to herd small fish before voraciously consuming them (Pooley and Gans, 1976). They are not, therefore, truly comparable to the Burmese python, which has been reported to fast for at least a year in captivity (Benedict, 1932). Consequently, more modest alteration of digestive anatomy and physiology between meals is in concert with the hypothesis (Secor, 2001) and evidence (Ott and Secor, 2007) that extremeness of gut regulation is proportional to the interplay of fasting duration and meal size.

Nevertheless, we choose to highlight the general weakness in all "grouped" experimental designs which are so common in this field. In grouped analyses, and particularly at the sample sizes found acceptable among comparative physiologists, sample means do not necessarily reflect population means, and more importantly, overlook the level to which these plastic responses vary individually. We therefore lobby for research to take advantage of emerging opportunities to utilize implantable telemetric devices and improved imaging modalities to facilitate paired, rather than grouped, analyses going forward. Until then, we are likely underestimating or overestimating the functional and/or anatomical plasticity of animals.

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CHAPTER 3:

Abolition of the crocodilian right-to-left shunt does not affect digestion despite profoundly altered intraventricular hemodynamics.

INTRODUCTION

While historically the cardiovascular systems of non-avian reptiles had been considered "imperfect" circulatory arrangements existing as evolutionary intermediates between the rudimentary cardiac morphologies of fish and amphibians and the more functionally-refined cardiac morphologies of relatively highly-aerobic endotherms (e.g. Foxon, 1955), reptilian cardiovascular physiology has more recently garnered appreciation for its remarkable complexity (see Burggren and Warburton, 1994; Axelsson, 2001; Hicks and Wang, 2012; Burggren et al.., 2014). This has been, to a sizable extent, a consequence of endeavors to determine the functional advantage(s) of cardiac shunting, a process common to all non-avian reptiles (hereafter "reptiles"). Due to varying degrees of anatomical and functional separation of the ventricle and the presence of the dual aortic arch system, these shunts permit differential distribution of blood between the pulmonary and systemic circulations (Hicks, 1998). Proximately, shunts result in delivery of saturated blood, otherwise bound for the systemic circuit, to the pulmonary circulation (left-to-right "systemic bypass shunt" shunt) and/or delivery of desaturated blood, otherwise bound for the pulmonary circuit, to the systemic circulation (right-to-left "pulmonary bypass" shunt) (Hicks, 1998). Shunting is actively controlled through autonomically regulated adjustments of vascular resistance in the systemic and pulmonary circuits and/or through utilization of anatomical features specific to the reptilian heart (e.g. White, 1969; Jones and Shelton, 1993; Axelsson et al.., 1996; Franklin and Axelsson, 2000; Jensen 2010a,b; reviewed in

Hicks, 1998). And while hypotheses regarding the evolutionary or functional advantage(s) of cardiac shunting have been debated among comparative organismal biologists for at least a century, empirical evidence supporting these hypotheses remains scarce (Hicks, 2002; Hicks and Wang, 2012).

Crocodilians, due to a unique cardiovascular morphology, are a particularly useful model for studying the potential functional advantage of cardiac shunting. The crocodilian cardiac arrangement is characterized by a fully-divided ventricular septum (otherwise found only in birds and mammals) and the presence of the dual aortic arches (present only in reptiles), with the right aorta (RAo) originating from the left ventricle (LV) and the left aorta (LAo) originating from the right ventricle (RV) immediately adjacent to the pulmonary artery (PA) (Webb, 1979; Fig. 3.1). While left-to-right (L-R) shunting cannot occur due to the presence of the ventricular septum, a right-to-left (R-L) shunt develops when blood is ejected into the LAo from the RV. This occurs when systemic vascular resistance is lower than pulmonary vascular resistance, which can be dramatically increased through obstructive action of the autonomically controlled cog-tooth valve found exclusively in the subpulmonary conus of crocodilians (many works reviewed in Hicks, 1998; Franklin and Axelsson, 2000). The R-L shunt can therefore be abolished through surgical occlusion of the LAo, and this phenotypic manipulation has been utilized to determine whether chronic elimination of the R-L shunt has deleterious effects (Farmer et al.., 2008; Eme et al.., 2009a; Eme et al.., 2009b; Eme et al.., 2010; Jones and Gardner, 2010; Gardner et al.. 2011)

Studies examining possible deleterious effects of chronic elimination of the R-L shunt have, as expected, revealed morphological and physiological responses to the surgical manipulations, but these changes have not resulted in obvious detriment to animal fitness. Notably, despite predictions that diversion of destaurated, hypercapnic, and acidic blood to the

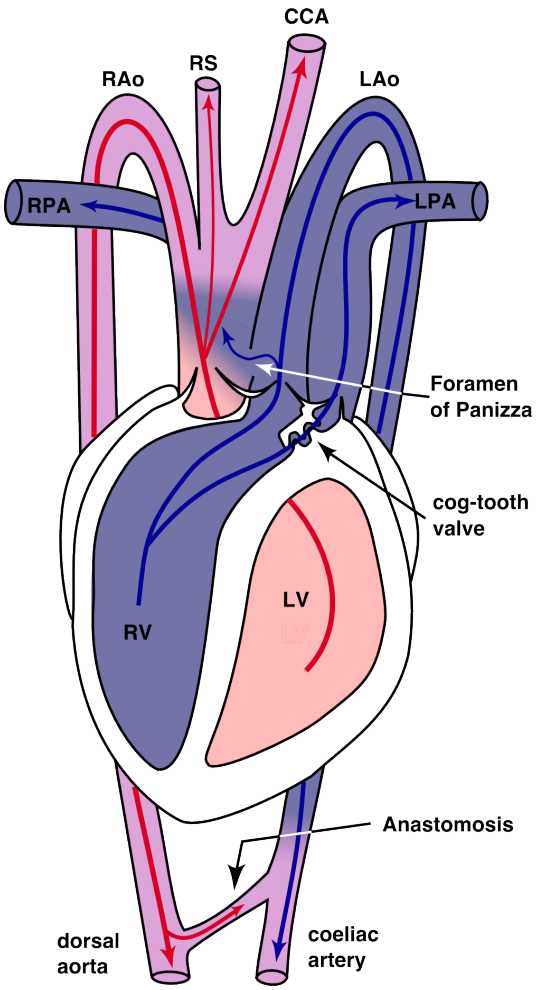

Figure 3.1. Crocodilian cardiovascular arrangement, with fully divided ventricle and presence of dual aortic arches, during late systole. The right aorta (RAo), right subclavian artery (RS), and common carotid artery exit the left ventricle (LV) and deliver blood to the systemic circulation. The right and left pulmonary arteries (RPA and LPA, respectively) exit the right ventricle and deliver desaturated blood to the lungs. The left aorta (LAo) also exits the right ventricle, and when pulmonary vascular resistance is greater than systemic vascular resistance and/or when the cog-tooth valve is actively obstructing the pulmonary arteries, a R-L shunt develops and desaturated blood is returned to the systemic circulation. Further downstream, the RAo becomes the dorsal aorta and the LAo becomes the coeliac artery, which delivers blood primarily to the digestive organs. Blood can be exchanged between the dorsal aorta and the coeliac artery at the arterial anastomosis, and between the RAo and LAo at the site of the actively controlled Foramen of Panizza. Figure from Eme et al.., 2010.

gut through the LAo would facilitate increased acid secretion into the lumen of the stomach (Jones and Shelton, 1993; Farmer et al.., 2008) or provide metabolic substrates in the liver (Farmer, 2011), and despite evidence that demineralization of bone does occur more slowly in

animals without the R-L shunt (Farmer et al.., 2008), neither growth rate nor food intake is dramatically impeded due to abolition of the shunt (Eme et al.., 2010; Jones and Gardner, 2010). It remains unclear, therefore, whether removal of the R-L shunt is detrimental to crocodilians (and, indeed, to reptiles), especially with regard to their ability to digest and assimilate ingested nutrients.

Here, we examined American alligators (*Alligator mississippiensis*) 7 years after surgical manipulation to determine whether there were negative consequences of "ultra-chronic" occlusion of the LAo. Given the debate about contributions of the R-L shunt to digestion and assimilation, we focused on digestive processes. We predicted, because growth rates are similar for animals with and without the R-L shunt (Eme et al.., 2010), that despite possible differences in stomach pH and rates of bone digestion (Farmer et al.., 2008), digestive efficiency would be similar in both groups. Furthermore, we predicted that this similarity would not be due to compensatory responses in the gut, and we predicted that gross anatomy of digestive organs, absorptive surface area, and digestive enzyme activity would be similar for both groups. Additionally, because these animals had experienced chronic occlusion of a major outflow tract and tolerated chronic RV hypertension, we aimed to characterize systemic and intraventricular hemodynamics.

MATERIALS AND METHODS

Animal acquisition and husbandry

In June, 2005, American Alligators (*Alligator mississippiensis*) were acquired as *in ovo* embryos from the Louisiana Department of Wildlife and Fisheries' Rockefeller Wildlife Refuge

(Grand Chenier, LA, USA) and reared in the vivarium facilities of University of California, Irvine. To ensure that specimens were sex-matched, eggs were incubated in moist vermiculite at 30 °C to stimulate female development via temperature dependent sex determination. After hatching, animals were housed in large (1m x 2.5m x 1.5m) stainless steel or fiberglass tanks, which contained water and were slanted to create an elevation gradient permitting both submersion and emersion, with the dry basking area in each tank warmed by a ceramic heat lamp. These tanks were housed in a vivarium which was maintained at 30 °C with a 12h:12, light:dark photocycle. For the first 2 years after hatching, animals were fed 2-3 times per week *ad libitum* meals of live goldfish or ground chicken, and from year 3 until euthanization (see below) in April, 2015, animals were fed 1-3 times per week *ad libitum* meals of commercial alligator chow (50% alligator starter; Texas Farm Products, Nacogdoches, TX, USA) or dead, previously-frozen mice.

Between May and July, 2007, approximately 18 months after hatching, animals were divided at random into two groups and underwent surgery (see details below) to occlude the LAo (henceforth "LAo-occluded" animals; N=16; 75±3 g) or a sham surgery which left the anatomy of the great vessels intact (henceforth "sham" animals; N=12; 74±4 g). Of these animals, N=14 (N=9 "LAo-occluded"; N=5 "sham") remained in the vivarium in October, 2014, when the present study began. A size-matched subset of this group ranging in mass from 2.7 kg to 8.8 kg (average mass = 4.4±0.7 kg) was used for these studies (N=5 sham, 4.32±1.01kg; N=5 LAo-occluded, 4.52±1.01kg). These animals had previously been used for diving, growth, and metabolic trials, for which food was occasionally withheld from each group for 4-8 weeks, and data from these 10 alligators have previously been published in an earlier study (Eme at al,

2010). Alligator husbandry, surgery, and experimental procedures were conducted as approved by UCI IACUC protocols 1999-2123 and 2009-2876.

Initial Surgical Procedure for Occlusion of the Left Aorta

Initial surgeries on this cohort of animals were performed in a similar fashion to those completed by Farmer et al.. (2008) and as described by Eme et al.. (2009, 2010; see the Eme et al.. papers for exact detail of surgeries performed on these 10 animals). To summarize, "LAo-occluded" animals were anesthetized using an inhaled anesthetic (Isoflo, Abbott Laboratories, North Chicago, IL, US), and an incision was made along the ventral midline which, following dissection of the underlying musculature and cutting of a small (~1 cm) section of the sternum and opening of the pericardium, revealed the heart and great vessels. The LAo was isolated, and using 6-0 suture (Deknatel, Research Triangle Park, NC, USA), occlusive ties were placed upstream and downstream of the Foramen of Panizza (the shared communication point of the LAo and RAo), and the LAo was ligated between these ties. Cessation of flow through the LAo was confirmed using an H_2 electrode technique (Clark et al.., 1960; Hicks and Comeau, 1994; Malvin et al.., 1995; Eme et al.., 2009; Eme et al.., 2010). Following confirmation of ablation of the LAo, the pericardium was closed with 6-0 silk suture, and the sternum and skin were closed with 3-0 suture (Ethicon, Somerville, NJ, USA). For the sham surgical group, the surgical protocol (including use of the H_2 tracer) was similar except the LAo was neither occluded nor ligated. Following termination of the surgical procedures, animals were treated with the analgesic flunixin meglumine (5 mg kg^{-1}; Flunixamine; Fort Dodge, Madison, NJ, USA) and the antibiotic enrofloxacin (10 mg kg^{-1}; Baytril; Bayer Corporation, Shawnee Mission, KS, USA).

Food was withheld for 5-7 days following surgery, but each animal was thereafter returned to its regular care and husbandry regimen.

Determination of apparent digestive efficiency and transit time

Apparent digestive efficiency (ADE) was calculated using formula 3.1 (e.g. Kitchel and Wendell, 1972; Johnson and Lilywhite, 1979) where ADE is energy absorbed via the gut, C is energy consumed, and F is energy contained in fecal waste. To calculate ADE, therefore, it was

$$\text{ADE (\%)} = \frac{C-F}{C} \times 100 \qquad \text{(Eq. 3.1)}$$

required to measure the energy content of a meal and feces derived from that meal. To determine energy content of food, previously killed mice (N=34) ranging from 2.7-38.7 g were weighed. dehydrated for >72 hours at 60 °C, weighed again, homogenized using a food processor (KFC3511; KitchenAid, Benton Harbor, MI, USA), compressed into cylindrical pellets of approximately 1.5 cm diameter x 1 cm height (approximately 1 g), and combusted in a Parr 6200 isoperibol calorimeter (Parr Instrument Company, Moline, IL, USA), with energy measurements repeated in triplicate. Individual whole mouse energy was calculated as the product of an individual's mass, dry mass to wet mass ratio, and mean energy content of the dried sample. For mice less than 8 grams, 3 mice of similar mass (within 0.1 g of one another) were dehydrated and homogenized together to yield enough material for combustion. A line was fit to a total energy vs. whole animal wet mass plot (see Figure 3.1), and the slope of this line (Equation 3.2) was used to calculate the energy content of a given meal size, where *y* is total energy content of meal mass *x*.

$$y = 10.97x - 29.56 \qquad \text{(Eq. 3.2)}$$

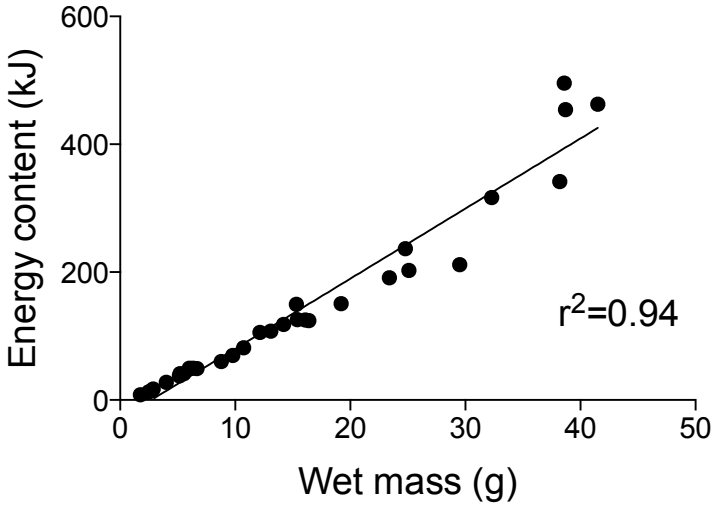

Figure 3.2. Whole animal energy content of mice used in marked alligator meals, as determined by bomb calorimetry. Energy content of homogenized samples of dried mouse (N=34) was measured in triplicate, averaged, and multiplied by an animal's dry mass to wet mass ratio and body mass to calculate whole animal energy content. The line fit to these data points (r^2=0.94, P<0.05) was used to calculate total energy content of a meal of a given size, a requisite for determining apparent digestive efficiency (ADE).

Beginning in October, 2014, alligators were placed in wire-bottomed stainless steel cages (61 cm x 61 cm x 38 cm for animals <3.5 kg; 95.3 cm x 90.2 cm x 81.3 cm for animals >3.5 kg) which were held in an environmental chamber at 30 °C and >95% relative humidity. Animals were provided shallow dishes of water, which they drank from with regularity. Alligators were given 72h to acclimate to these cages, and were subsequently fed weekly with rodent meals equivalent to 2.5% body mass, which were marked alternately with inert indigestible markers used to identify the source of feces: green chromic oxide (Sigma-Aldrich, St. Louis, MO, USA; each mouse injected IP with 1 ml $kg^{=1}$ of a 20% suspension) and red carmine (Sigma-Aldrich, St. Louis, MO, USA; each mouse injected IP with 1 ml $kg^{=1}$ of a 10% suspension) (e.g. Owens and Hanson, 1992; Barboza, 1995; Rozin and Meyer, 1964). As animals digested, they were monitored using a high definition security camera and digital video recorder system (QC908; Q-See, Anaheim, CA, USA) with cameras mounted above and below the wire cage bottoms. This, along with remote monitoring, allowed for accurate calculation of transit time and ensured that

fecal collection took place no later than 4 h following defecation. Feces (nitrogenous waste was not collected because it was unmarked) derived from the second meal (red) was collected shortly after appearance, frozen at -20 °C, dehydrated at 60 °C for >72 h, weighed, homogenized using a mortar and pestle, compressed into a ~1g pellet, and combusted using bomb calorimetry as described above. Assuming that 100% of carmine was excreted and collected, energy attributable to red carmine (found to be 11.3 kJ $g^{=1}$ of dry carmine using calorimetric techniques as described above) was subtracted from the total energy content of feces. To determine whether alligators were differentially extracting mass from meals, we calculated and have reported relative fecal mass as the ratio of total fecal (dry) mass to total meal (wet) mass for both groups. We expressed total fecal energy as the product of fecal dry mass (g) and mean mass-specific energy content (kJ g^{-1}).

Using the aforementioned camera and digital video recorder system, it was possible to measure time to defecation to the nearest 1 s. Here, we report "time to fist appearance" as the time elapsed between feeding and initial appearance of marked feces and "time to last appearance" as the time elapsed between feeding and final appearance of marked feces. Transit times from the second (red) and third (green) meals were averaged to calculate these values.

Cannulation for Measurement of Resting Blood Pressure and Heart Rate

To measure resting MAP and f_H in resting alligators of both groups, each animal was cannulated with an occlusive femoral arterial catheter as follows. After collection of the third marked meal, all animals were fasted for 56 days and then underwent surgical implantation of catheters. To induce anesthesia, the head of each animal was placed in a sealed container containing isoflurane-soaked gauze. Upon loss of the vestibulo-ocular reflex, each animal was

intubated and artificially ventilated at 5 breaths min^{-1} and 50 ml kg^{-1} tidal volume (SAR-830 ventilator; CWE, Ardmore, PA, USA), initially with 4% isoflurane in room air, maintained with a fluroethane vaporizer (Foregger Fluomatic, Smithtown, NY, USA), until the "paw pinch" reflex was lost, whereafter they were ventilated with 1.5-2% isoflurane. The ventral surface of the proximal left lower limb was scrubbed with an iodine solution (Prepodyne iodine scrub; WestAgro, Kansas City, MO, USA) and ~0.5 ml of lidocaine (2%) was injected to induce local anesthesia near the surgical site. An incision of ~2 cm was used to expose underlying musculature, which was blunt dissected away to reveal the femoral artery. A polyethylene catheter (PE 50, SAI Infusion Technologies, Chicago, IL, USA) containing 50 IU ml^{-1} heparinized saline was conclusively placed in the artery, was externalized on the dorsal side of the proximal lower limb, and was secured using 2-0 suture (Ethicon, Somerville, NJ, USA). The incision was then closed using 3-0 suture, and the animal was artificially ventilated with room air until resumption of voluntary breathing. Animals were placed in individual opaque containers which were housed in a 30 °C environmental chamber. Catheters were led through breathing holes in these containers and secured to helium-filled Mylar balloons so that catheters could be retrieved while minimally disturbing the animals. Catheters were flushed daily with 50 IU ml^{-1} heparinized saline.

24 and 48 hours after surgery, resting MAP and f_H were measured in each animal by connecting these femoral arterial catheters via saline-filled PE-50 tubing to pressure transducers (PX600, Baxter Edwards, Irvine, CA, USA) which were calibrated against a saline-filled column, amplified using a bridge amplifier (ADInstruments, Colorado Springs, CO, USA), and sampled at 120 Hz (MP100, BioPac Systems, Inc., Goleta, CA, USA). Data were recorded for 2-5 h using the Acq*Knowledge* software package (Version 3.8.1, BioPac Systems Inc., Goleta, CA,

USA), and MAP and f_H were analyzed by averaging steady and representative 5-10 minute traces.

In-situ *Acute Preps, Dissections, and Preservation of Tissues*

Following measurements of resting MAP and f_H, animals were returned to their enclosures in the vivarium and fed 2.5% body mass on alternating days for 5 days to compensate for potential downregulation of digestive processes (e.g. Starck et al.., 2007). On the seventh day following cannulation, all animals were anesthetized for terminal procedures during which *in-situ* measurements of intraventricular, right aortic, and pulmonary arterial pressures were made. To do so, anesthesia was induced, as above, and following induction of anesthesia, a 15-20 cm incision was made along an animal's ventral surface, superficial to the heart and great vessels. After blunt dissection of underlying musculature and connective tissue, the pericardium was exposed, which was entirely opened (often by cutting through sutures placed in the early summer of 2007) to expose the heart and outflow tract. Heparinized saline-filled polyethylene catheters (PE-90, SAI Infusion Technologies, Chicago, IL, USA) were simultaneously introduced non-occlusively in the RAo and LPA, through which they were passed, respectively, into the LV and RV. Intraventricular pressures were recorded for >2 minutes, after which the catheters were systematically moved downstream of the ventricles across the valves back into the RAo and LPA, where pressures were again recorded for >2 min.

Following hemodynamic assessment, the heart was removed, rinsed thoroughly with heparinized saline, blotted dry, separated into atria and ventricles, and weighed. Ventricles were saved in 10% neutrally buffered formalin for forthcoming diffusion tensor magnetic resonance imaging (DT-MRI) analyses. Sequentially, liver, stomach, intestines, and kidneys were removed,

thoroughly rinsed with saline, blotted dry and weighed. The intestine was measured lengthwise to the nearest 0.1 cm, after which it was separated into small and large intestines. At the midpoint of the small intestine, the small intestine was separated into "proximal intestine" (PI) and "middle intestine" (MI) and from the middle of each of these sections small (~0.5 cm) biopsies were removed and fixed in Trump's fixative (4% formaldehyde, 1% glutaraldehyde, in 10mM monobasic sodium phosphate and 6.75 mM sodium hydroxide; McDowell and Trump 1976) for histological analysis. Adjacent to these sections, additional ~1 cm sections were cut longitudinally to expose the mucosa, which was delicately rinsed with saline and scraped using a microscope slide into a centrifuge tube and immediately snap-frozen in liquid nitrogen and stored at -80 °C for enzymatic assays (see below). Biopsies were also removed from the middle of the large ("distal") intestine (DI) and were preserved for histology and enzyme assays as described above.

Preparation of gut tissue homogenates and enzyme assays

Frozen mucosal scrapings from each region were weighed (mass ± 0.001g) and homogenized as descried by German and Bittong (2009) and German et al.. (2014). Scrapings were homogenized in 3-15 volumes of 25 mM Tris-HCl, pH 7.5, with the supernatants of centrifuged homogenates preserved in 100-200 ul aliquots and frozen at -80 °C until thawed for use in maltase, aminopeptidase, and lipase assays as summarized below and as described in detail elsewhere (German and Bittong, 2009; German et al.., 2014).

All assays were spectrophotometric and absorbance measurements were recorded in a BioTek Synergy H1 Hybrid spectrophotometer/fluorometer (BioTek, Winooski, VT, USA), all assays were run at 30 °C (the temperature to which animals were acclimated), and all reagents

were acquired from Sigma-Aldrich (St. Louis, Mo, USA). Maltase activity was measured as described by German et al.. (2014) except that proximal and middle mucosal samples ran for 10 min before addition of assay reagent (Sigma GAGO20), whereas distal samples ran for 20 min due to low predicted maltase activity in that region of the gut. Maltase activity is expressed in U (nmol glucose liberated min^{-1}) per gram of mucosal scraping (Dalhqvist, 1968; German and Bittong 2009; German et al.., 2014). Aminopeptidase and lipase assays were performed exactly as prescribed by German and Biting (2009) and German et al.. (2014), where aminopeptidase activity is expressed as U (μmol p-nitroaniline liberated min^{-1}) per gram of scraping and lipase activity is expressed as U (μmol p-nitrophenol liberated min^{-1}) per gram of scraping.

Histological analysis

All tissues remained fixed in Trump's fixative >24 h, after which they were sequentially rinsed at 4 degrees C in 0.1 M phosphate buffered saline (thrice at 20 min per rinse), circulating ultra-pure water (overnight, ~12 h), and serially dehydrated in 25%, 50%, and 70% ethanol (30 min each) (German et al.., 2010). Samples were embedded in paraffin and sliced via microtome into 5 um sections, which were mounted and stained with hematoxylin and eosin (Mass Histology Services, Worcester, MA, USA). Slides were digitally imaged at 40x (Axioplan 2; Carl Zeiss AG, Oberkochen, Germany), and these images were "stitched" together using the Photomerge command in Adobe Photoshop (Version 13.0; Adobe Systems Inc., San José, CA, USA). The uncompressed merged images were imported into ImageJ, where absorptive surface area (the interface between brush border and lumen) was measured using the Wand tool and the smoothbore cylinder area was measured using the Segmented Line tool (Schneider and Rasband,

2012). We expressed relative surface area of gut regions as the ratio of absorptive surface area to the area of the smoothbore cylinder.

Statistical analyses

All variables except for small intestinal and pancreatic mass were analyzed with an analysis of covariance (ANCOVA) model in JMP (Version 7, SAS Institute, Inc., Cary, NC, USA) with animal mass as the covariate. Large intestine length was first log transformed and apparent digestive efficiency and hematocrit were first arcsine square root transformed before analysis with ANCOVA to meet the assumption of homogeneity of variance. Small intestinal and pancreatic mass data did not display homogeneity of slopes, so mass specific values were analyzed using ANOVA in JMP. Enzyme activities and relative gut surface area were analyzed using two-way ANCOVA with post-hoc Tukey's test in SPSS (Version 21, International Business Machines, Armonk, NY, USA). All models were considered significant at the level of $P<0.05$. The mouse energy regression plot and formula was produced using GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA, USA), and the slope (considered significantly different from 0 at the level of $P<0.05$) was analyzed using the software's linear regression analysis. All variables are reported as means ± s.e.m.

RESULTS

Digestion

There were no differences in any digestive metric between LAo-occluded animals and sham-operated controls (Table 3.1). There was no difference in relative fecal mass

($M_{feces(dry)}$:$M_{meal(wet)}$; $F_{2,7}$=1.9, *NS*) nor mass-specific energy content of feces ($F_{2,7}$=3.8, *NS*), indicating that neither absorption of nutrient mass nor nutrient energy was hindered by abolition of the R-L shunt. Because all animals were fed meals of similar energetic content, these results, in accordance with Equation 3.1, indicate that apparent digestive efficiencies of these two

Table 3.1. Nutrient absorption and meal transport

Digestive metric (unit)	Sham	LAo-occluded
Relative fecal mass ($M_{feces(dry)}$:$M_{meal(wet)}$)	0.036±0.004	0.037±0.003
Fecal energy content (kJ g^{-1})	14.4±1.3	13.2±0.8
Initial defecation (h post feeding)	58.9±7.6	83.4±30.4
Final defecation (h post feeding)	193.2±44.0	273.8±42.3

N=5 per group. An asterisk indicates a difference due to a significant effect of group (i.e. sham vs. surgical). Values are means ± s.e.m.

experimental groups were virtually identical (Fig. 3.3). The nearly-significant ANCOVA model for apparent digestive efficiency ($F_{2,7}$=4.3, *P*=0.6), suggested an effect of mass ($F_{1,7}$=8.5, *P*<0.05) but not group ($F_{1,7}$=0.03, *NS*) on ADE, i.e., larger animals from each group tended to have the highest ADE. Transit time was similar for the two groups, as both time to first defecation ($F_{2,7}$=1.3, *NS*) and time to final defecation ($F_{2,7}$=0.8, *NS*) were not significantly different (Table 3.1).

There were no differences in the size (length or mass) of digestive organs attributable to group effects (Table 3.2). Specifically, there were no differences in small intestine length ($F_{2,7}$=0.1, *NS*), large intestine length ($F_{2,7}$=0.5, *NS*), small intestine mass ($F_{1,8}$=0.2, *NS*), nor pancreas mass ($F_{1,8}$=0.2, *NS*). The ANCOVA model yielded significance for total intestinal length ($F_{2,7}$=6.0, *P*<0.05) due to an effect of mass ($F_{1,7}$=12.0, *P*<0.05) but not group ($F_{1,7}$=0.1,

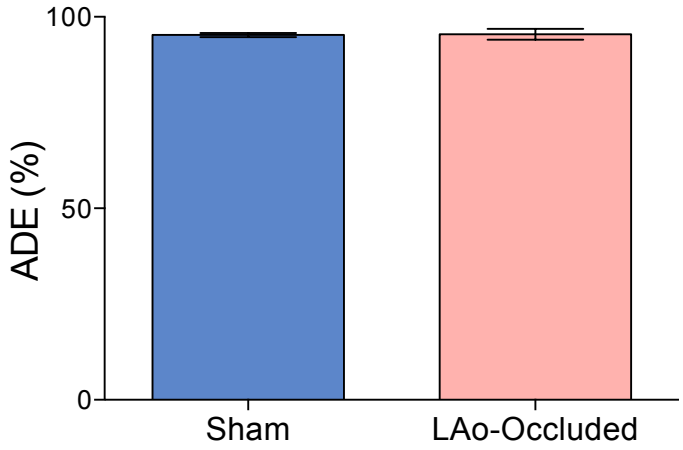

Figure 3.3. Apparent digestive efficiency in alligators with (Sham) and without (LAo-occluded) the pulmonary bypass (R-L) shunt. ADE was calculated according to Equation 3.1, where energy content of meals and feces were determined using bomb calorimetry. Average values (mean±s.e.m.) were virtually identical between groups (N=5 per group). There was a nearly-significant effect of size, but not group, indicating that larger animals tended to have higher ADE, but there was no consequence of surgically abolishing the LAo and R-L shunt.

NS), with similar findings for stomach mass (model - $F_{2,7}$=49.0, P<0.05; mass effect - $F_{1,7}$=97.8, P<0.05; group effect - $F_{1,7}$=0.0, *NS*), large intestine mass (model - $F_{2,7}$=18.1, P<0.05; mass effect - $F_{1,7}$=35.8, P<0.05; - $F_{1,6}$=1025.6, P<0.05; group effect - $F_{1,6}$=0.3, *NS*), and kidney mass (model - $F_{2,6}$=104.8, P<0.05; mass effect - $F_{1,6}$=184.7, P<0.05; group effect - $F_{1,6}$=4.0, *NS*). Clearly, removal of the LAo did not affect the gross anatomy of digestive organs.

At the tissue level, there were no differences in gut surface area revealed histologically (Fig. 3.4A). Differences in relative surface area ($F_{3,23}$=21.32, P<0.05) were due to effects of gut region ($F_{2,23}$=58.289, P<0.05) and mass ($F_{1,23}$=4.188, P=0.05), but not group ($F_{1,23}$=1.692, *NS*) nor an interaction of region and group ($F_{1,23}$=2.877, *NS*). On average, PI had roughly 470% more

Table 3.2. Gross anatomy of digestive organs

Organ size (unit)	Sham	Lao-occluded
Total gut length (cm)	61.2±6.7	60.6±6.1
Small intestine length (cm)	51.3±6.8	53.2±5.8
Large intestine length (cm)	8.8±0.6	7.4±1.4
Stomach mass (g)	11.8±0.8	11.4±0.9
Small intestine mass (g kg^{-1})	9.5±0.7	9.2±0.5
Large intestine mass (g)	19.4±4.1	17.6±4.0
Liver mass (g)	36.1±9.9	41.2±9.1
Pancreas mass (g kg^{-1})	0.45±0.095	0.60±0.070
Kidney mass (g)	10.2±2.5	13.0±2.5

N=5 per group, except N=4 for sham liver mass and N=4 for LAo-occluded kidney mass. An asterisk indicates a difference due to a significant effect of group (i.e. sham vs. surgical). Values are means ± s.e.m.

and 1000% more relative surface area than MI and DI, respectively, and MI had 540% more relative surface area than DI. We can therefore characterize the alligator gut as having progressively less absorptive surface area in the intestine, decreasing proximally to distally, but there was not a response to removal of the R-L shunt.

As with relative surface area, activity of the three digestive enzymes we assayed did not differ between groups, but two differed by gut region (Fig. 3.4B-D). There were differences in aminopeptidase activity (Fig. 3.4B; $F_{6,23}$=7.8, P<0.05), attributable to an effect of region ($F_{1,23}$=19.2, P<0.05), but not group ($F_{1,23}$=5.9, *NS*), an interaction between group and region ($F_{2,23}$=2.4, *NS*), nor mass ($F_{1,23}$=0.9, *NS*). Aminopeptidase activity was highest in the MI, which had 1.6-fold more activity than PI and 4.1-fold more activity than DI. The PI had 2.5-fold more activity than DI. There were nearly-significant differences in lipase activity (Fig. 3.4C, $F_{6,23}$=2.3, P=0.07), driven primarily by an effect of region ($F_{1,23}$=6.3, P<0.05), but not group ($F_{1,23}$=0.3,

NS), interaction of group and region ($F_{2,23}$=0.4, *NS*), nor mass ($F_{1,23}$=0.0, *NS*). Lipase activity was highest in the PI, with activity 1.8-fold higher than in MI and 2-fold higher than in DI. Again, while digestive enzyme activity differed by region of the gut, removal of the R-L shunt did not result in enzymatic differences between groups. Maltase activity (Fig 3.4D) differed significantly ($F_{6,23}$=8.5, P<0.05) by region ($F_{1,23}$=24.0, *P*<0.05), but not group ($F_{1,23}$=0.5, *NS*), interaction of group and region ($F_{2,23}$=0.9, *NS*), nor mass ($F_{1,23}$=0.9, *NS*).

Hemodynamics

We identified a suite of cardiovascular differences in those animals with chronically occluded left aortae (Table 3.3). 24 hours after surgical implantation of femoral arterial catheters, the LAo-occluded group exhibited a 77% higher resting f_H (model - $F_{2,7}$=12.1, *P*<0.05; mass - $F_{1,7}$=0.0, *NS*; group - $F_{1,7}$=24.3, *P*<0.05) and a 16% reduction in P_{sys} as compared to sham-operated controls (model - $F_{2,7}$=13.7, *P*<0.05; mass - $F_{1,7}$=22.2, *P*<0.05; group - $F_{1,7}$=6.4, *P*<0.05). These statistical differences did not persist 48 hours after surgery (f_H - F2,6=2.4, *NS*; P_{sys} - $F_{2,6}$=2.3, *NS*), but the trends were similar.

Within the heart, *in situ* acute preps (Figs. 3.4 and 3.5) revealed no differences in LV pressure ($F_{2,7}$=0.2 *NS*). RV pressure, however, was dramatically increased in the LAo-occluded animals, with peak RV systolic pressure 3.5-fold higher than in sham-operated controls (model - $F_{2,7}$=45.6, *P*<0.05; mass – $F_{1,7}$=0.6, *NS*; group – $F_{1,7}$=96.7, *P*<0.05). Immediately downstream of the LV, there was no difference in peak P_{RAo} between groups ($F_{2,7}$=0.1, *NS*) and immediately downstream of the RV, there was no difference in P_{LPA} between groups. Considering the markedly increased P_{RV} in LAo-occluded animals and the unchanged P_{LPA}, the P_{RV}-P_{LPA} difference (essentially the pressure difference across the cog-tooth valve), was 6-fold higher in

LAo-occluded animals than in controls (model – $F_{2,7}$=44.1, *P*<.0001; mass – $F_{1,7}$=0.7, *NS*; group – $F_{1,7}$=88.1, *P*<0.05). The pressure-overloaded RV of LAo-occluded animals resulted in an approximate doubling of ventricular mass (model - $F_{2,7}$=19,0 *P*<0.05; mass - $F_{1,7}$=20.3, *P*<0.05; group $F_{1,7}$=15.9, *P*<0.05). Differences in atrial mass ($F_{2,7}$=24.5, *P*<0.05) were largely due to an effect of animal mass ($F_{1,7}$=42.4, *P*<0.05), but there was a nearly significant effect of group ($F_{1,7}$=5.0, P=0.06). The LAo-occluded group had a Hct 33% lower than the sham group (model - $F_{2,7}$=5.0, *P*<0.05; mass - $F_{1,7}$=0.1, *P*<0.05; group $F_{1,7}$=129.0, *P*<0.05)

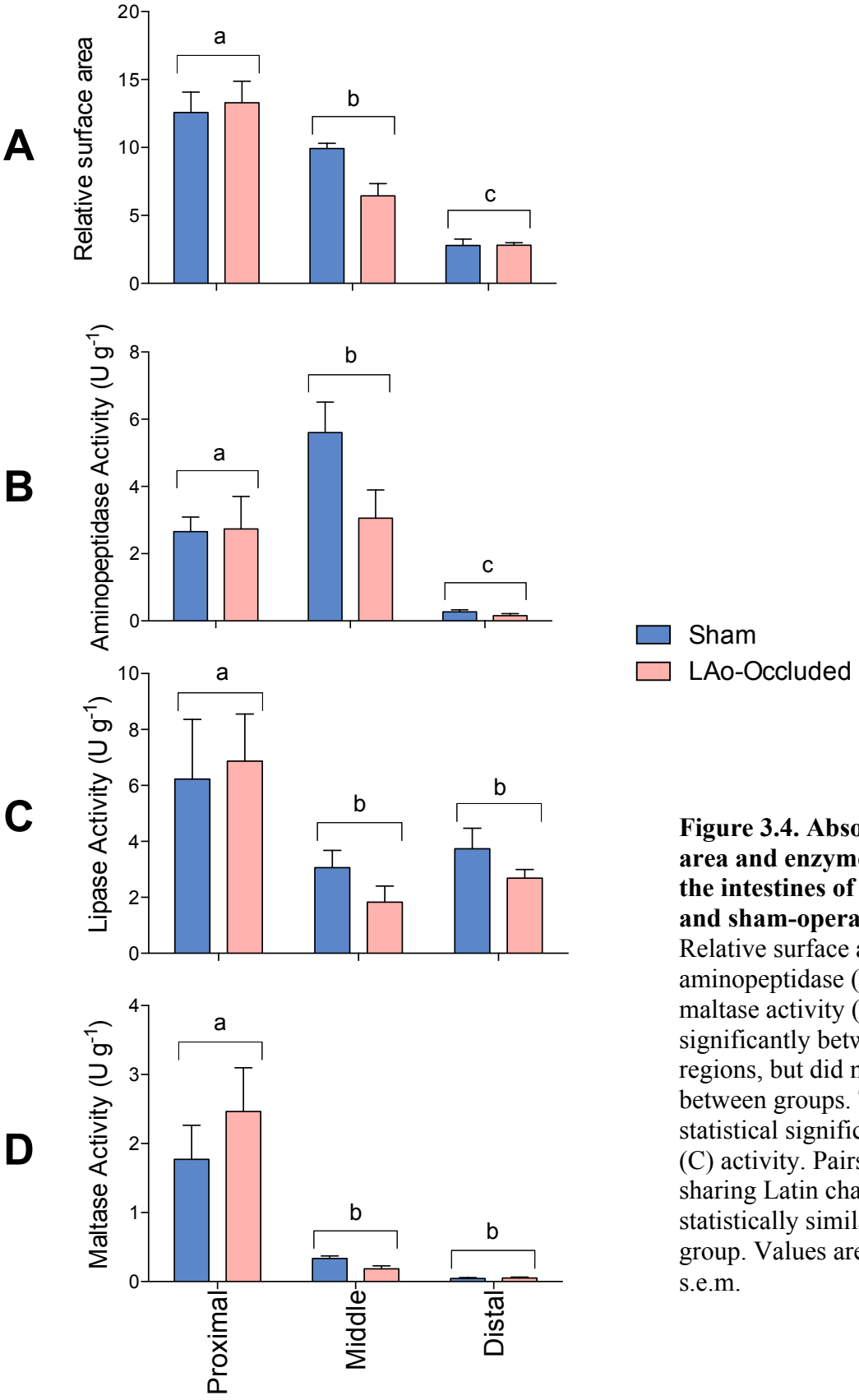

Figure 3.4. Absorptive surface area and enzyme activity in the intestines of LAo-occluded and sham-operated alligators. Relative surface area (A), aminopeptidase (B), and maltase activity (D) differed significantly between gut regions, but did not differ between groups. There was no statistical significance for lipase (C) activity. Pairs of bars sharing Latin characters are statistically similar. N=5 per group. Values are means ± s.e.m.

Table 3.3. Cardiovascular and hemodynamic parameters.

CV Parameter	Sham	Lao-occluded
Resting f_H – 24 hours (beats min^{-1})	7.8±0.4	13.8±1.1*
Resting P_{sys} – 24 hours (kPa)	3.1±0.3	2.6±0.2*
Resting f_H – 48 hours (beats min^{-1})	6.9±0.1	15.4±2.5
Resting P_{sys} – 48 hours (kPa)	3.0±0.3	2.0±0.7
Hct (%)	21.2±1.7	14.2±1.0*
Ventricle wet mass (g)	5.0±1.0	10.6±2.2*
Atrial wet mass (g)	1.6±0.4	2.2±0.4†

N=5 per group. An asterisk indicates a difference due to a significant effect of group (i.e. sham vs. surgical). A dagger represents a nearly-significant effect of group. Values are means ± s.e.m.

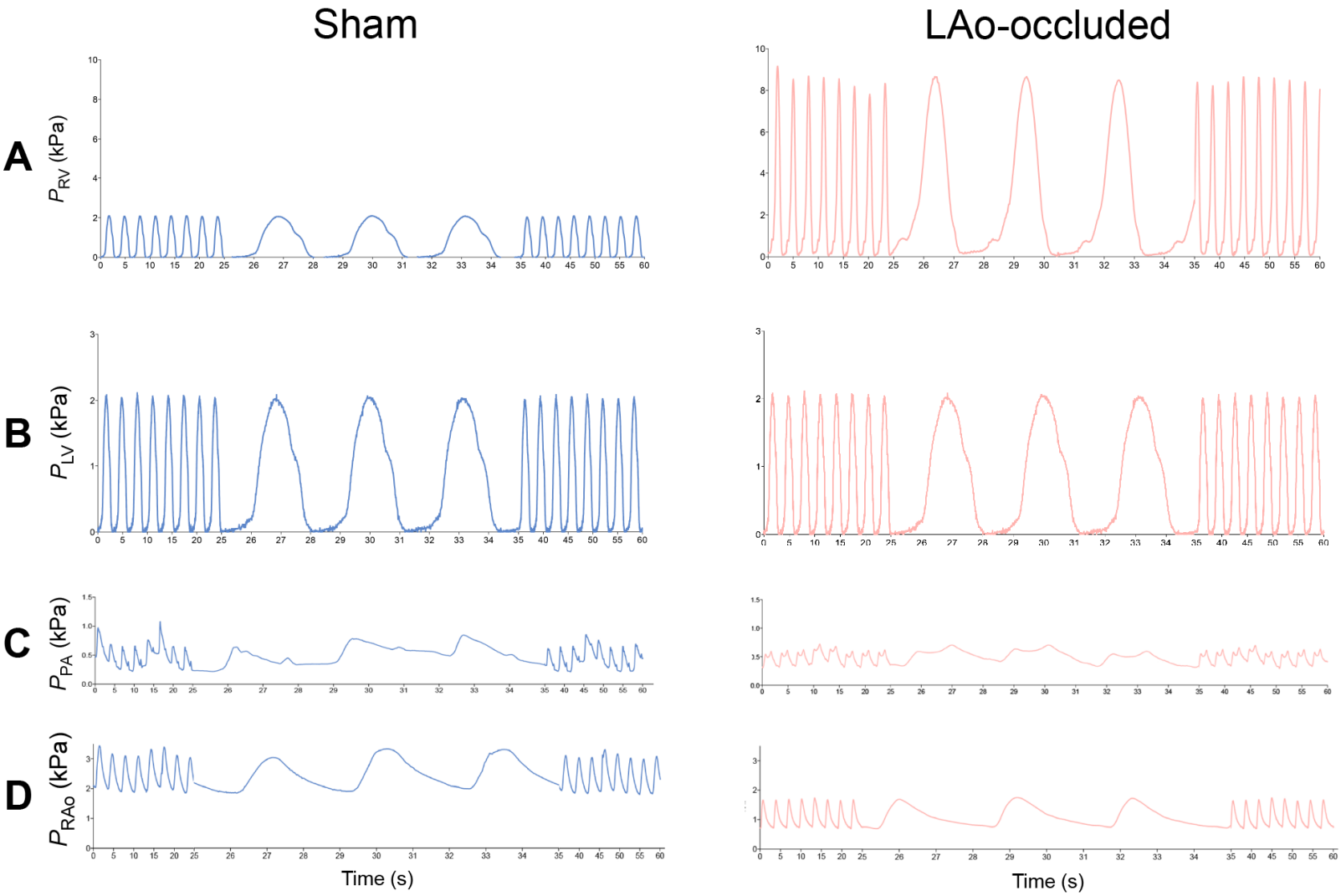
Sham
LAo-occluded
A
B
C
D
P_{RV} (kPa)
P_{LV} (kPa)
P_{PA} (kPa)
P_{RAo} (kPa)
Time (s)
Time (s)

Figure 3.5. (Previous Page) Pressure traces from *in-situ* acute preps of resting anesthetized alligators. In these representative traces, the 3-fold difference in peak right ventricular pressure (P_{RV}; A) between sham and LAo-occluded animals is clear. There are no significant differences, however, in peak left ventricular pressure (P_{LV}; B), peak pulmonary arterial pressure (P_{PA}; C), or peak right aortic pressure (P_{RAo}; D) between groups. In LAo-occluded animals, the P_{RV} trace represents a right ventricular hypertension presumably due to obstruction of the PA by the cog-tooth valve, subsequent isovolumic contraction, and the absence of the "pressure relief" function of the LAo. Despite statistically similar peak P_{PA}, the waveform found in LAo-occluded animals is characterized by a biphasic peak. We attribute this waveform to the high RV pressure which either briefly forces open the cog-tooth valve or is immediately transmitted to the pulmonary circuit following active opening of the cog-tooth valve. In any case, the cog-tooth valve is capable of protecting the pulmonary circulation from RV pressures >3-fold higher than normal physiologic values. P_{RV} and P_{LV} traces for each group are from the same animal and are synchronous. Likewise, P_{PA} and P_{RAo} recordings are synchronous traces from the same animal.

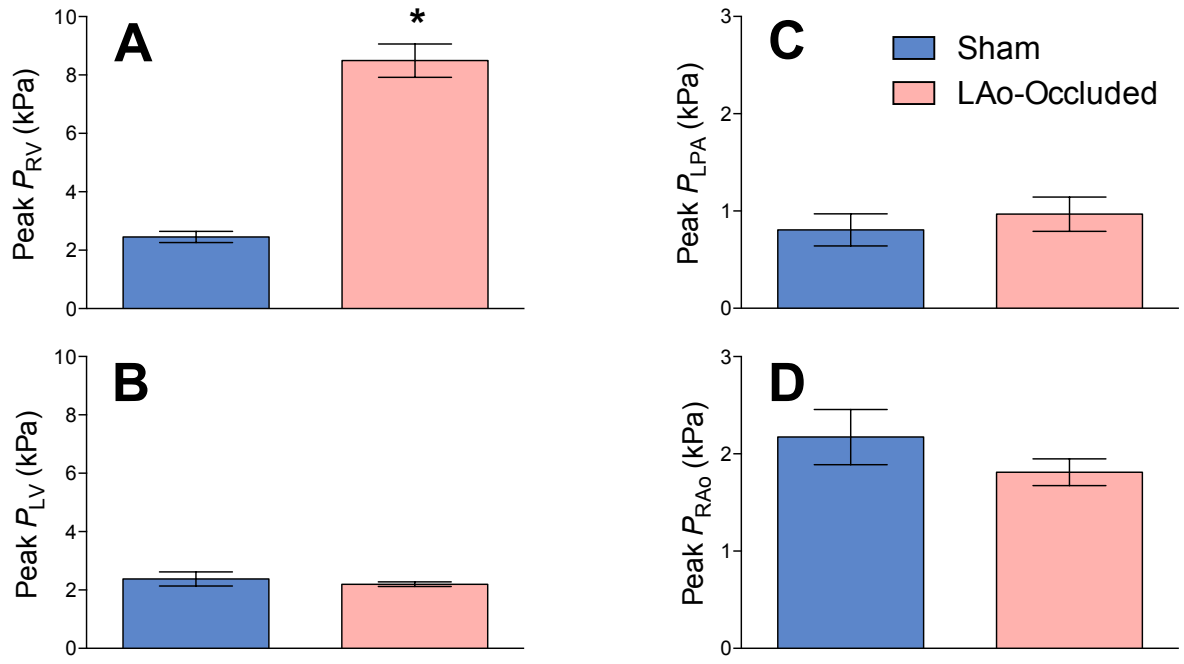

Figure 3.6. Peak pressures within the ventricles and great vessels of alligators with (sham) and without (LAo-occluded) the capacity for R-L shunting. Only peak right ventricular pressure (P_{RV}, A) differed significantly between groups and, combined with the similarity in peak left pulmonary arterial pressure (P_{RV} ,C), resulted in a statistically higher trans-valvular pressure gradient in LAo-occluded animals (see text). Neither peak left ventricular pressure (P_{RV}, B) nor peak right aortic pressure (P_{RAo}, D) differed between groups. N=5 per group for all measurements. Values are means ± s.e.m

DISCUSSION

This study provides additional evidence that the R-L pulmonary bypass shunt does not critically affect digestive processes by demonstrating that digestive performance is similar in groups of alligators that can and cannot generate a R-L shunt. This similarity in digestive performance is not due to differences in ADE and is not a consequence of structural or functional compensation by the digestive organs. Additionally, we provide evidence that alligators can tolerate even "ultra chronic" occlusion of the LAo; seven years after initial manipulation, outside of expected differences in intraventricular hemodynamics, there are no obvious deleterious consequences of ligating a major outflow tract and preventing a R-L cardiac shunt.

Digestive Similarities

During R-L shunting in crocodilians, the LAo delivers desaturated blood that is relatively acidic and hypercapnic to the digestive organs (Webb, 1979; Jones and Shelton, 1993). Because CO_2 availability is argued to be a primary determinant of the rate of gastric acid secretion (Kidder and Montgomery, 1974), the R-L shunt may enable faster rates of acid secretion in crocodilians (Jones and Shelton, 1993; Farmer et al.., 2008; Farmer, 2011) and therefore serve as an adaptive advantage of the pulmonary bypass shunt. Indeed, Farmer et al.. (2008) have demonstrated that surgical occlusion of the alligator LAo impedes digestion of bone in the relatively less acidic stomach lumens of manipulated animals. These ideas and evidence conflict, somewhat, with the work of others, who have demonstrated that the loss of the R-L shunt is either modestly (Eme et al.., 2010) or not (Jones and Gardner, 2010) detrimental to growth rate in crocodilians.

Here we have provided some explanations for the similarity in growth rates, namely, that digestive efficiency was virtually identical in those animals that can and cannot produce a R-L shunt (Fig. 3.3). Regardless, therefore, of what differences might occur in the stomach, animals with and without the R-L shunt are removing nearly identical amounts of both mass and energy from ingested meals. In fact, the ADE we report for LAo-occluded animals (95.2%) is not only statistically similar to that of sham animals (95.4%), but is quite high among vertebrates. These values are higher, for example, than those reported for captive estuarine crocodiles (*Crocodylus porosus*) (86.4% - Garnett, 1987; and 85.2% - Davenport et al.., 1989). These relatively high ADE figures are likely due to enzyme expression and activity optimized for the constant diet of these animals.

While digestive efficiency is an important indicator of digestive performance, the rate of net energy gain is more ecologically relevant, and is optimized by trade-offs between digestive efficiency and mean retention time (Sibly, 1981; Hume, 2005). In the present study, there were no significant differences in time to first or final appearance of feces associated with a given meal (Table 3.1). Mean retention time, therefore, did not differ between groups, but it was possible that this was due to compensatory responses in several of the determinants of retention time. These determinants include the particle size of digesta (as determined by oral processing), food intake rate, gut capacity and morphology, digestive enzyme activity, and area-specific intestinal absorption rates (Karasov and Diamond, 1985; Hume, 2005). Of these, oral processing (chewing) is minimal in reptiles and particle size would not be expected to differ between groups. Additionally, during *ad libitum* feeding, food intake rates in these groups of animals did not appear to differ (personal observation), and were statistically similar in another study (Jones

and Gardner, 2010). We did not assess rates of epithelial transport, but did examine the remaining variables: gut morphology and digestive enzyme activity.

We removed all digestive organs and found no differences in size (length or mass) of any organ (Table 3.2). The similarity in length and mass of all segments of the intestine suggested there was no difference in absorptive surface area between groups. This was confirmed histologically, and we report that there are no differences in relative surface area in any gut region attributable to removal of the R-L shunt (Fig. 3.4A). We can, however, report that absorptive surface area decreases on average along the length of the gut in alligators, which is in agreement with another recent publication (Tracy et al.., 2015). Given their similar food intake rates, and because we have found no differences in retention time or gut morphology between groups, digestive tract capacity (i.e. total digesta volume) should be considered similar in these two groups of animals. Lastly, we assessed enzyme activity of aminopeptidase, lipase, and maltase, which catalyze the breakdown of proteins, lipids, and disaccharides, respectively. Again, we found no differences in enzyme activity attributable to loss of the R-L shunt, but report differences along the length of the intestine, generally decreasing distally, similar to those found in crocodilians and other vertebrates (e.g. Tracy et al.., 2015; Fig. 3.4B-D).

Ultimately, we measured no differences in these determinants of retention time in animals with and without the R-L shunt. This suggests that the similarities we describe in transit time and ADE are not due to compensatory responses of animals suffering detriments associated with loss of the shunt. Clearly, there does not appear to be an effect of the pulmonary bypass shunt on rate of net energy gain, the most relevant metric of digestive performance.

Hemodynamics and Cardiovascular Responses to Ultra-Chronic LAo-Occlusion

Right ventricular pressure is known to increase almost immediately following surgical occlusion of the LAo, approaching values 300% higher than in unmanipulated controls (Eme et al.., 2010). In this study, we find that right ventricular hypertension has persisted since initial occlusion, with peak P_{RV} 3.5-fold higher than in unmanipulated animals (Fig. 3.4A). Impressively, the animals in this study tolerated this right ventricular hypertension for approximately seven years without experiencing any obvious detriment to overall health. This is somewhat surprising, because the typical response to elevated afterload in vertebrates is a compensatory enlargement of the ventricle serving to reduce wall stress associated with hypertension. This "pathologic" cardiac hypertrophy, in mammalian models, is characterized by irreversible alterations in chamber architecture, which are eventually subject to decompensating and subsequent failure (Dorn, 2007; Hill and Olson, 2008). Severe increases in pulmonary vascular resistance, i.e. restrictive pulmonary banding or, clinically, pulmonary embolisms, can result in almost immediate failure of the right ventricle. It is somewhat surprising, then, that in this model of dramatic right ventricular hypertension, there is not an obvious progression towards failure.

This right ventricular hypertension provides insight into what could be considered the most complex cardiovascular arrangement among vertebrates. Because both the PA and LAo stem from the RV, occlusion of the LAo might be expected to result in complete diversion of blood from the right ventricle into the pulmonary artery during systole, with predicted peak P_{RV} and peak P_{PA} being essentially equal. In crocodilians, however, the right ventricle is capable of generating pressures adequate for systemic perfusion, (doing so with flow through the autonomically-controlled Foramen of Panizza; e.g. Shelton and Jones, 1991; Jones and Shelton,

1993; Axelsson et al.., 1996; Fig. 3.1), but transmission of this systemic pressure into the pulmonary circuit would likely result in pulmonary edema and reduced pulmonary gas exchange (Burggren, 1982). It is the action of the cog-tooth valve, a morphological feature specific to the crocodilians, which prevents transmission of elevated P_{RV} into the pulmonary circulation. This valve, with nodules of connective tissue fitting together like cog teeth, can obstruct the pulmonary artery, enormously increasing pulmonary vascular resistance and preventing pulmonary arterial flow (e.g. Franklin and Axelsson, 2000; Axelsson et al.., 2006). In this capacity, the LAo and, consequently, the R-L shunt, serves as a "pressure release," preventing the pulmonary circulation from exposure to systemic pressures. Of course, occlusion of this vessel results in loss of this pressure release. Chronic occlusion of the LAo, therefore, also serves as a model for determining the protective capacity of the cog-tooth valve. In this study, in addition to the RV hypertension of LAo-occluded animals, we report no differences in peak pulmonary arterial pressures between groups and, consequently, a significant difference in P_{RV}-P_{PA} between groups, with peak pressure across the cog-tooth valve approximately 5-fold higher in LAo-occluded animals (Table 3.3). This indicates that, indeed, the cog-tooth valve appears capable of protecting the pulmonary circulation from pressures 3.5-fold higher than "typical" P_{RV}, and is capable of doing so for an extended period of time (~7 years).

Consequently, however, the right ventricle has been chronically exposed to markedly elevated peak systolic pressures, and the compensatory response is impressive. We found an approximate doubling of ventricular mass in animals with ligated left aortae as compared to sham-operated controls. This change in ventricular mass is among the highest reported for vertebrates, and is likely due primarily to afterload-induced compensatory hypertrophy which should probably be characterized as "pathologic" despite the absence of any apparent deleterious

effects. Evidence suggests this ventricular enlargement may also involve some degree of hypoplasia (Eme et al.., 2010).

Lastly, we examined f_H and P_{sys} in animals with and without the capacity to generate a R-L shunt. We found a modest difference in P_{sys} at 24 hours after surgical implantation of catheters, with RAo-occluded animals exhibiting a 16% lower P_{sys} as compared to sham-operated controls. By 48 hours post-surgery, there were no significant differences between groups, but a similar trend persisted. The relative hypotension may be due to action of B-type (Brain) natriuretic peptide (BNP), which is secreted by ventricular myocytes in response to afterload-mediated stretch and serves to reduce blood volume and blood pressure via natriuresis and diuresis (Toop and Donald, 2004). The relative tachycardia in LAo-occluded animals (78% difference between groups 24 h after cannulation) may be a barostatic response to this BNP-mediated hypotension, but is more likely due to the enigmatic anemia we found in LAo-occluded animals (33% reduction in Hct as compared to shams). This reduction in Hct is probably not due to the action of BNP which serves only to reduce blood volume and, if anything, increase Hct. We have considered that loss of the R-L shunt may affect iron metabolism, and stomach pH does have an effect on iron liberation and absorption in reptiles, but this is apparently only true for herbivorous species (Koelz, 1992). It is unlikely, then, that loss of the R-L shunt affects iron absorption and hematopoiesis. Reductions in Hct can serve to reduce total peripheral resistance, but we do not propose a mechanism linking right ventricular hypotension to plasma volume.

General conclusions

In this series of studies, we examined digestive performance and hemodynamics of animals with 7-year "ultra-chronic" occlusion of the LAo, the major outflow tract usually

permitting establishment of a R-L shunt, and we report no obvious detriment to animal health. This includes the presence of a sizable right ventricular hypertension which is apparently tolerated, with no direct impact on pulmonary arterial pressure, via compensatory enlargement of the ventricle. Despite the prevalent hypothesis that the R-L shunt facilitates digestion through delivery of hypercapnic and acidic blood to the digestive organs, we find no differences in digestive efficiency or transit time (which is, itself, a product of similar gut morphology and enzyme activities).

These findings dovetail nicely with earlier work demonstrating that despite proximate results of occlusion of the LAo, there have been no long-term deleterious consequences of prohibiting R-L shunting in crocodilians. For example, removal of the shunt affects stomach pH (Farmer et al.., 2008), but not net rates of energy intake (this study) or growth rate (Eme et al.., 2010; Jones and Gardner, 2010). Removal of the shunt affects cardiovascular morphology (Eme et al.., 2010; this study) but not gas exchange while diving (Eme et al.., 2009). Removal in the shunt of embryos affects blood flow to the gas exchange organ, but not overall oxygen consumption (Eme et al.., 2011). Similarly, surgical ablation of the R-L shunt in rattlesnakes (*Crotalus durissus*) failed to cause any detriment to metabolic rate or growth rate (Leite et al.., 2013). Even surgical occlusion of the RAo, the "major" outflow tract, in caimans (*Caiman crocodilus*), does not result immediately in reduced food intake and growth rates (Jones and Gardner, 2010; though communication between LAo and RAo was still permitted at the FoP and anastomosis in this study). Summarily, these studies provide support for the idea that cardiac shunting is not an adaptive trait conveying fitness but rather an ancestral condition against which no selective pressures have acted.

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CONCLUSIONS

The response to digestion of sit-and-wait predators is determined by complex, actively regulated relationships between visceral organs. Along the cardiac-gastrointestinal axis in these intermittently-feeding ectotherms, in addition to the well-described plasticity of digestive organs, both evolutionarily-derived cardiovascular morphologies and cardiac plasticity have been hypothesized to support the sometimes-monumental processes of digesting large intact meals, absorbing their nutrients, and synthesizing new biomolecules.

The bulk of these energetically expensive processes are "fueled" aerobically, and the digestive and accessory digestive organs are therefore expected to experience high oxygen demand during digestion of large prey. Furthermore, in the presence of the large influx of nutrients, there is a significant need to distribute both substrates and newly-synthesized molecules throughout the splanchnic bed and the systemic circulation. To support this demand, postprandial gastrointestinal hyperemia occurs across a large number of lower vertebrates, which, in addition to increasing oxygen delivery, provides the requisite flow for distribution of metabolic substrates and products. This hyperemia is generally accomplished by increasing total cardiac output (which occurs as result of both elevated heart rate and stroke volume) and/or by preferential distribution of flow to the gut. It is in determining the quantity and quality of blood flow to the gut that both cardiac plasticity and innate cardiovascular arrangements are hypothesized to confer adaptive benefits. In this dissertation I have investigated both of these potential mechanisms.

What triggers postprandial cardiac hypertrophy in Burmese pythons and other animals?

Gastrointestinal hyperemia can be supported by actively-controlled increases in cardiac output. As is common in mammalian models, the heart, when confronted with long-term elevations in preload, exhibits hypertrophy, a phenomenon which results in pronounced changes in ventricular architecture and a more long-term (but reversible) elevation in stroke volume. Among vertebrates, the most impressive cardiac hypertrophy occurs (at least occasionally) in Burmese pythons, where, following feeding, the heart can grow by as much as 40% within 48 hours. While work has indicated that high circulating levels of free fatty acids are a trigger for postprandial cardiac hypertrophy in pythons, postprandial cardiac hypertrophy is not universal among Burmese pythons subjected to virtually identical experimental protocols. In considering why this response may be facultative rather than obligatory, it seemed worthwhile to consider that the heart might respond directly to hemodynamic challenges, as it does in other vertebrate models. In the first chapter of this dissertation, we hypothesized that postprandial cardiac hypertrophy could be triggered by a "low O_2" signal which, as mediated through autonomic control, would affect postprandial hemodynamics. I found, in those animals confronted simultaneously with high oxygen demands (during digestion) and low oxygen supply (because they had been rendered anemic), a 40% ventricular enlargement as compared to fasted controls. My hypothesis that postprandial cardiac hypertrophy occurs in response to low oxygen availability was supported; there is likely a "low O_2" threshold below which postprandial cardiac hypertrophy is stimulated due to dramatically altered postprandial hemodynamics.

While I successfully determined an additional (and perhaps the primary) trigger for postprandial cardiac hypertrophy, it remained unclear to what extent other animals could utilize this approach to augment cardiac output during digestion of large prey items. I elected, in the

research presented in the second chapter of this dissertation, to examine postprandial American alligators. I hypothesized that alligators would exhibit postprandial cardiac hypertrophy, if not innately, than when confronted with a hypoxic challenge during digestion. Alligators do feed intermittently and show some similarities to pythons in their digestive response, but ultimately digestion of a meal is not as dramatic in alligators as it is in pythons. We found only modest differences in the cardiovascular performance of fed anemic animals as compared to fasting controls, and those differences were surprisingly due to feeding alone, and not to anemia, indicating that for a given state, alligators have excess delivery capacity. Reflecting the modest differences in cardiovascular function, there were no differences in the gross anatomy of the heart. Essentially, alligators are operating well above the threshold level of O_2 depletion required to stimulate postprandial cardiac hypertrophy. Surprisingly, there were very few differences in the gross anatomy of visceral organs throughout the cycle of fasting and feeding, indicating that alligators do not down-regulate the size and function of their visceral organs to the same extent as other renowned sit-and-wait predators.

Does the R-L shunt affect digestive performance?

While alligators apparently do not utilize phenotypic plasticity to facilitate and/or improve digestive performance, they are hypothesized to utilize the R-L shunt, a character found in all non-avian reptiles, to aid digetion. They are hypothesized to do so by altering either the quantity or quality of blood delivered to the splanchnic bed, providing substrates for acid secretion in the stomach and anabolism in the liver, or by differentially providing additional blood supply to the digestive organs through the use of the left aorta. In the third chapter of this dissertation, using animals with “ultra-chronic” (7 year) occlusion of the left aorta, I examined

digestive performance in animals fed intact rodent meals. Ultimately, we found no differences in any metric of digestive performance from the whole organism level down to the molecular level. In fact outside of the right ventricle, which was massively hypertrophied, there seemed to be no consequence of chronic occlusion of a major outflow tract. This supports earlier studies demonstrating no effect of abolishment of the R-L shunt on growth or food intake, and contributes to an expanding body of literature arguing that there may be no functional consequence or adaptive advantage to the utilization intracardiac shunts.

While these two potential adaptive mechanisms (postprandial cardiac hypertrophy and R-L shunting) are discussed here in isolation, they are not mutually exclusive and may or may not occur simultaneously in individuals of various species of non-avian reptile. I mention this here to illustrate how remarkably complex and diverse the morphology, physiology, and regulation of the reptilian heart actually is. It is, therefore, worthwhile to study the field of comparative cardiorespiratory physiology not because it has implications for human health (which it might), but because over time "traditional" physiological and surgical manipulations along with molecular, imaging, telemetric, and still-uninvented techniques are sure to reveal remarkable detail about the evolution, function, and regulation of the vertebrate heart. This dissertation, I hope, contributes to that discovery.