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The Interaction of Elevated Metabolic States in Snakes

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Alexander G. S. Jackson

Dissertation Committee:
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2014

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ABSTRACT OF THE DISSERTATION

The interaction of elevated metabolic states in snakes

By

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Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor James W. Hicks, Chair

Physiologists have traditionally studied elevated metabolic states in isolation (e.g., the physiological response to physical activity). However, under natural conditions animals may perform more than one elevated metabolic state simultaneously (e.g., predator avoidance while processing a recently ingested meal) and the integrated response to such a condition is not well understood.

For my dissertation work, I used oviparous (*Lamprophis fuliginosus*) and viviparous (*Thamnophis marcianus*) snakes to test whether the O_2 delivery capacity of these species' cardiopulmonary systems would be sufficient to meet the O_2 demands from reproduction, physical activity, and digestion simultaneously (i.e., additivity). Flow-through respirometry was used to measure levels of gas exchange (i.e., oxygen consumption, $\dot{V}O_2$; and carbon dioxide production, $\dot{V}CO_2$) during standard conditions (i.e., standard metabolic rate, SMR), and then during reproduction, activity (while fasting), digestion of a large (i.e., 10 to 20% body mass) meal, and post-prandial activity.

Results indicate that reproduction causes significant increments in $\dot{V}O_2$ for females of both species. Levels ranged from 1.4 to 3.2 fold above SMR. During physical activity (while

fasting), males and females of both species exhibited peak factorial increments in $\dot{V}O_2$ that ranged from ~6 to 9.6 fold above SMR; digestion resulted in peak factorial increments of ~3 to 6 fold above SMR. Contrary to predictions, males and non-reproductive females of both species exhibited a prioritization pattern of O_2 delivery during post-prandial activity (i.e., factorial increments in $\dot{V}O_2$ were similar to or lower than levels attained during activity while fasting). When reproduction and digestion were combined, female *Lamprophis fuliginosus* exhibited a prioritization pattern of O_2 delivery, while *Thamnophis marcianus* exhibited a more-than-additive pattern of O_2 delivery (i.e., there was a 30-50% increase in $\dot{V}O_2$ above non-reproductive levels). Contrary to predictions, during the combination of reproduction and post-prandial activity females of both species exhibited a prioritization pattern of O_2 delivery.

Overall, results from my dissertation work indicate that these species' cardiopulmonary systems' capacity for O_2 delivery is limited to increments of approximately 9 to 10 fold above SMR. In most cases, activity performance (i.e., time to exhaustion, TTE) was maintained, possibly at the expense of digestive and reproductive processes.

INTRODUCTION

Animals are open systems that are constantly in contact with a fluctuating external environment; however the essential biosynthetic, transport, and mechanical functions that are necessary to support life operate optimally under narrow physical conditions (e.g., ion, nutrient, and gas concentrations; temperature and pH). Therefore, animals have evolved redundant feedback loops between co-operating organ systems to maintain fairly stable conditions around biologically relevant set points – termed homeostasis (Bernard, 1878; Cannon, 1929). Much of the energy required to maintain internal conditions at fairly stable levels, in spite of environmental (e.g., cold weather, hypoxia, etc.) and physiological (e.g., illness, digestion, intense physical activity) perturbations is derived from the hydrolysis of adenosine tri-phosphate (ATP). However, because ATP is not stored in large quantities, there is continual transfer of energy from stored macromolecules (i.e., carbohydrates, lipids, and proteins) into the recycling of ATP through a complex series of enzyme mediated oxidation reactions (Atkinson, 1977). Under aerobic conditions, the vast majority of ATP synthesis takes place through oxidative phosphorylation, where the products from the above mentioned reactions (i.e., nicotinamide adenine dinucleotide, NADH; and flavin adenine dinucleotide, FADH) are oxidized in the electron transport system (where O_2 serves as a terminal electron acceptor) (Mitchell, 1961).

Based on the Law of Hess, if the quantity of heat produced and O_2 consumed in the complete oxidation of carbohydrates, fats, and proteins (i.e., 18.8 to 20.9 kJ per liter of O_2; Brown and Brengelmann, 1965) is known, the rate of whole-animal uptake of O_2 (i.e., rate of oxygen consumption, $\dot{V}O_2$) by the respiratory organs (i.e., skin or lungs) can be measured using indirect calorimetry, and converted into energy equivalents (i.e., Kilojoules) (Lusk, 1928; Kleiber, 1961). Indirect calorimetry can also be used to estimate the primary metabolic substrate

currently being utilized to form ATP (i.e., carbohydrates, fats, or proteins) if CO_2 production per

unit time (i.e., $\dot{V}CO_2$) is simultaneously measured (Kleiber, 1961). In vertebrates with a closed

circulatory system, O_2 is transported from the atmosphere to individual cells (and vice versa for

CO_2) by the cardiopulmonary system, which consists of four transport steps that function in

series: ventilation, diffusion of oxygen from the atmospheric into the blood, circulation, and

diffusion of oxygen from the blood into individual cells (Wang and Hicks, 2001). The structures

and functions of the oxygen transport cascade for vertebrates with a closed circulatory system are

presented in Figure 1.1.

The stead-state $\dot{V}O_2$ that is utilized by a vertebrate to maintain homeostasis under

"standard" conditions is defined as the standard metabolic rate (SMR) (Kleiber, 1961;

Hochachka and Somero, 2002). The standard conditions under which SMR is measured

typically include: adult individuals that are non-reproductive, inactive, and postabsorptive;

measurements take place within the species' thermal neutral zone (or preferred body

temperature) and during the inactive phase of the photocycle. A number of factors can influence

SMR, including: body size (i.e., SMR scales to the ~0.75 power of the animals mass; Kleiber,

1932; Bennett and Dawson, 1976) ontogenetic stage (i.e., neonates and juveniles tend to have

larger SMR values than sexually mature and reproductively inactive adults; Bennett and

Dawson, 1976), and species. Regarding this latter factor, one of the most fundamental

differences in SMR exists between endothermic vertebrates (i.e., mammals and birds) and

ectothermic vertebrates (i.e., fish, amphibians, reptiles). The SMR of an endothermic vertebrate

is generally 5 to 10 fold larger than a similarly sized ectotherm - a difference that is related to

higher rates of heat production coupled with morphological features (i.e., fur, subcutaneous fat,

feathers) that serve to lower thermal conductance (Kleiber, 1961; Bennett and Dawson, 1976).

To simplify discussion, the remainder of this introductory chapter will focus on ectothermic vertebrates, with a specific emphasis on reptiles (i.e., Crocodilians, Testudines, and Squamates).

Under natural conditions, ectothermic vertebrates rarely function under "standard" conditions. Instead, they exist within dynamic natural habitats (e.g., fluctuating levels of temperature, humidity, sunlight, and gas partial pressures) and must perform a suite of functions (e.g., physical activity, digestion, and reproduction), sometimes simultaneously, in order to survive and ultimately procreate. For most species, an elevation of body temperature by 10ºC results in an approximately two to three fold increase in O_2 demand by metabolically active tissues (Bennett and Dawson, 1976). More profound increments in tissue oxygen demand (i.e., ~5 to 10 fold) are elicited in during the functional states of physical activity, and in some species, digestion (Bennett and Dawson, 1976; Gleeson and Bennett, 1985; Secor, 2009). The physiological processes associated with reproduction also elevate O_2 demand above SMR levels (i.e., ~1.3 to 2.9 fold) (e.g., Boehlert et al., 1981; Birchard et al., 1984; Ellis and Chappell, 1987).

To match tissue metabolic demand with O_2 supply, rapid adjustments in the convective components of the oxygen transport cascade occur. These adjustments include elevations in the rates of ventilation (the product of breathing frequency and tidal volume) and circulation (the product of heart rate and stroke volume). Moreover, by elevating perfusion of the pulmonary surface (e.g., reduced ventilation/perfusion heterogeneity in the lungs) and/or decreased R-L shunt fraction, extraction of O_2 from ventilated air may increase, resulting in higher arterial PO_2, and thus a steeper O_2 diffusion gradient at the tissues (Gleeson and Bennett, 1985; Hicks and Wang, 1996). If O_2 demand is chronically elevated (i.e., for days to weeks) phenotypic adjustments (e.g., ventricular hypertrophy, increased blood volume, increased hematocrit,

increased capillary density) may serve to increase tissue diffusive capacity and O_2 delivery (Wagner, 1996).

Under natural conditions, ectothermic vertebrates may perform more than one functional state simultaneously (e.g., locomotion while digesting a large meal), but the integrated response to simultaneously occurring functional demands is not well understood (Jackson, 1987). This introductory chapter will first review the individual factors that influence metabolism, and then examine the patterns of interaction that may occur when more than one functional state is combined simultaneously. More specifically, the effects of temperature and body size on metabolism will be reviewed. Next, the metabolic consequences and physiological mechanisms associated with functional states (i.e., physical activity, digestion, and reproduction) will be reviewed. Finally, the integrated response to simultaneously occurring functional states will be reviewed, and a model system for such examinations (i.e., snakes) will be proposed.

Temperature

Many physical and biochemical processes are strongly influenced by temperature and generally double or triple in rate with every 10°C change (i.e., a Q^{10} of ~2 to 3) (Hochachka and Somero, 2002). For example, $\dot{V}O_2$ increases by a factor of 2 to 3 for every 10°C increase in body temperature in reptiles (Bennett and Dawson, 1976). This generalization can be extrapolated to many whole-animal functions. For instance, maximal sustainable locomotor speed/endurance of *Iguana Iguana* increases approximately three fold between 25ºC and 35ºC (Moberly, 1968). Similarly, rates of digestion and passage rate of food through the stomach/intestine increased by approximately two fold between 20ºC and 30ºC in *Charina bottae* (Dorcas et al., 1997).

Unlike endotherms, which produce sufficient metabolic heat and exhibit morphological features for its retention (e.g., subcutaneous fat, fur, and feathers), ectothermic vertebrates must behaviorally thermoregulate to attain appropriate body temperature values that optimize physiological functions. Reptilian thermal optima are not static for all functional states. For example, previous work on *Thamnophis elegans* demonstrated that maximal rates of digestion, crawling, and swimming occur at Tb values that range from 28.5°C to 34.5°C (Stevenson, et al., 1985). A commonly used index to explore thermal optima in reptiles is through measurement of the species' preferred body temperature (PBT) - defined as the mean Tb selected by a reptile located within a linear thermal gradient (Dawson, 1975; Huey, 1982). To illustrate the relationship between body temperature, performance, and PBT, Figure 1.2 presents results for the lizard *Sceloporus undulates* (Angilletta et al., 2002) that is typical of many species of squamate reptiles.

Reproductive processes are sensitive to temperature. For instance, the thermal regimen selected by (or forced upon) reproductive females affects reproductive cycle time (Beuchat, 1988) and immune function (Michel et al., 2013). Moreover, during development, the thermal regimen experienced by embryos affects offspring morphology (i.e., body size and shape; Shine, 1995), behavior (i.e., activity levels and basking behavior; Burger, 1989), and performance characteristics (i.e., running speed and endurance; van Damme, et al., 1992). The PBT values associated with the reproductive processes may differ from non-reproductive values, and a number of studies have investigated these shifts (see Table 1.3). However, results from this work do not indicate a consistent trend. For example, *Natrix natrix* exhibits a 2.2ºC decrease in PBT, *Crotalus viridis oreganus* does not change PBT, and *Crotalus horridus* exhibits a 4.4ºC increase in PBT during reproduction (Issac and Gregory, 2004; Gardner-Santana and Beaupre,

2009; Charland and Gregory, 1990). General conclusions from this work are difficult to make

due to variations in experimental technique (e.g., cloacal probes *versus* surgically implanted data

loggers), operative temperature range (i.e., field *versus* laboratory gradients), frequency of

measurements (e.g., at just one point during the reproductive cycle), and the lack of a correlation

between the measurement and the underlying phase of the reproductive cycle (e.g., using

ultrasonography; Tu and Hutchinson, 1994).

Body Size

In vertebrates, whole-animal metabolic rate (i.e., $\dot{V}O_2$) scales with body mass according

to the following equation: $VO=aM^b$, where *a* is the mass coefficient, *M* is body mass, and *b* is

the scaling exponent (Rubner, 1883; Kleiber, 1932). For reptiles, the scaling exponent in this

relationship varies between 0.77 and 0.80 (depending on the temperature of the measurements)

(Bennett and Dawson, 1976). This relationship is similar (i.e., with a scaling exponent of ~0.75)

for most vertebrate taxa, and for most organisms in general (Kleiber, 1961; Hemmingsen, 1960;

Schmidt-Nielsen, 1984). Thus, the SMR of endotherms and ectotherm may differ by a factor of

five to ten, but species within these groups have convergently evolved a similarity in the

relationship (i.e., the scaling exponent, *b*) between metabolic rate and body size. As a

consequence of this relationship, a considerable fraction (e.g., up to ~90% in squamate reptiles;

Bennett and Dawson, 1976; Andrews and Pough, 1985) of intra and interspecific variation in

metabolic rate (assuming standard conditions) can be attributed to discrepancies in body size.

Physical Activity

Most species of reptiles are tetrapods and propel themselves via a sprawling locomotion

modality in which the limbs sweep laterally along the body in various gait patterns (e.g., lateral

sequence walking, trots, and diagonal sequence runs) (Reilly and Delancey, 1997). On the other hand, snakes (in addition to amphisbaens and some species of lizards) have evolved elongate bodies and a reduction in limbs, and therefore possess unique mechanisms to perform bodily movements in terrestrial habitats. Snakes propel themselves using lateral undulation, concertina locomotion, and/or sidewinding movement modalities (Mosauer, 1932). Most species of snakes perform more than one locomotion modality simultaneously, but lateral undulation is used dominantly in most above-ground environments, while concertina locomotion occurs dominantly in subterranean habitats (e.g., tunnels) (Gans, 1974). For lateral undulation, axial bends form around objects on the substratum (e.g., rocks, grass etc.) and the snake is ultimately propelled forward through sinusoidal waves of posteriorly propagating axial musculature activity along the trunk (Gray, 1946; Jayne, 1986; Jayne, 1988; Moon and Gans, 1998). In contrast, during concertina and sidewinding locomotion, certain portions of the body are moved forward while others maintain static contact with the substrate (Gans, 1974; Jayne, 1986).

For all locomotion modalities, propulsion is derived from muscular forces acting on the skeletal lever system, causing bones to move about on the joint axes (McArdle et al., 1986). The formation of muscular tension is ultimately derived from active shortening of the muscle fibers through the mechanism of cross bridge cycling (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). As activity performance increases (i.e., speed/exertion) additional motor units are recruited, resulting in proportionally larger energetic demands by the active skeletal muscle fibers (Taylor et al., 1970). This relationship is illustrated in Figure 1.3 for lizards of the genus *Varanus* under a typical treadmill activity protocol. Decrements in skeletal muscle tissue arterial PO_2 and the release of local factors (i.e., CO_2, adenosine, lactate, K+ ions) trigger arterial dilation (i.e., active hyperemia), resulting in considerable increments in muscle blood flow

(Bockman, 1976; Musch, et al., 1987; Williams and Leggett, 1989). To match O_2 delivery with tissue demand, cardiopulmonary output increases proportionally with $\dot{V}O_2$. Reptiles exhibit significant increments in both cardiac output (e.g., 3 fold in *Python molurus;* ~2 to 3 fold in *Varanus exanthematicus* and *Iguana iguana*) and minute ventilation (e.g., 24 fold increase in *Python molurus*; ~2 to 8 fold increase in *Varanus exanthematicus* and *Iguana iguana*) during physical activity (Secor et al., 2000; Gleeson et al., 1980).

Like most vertebrates, reptiles exhibit peak factorial increments in $\dot{V}O_2$ during physical activity of 5 to 10 fold above SMR (Brett, 1972; Gleeson and Bennett, 1985; Jones, 1994; Bishop, 1999). A list of previously published values for snakes is presented in Table 1.4. Although the factorial increments in $\dot{V}O_2$ are similar between vertebrate lineages, the aerobic capacity of a reptile is approximately $1/10^{th}$ the magnitude of a similarly sized mammal or bird (Bennett, 1994). The remaining energy requirements for activity performance are derived from anaerobic metabolic pathways (e.g., in snakes ~50 to 90% of energy for activity is produced from anaerobic glycolysis) (Ruben, 1976; Gleeson and Bennett, 1982; Gratz and Hutchison, 1977). As a result, muscle and liver glycogen stores are rapidly depleted, and lactic acid accumulate in the muscles and bloodstream (e.g., ~5 to 15 fold increase in blood lactate concentration; Ruben, 1976; Gratz and Hutchison, 1977; Gleeson and Bennett, 1982).

To buffer the metabolic acidosis, reptiles release bicarbonate-derived CO_2 from the lungs. As a consequence, RQ values during physical activity in reptiles routinely exceed 1.0 (e.g., RQ values of 1.5 to 2.2 in a number of lizard species; see references in Gleeson and Bennett, 1982). Moreover, the air convection requirement (VE/ $\dot{V}O_2$) nearly doubles over resting values (Wang et al., 1997; Hicks et al., 2000; Secor et al., 2000). Following the

completion of physical activity, $\dot{V}O_2$ values do not return to baseline levels for up to several hours (e.g., Gleeson, 1982; Hartzler, et al. 2006). This excess post exercise oxygen consumption (EPOC) is associated with lactate oxidation, glycogenesis, replenishing muscle phosphocreatine and ATP stores, and the resaturation of myoglobin (Gleeson, 1991; Gleeson and Hancock, 2002).

Digestion

The post-prandial increase in energy production associated with the ingestion, breakdown, absorption, and assimilation of foodstuffs is termed specific dynamic action (SDA; Rubner, 1902). Most species of ectothermic vertebrates experience a modest (i.e., ~1.5 to 3.0 fold) $\dot{V}O_2$ increment during the SDA response (Secor, 2009). On the other hand, some species of carnivorous reptiles that ingest very large (i.e., over 10% of body mass) and protein rich meals at infrequent intervals may experience peak $\dot{V}O_2$ increments during the SDA response that approach or even exceed $\dot{V}O_2$ values elicited by physical activity. For example, Benedict (1932) demonstrated that Indian pythons (*Python molurus*), Boa Constrictors (*Boa constrictor*), and Gopher Snakes (*Pituophis catenifer*) exhibited $\dot{V}O_2$ levels up to seven fold above SMR.

Since Benedict (1932)'s work, the metabolic consequences, physiological responses, and mechanisms associated with this large SDA response have become fairly well elucidated (Wang et al., 2001; McCue, 2006; Secor, 2009). Rapid increments in $\dot{V}O_2$ occur within hours following ingestion, which progressively continue, until reaching peak levels between 12 and 48 hours following ingestion; peak $\dot{V}O_2$ increments range from approximately 3 to 19 fold above SMR, and then progressively return to SMR levels over the next two days to three weeks (Wang et al., 2001; McCue, 2006; Secor, 2009). A visual framework describing the components of the SDA response in snakes is presented in Figure 1.4. A summary of previously published $\dot{V}O_2$

increments for snakes (for the meal size treatments used in my dissertation work) is presented in Table 1.4.

The components of the SDA response (i.e., time to peak, the factorial increment, and duration) are influenced by a number of factors. The magnitude of the response (i.e., peak $\dot{V}O_2$) correlates linearly with the size of the ingested meal (Andrade et al., 1997; Secor and Diamond, 1997; Bessler et al., 2010). Meals that are high in protein content and fully intact (e.g., whole rodents) tend to result greater SDA costs (i.e., larger peak values and longer durations) than small meals or those that have lower protein content and larger surface-to-volume ratios (Benedict, 1932; McCue, et al., 2005; Boback et al., 2007). Elevations in body temperature cause quicker rates of gastric emptying and gut passage rates (Dorcas et al., 1997). Both peak $\dot{V}O_2$ levels and the duration of the SDA response are shorter at high temperatures while lower temperatures have an opposite effect – though the total SDA cost is similar, regardless of the temperature treatment (Wang et al., 2003; Secor, 2009). A number of other factors, such as ontogenetic stage, sex, body size, and meal type have also been demonstrated to affect the SDA response (Secor, 2009).

The presence of a meal within the stomach, and later chyme within the small intestine, triggers substantial increments in stomach, intestinal, pancreatic, liver, and kidney activity within 24 to 72 hours following ingestion (Pawlik et al., 1980; Gallavan and Chou, 1985; Secor and Diamond, 1995). These organs also exhibit considerable (i.e., up to two fold) increments in wet mass (Secor and Diamond, 1995). As a result of increased metabolic activity, the PO_2 of gastrointestinal tract arteries decreases, triggering vasodilation, which causes a substantial influx of blood flow (e.g., up to 14 fold increase in *Python molurus*) (Secor and White, 2010). Cardiopulmonary adjustments occur to match O_2 demand, manifesting as a substantial elevations

in cardiac output (e.g., 2.9 to 3.6 fold increase in *Python molurus*; and 2 fold in *Varanus exanthematicus*) and elevations in minute ventilation (e.g., 4 fold in *Python molurus;* and 2.5 fold in *Varanus exanthematicus*) (Hicks et al., 2000; Secor et al., 2000; Secor and White, 2010). As a result of increased plasma HCO_3^- (related to H+ and Cl^- secretion into the stomach), ventilatory control is adjusted (i.e., decreasing air convection requirement to half of resting levels) to maintain arterial pH (Overgaard et al., 1999; Secor et al., 2000).

The increase in $\dot{V}O_2$ following digestion is a result of a combination of factors, including the increased workload of the gastrointestinal organs (e.g., upregulation of intestinal brush border enzymes/nutrient transporters), the kidneys (i.e., increased processing of metabolic waste), the heart, and lungs (i.e., increased cardiopulmonary output) (Secor, 2009). A previous study indicated that increased H+ ion secretion into the stomach during the gastric phase of digestion was responsible for up to 50% of the SDA cost in *Python molurus* (Secor, 2003) but in subsequent work (abolishing H+ secretion with the use of omeprazole) this finding was refuted (i.e., SDA was unchanged) (Andrade et al., 2004). Ultimately, the majority of the SDA response is caused by elevations in the rate of whole-animal protein synthesis (Wang et al., 2001; Secor, 2009). For example, administration of the protein synthesis inhibitor cyclohexamine reduced the SDA of *Python molurus* by 71% (McCue et al., 2005). Similar findings have also been reported for fish (Brown and Cameron, 1991).

Reproduction

Ectothermic vertebrates exhibit two generally recognized modes of reproduction: oviparity (egg-laying) and viviparity (live-bearing) (Weekes, 1927; Fitch, 1970). Oviparity is considered to represent the ancestral mode of reproduction, and occurs in all crocodilians,

testudines, most species of fish and amphibians, and in approximately 80% of squamate reptiles (Amoroso, 1968; Blackburn, 1985). Squamate reptiles are considered to represent a model system for studies on the evolution of viviparity because this mode of reproduction has convergently evolved from oviparity in this group more frequently than in all other vertebrate lineages (i.e., more than 76% of vertebrate viviparous origins are in the order Squamata; Blackburn, 1985), occurs in most lineages (e.g., in snakes viviparity occurs in Colubridae, Boidae, Viperidae, and Elapidae; Neill, 1964), and at low taxonomic levels (i.e., family, genus and species; Blackburn, 2006). Species of both parity modes also share similar morphological features (e.g., oviduct, embryonic egg tooth) and physiological processes (i.e., vitellogenesis and embryogenesis) (Neill, 1967; Stewart, 1992).

All oviparous and most viviparous squamates (with the exception of some species of *Scincidae* lizards) are leithotrophic, that is, energy for embryonic development is derived from stored yolk (Stewart, 1992). Yolk is allocated to the ovarian follicles during the physiological process of vitellogenesis, where estradiol triggers the synthesis of the yolk precursor molecule (vitellogenin), very low density lipoproteins (VLDLs), and a variety of vitamin and trace mineral binding proteins (White, 1991). These biomolecules are transported by the blood to the ovarian follicles, and incorporated via endocytosis (Wallace, 1985; White, 1991). Approximately half-way through the reproductive cycle, the vitellogenic processes are rapidly down regulated, the mature ova are fertilized, and embryogenesis begins (Dessauer, and Fox, 1959). Unlike turtles and crocodilians, which oviposit eggs containing embryos at very early stages of development (i.e., blastulae to neurulae), oviparous snakes retain embryos within the oviduct for up to five weeks (i.e., until the limb bud stages of development) before oviposition (Shine, 1983). At this point embryonic gas exchange requirements are still very low. On the other hand, viviparous

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species retain embryos within the oviduct until term, where all fetal gas exchange requirements (and in some species, nutrient transfer; Stewart, 1992; Van Dyke and Beaupre, 2012) must be met by the mother. To facilitate these requirements, viviparous species have evolved a number of oviduct structural adaptations that include: a closer morphological association of fetal and oviduct epithelial tissues (Blackburn and Stewart, 2011), extended functions of the omphalopleure (Blackburn, 1998), increased vascularity of the oviduct (Masson and Guillette, 1987), and in some species, changes in fetal and maternal blood-oxygen affinity (Grigg and Harlow, 2006).

For species of both parity modes the reproductive cycle period can last for multiple months (Fitch, 1970) and during this time the female is expected to incur direct energetic costs. Clausen's (1936) work in the viviparous De Kay's Snake (*Storeria dekayi*) was one of the first to demonstrate these costs (i.e., a ~1.1 to 1.8 fold increase in maternal $\dot{V}O_2$ during the reproductive cycle), but subsequent studies were not carried out until Guillette (1982) and Birchard et al., (1984)'s work on *Thamnophis sirtalis* and *Sceloporus aeneus*. Since this time, however, a number of subsequent examinations have been carried out, and a summary of these findings (for snakes) is presented in Table 1.1. A hypothetical framework model depicting the general phases of the reproductive cycle, major cycle events, and the general flow of nutrients from the mother to the gametes is presented in Figure 1.5.

Females of most species exhibit increments in $\dot{V}O_2$ during both the vitellogenic (i.e., ~1.3-1.5 fold) and embryogenic (i.e., 1.5-2.9 fold) phases of the reproductive cycle. Although the physiological mechanisms responsible for these direct costs have not been elucidated, it is likely that elevated rates of whole-animal protein synthesis are a major source during vitellogenesis (i.e., related to biomolecule synthesis and organ remodeling; Houlihan, 1991).

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During early embryogenesis, fetal metabolism is still relatively low, but after the limb bud stages of development (i.e., the point in which oviparous species lay eggs) fetal metabolism increases progressively until parturition (Andrews, 2004; Thompson and Stewart, 1997). Therefore, increased rates of protein synthesis, related to the maintenance of reproductive support structures, is a major source of energy demand during early to mid-embryogenesis, while fetal gas exchange requirements contribute to much a greater extent (i.e., at least 30% and as much as 100%; Schultz, 2008; Van Dyke and Beaupre, 2011) prior to parturition. Compensatory elevations in cardiopulmonary output may also contribute to the direct energetic costs of maintaining the reproductive condition (Birchad et al., 1984), but few studies have been carried out in squamate reptiles.

Interaction of Elevated Metabolic States

Before considering the conflicts for O_2 between activity, digestive and reproductive functions, it is important to examine the physical/structural consequences of combining them simultaneously. The ingestion of a meal results in a direct increase in body weight, and reproductive females experience an additional 20-60% increment in weight associated with the enlarged ovarian follicles (see Table 2.2 in Chapter 1). Any increase in body weight will increase the drag force that the snake must overcome to propel itself, and therefore the overall metabolic cost of locomotion will increase (Taylor et al., 1980; Moon and Gans 1998). The ingested meal size and enlarged ovarian follicles may also lead to the compression of visceral organs. As a result, less volume exists for lung expansion (thereby limiting O_2 and CO_2 exchange, and possibly elevating the cost of ventilation) and vascular resistance may increase, leading to decreased venous return (thereby limiting cardiac output) (Munns et al., 2004; Munns and Daniels, 2007; Gilman et al, 2013). Based on Taylor (1980)'s work in large and small

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mammals, each 10% increase in body weight resulted in a 10% increase in the energetic cost of locomotion. Thus, a reproductive female that has recently ingested a meal may experience up to a 40% increase in the energetic costs of locomotion, while simultaneously confronted with the consequences of decreased venous return and limited lung expansion.

The functional states of digestion and reproductive do not, however, function as a simple metabolically inert bolus of material placed within the snake during locomotion. Instead, there are considerable conflicts for O_2 that exist between the metabolically active tissues associated with physical activity (~5 to 10 fold increase in $\dot{V}O_2$), digestion (~3 to 10 fold increase in $\dot{V}O_2$), and reproduction (~1.3 to 2.9 fold increase in $\dot{V}O_2$). Although reproduction, digestion, and activity utilize the same cardiopulmonary mechanisms for oxygen transport, there are considerable differences between the active tissue locations (i.e., GI tract *versus* skeletal muscle *versus* oviduct/liver), metabolic patterns (i.e., anabolic *versus* catabolic), acid-base status (alkalosis *versus* acidosis), and time course (minutes *versus* days *versus* months) of these functional states (Hicks et al. 2000). Moreover, like most vertebrates, reptiles have a limited volume of blood (relative to the potential volume of all maximally dilated arterial vascular beds) by which O_2 delivery can be attained.

Therefore, in order to 1) supply O_2 demands to metabolically active organs simultaneously, 2) meet any additional O_2 requirements imposed by physical/structural alterations, and 3) maintain performance of these simultaneously occurring functional states (i.e., continued digestion of the meal, provisioning embryos with O_2, and speed/endurance levels) the individual must somehow increase cardiopulmonary output and/or adjust oxygen transport parameters (e.g., reduction in R-L shunts, reduced ventilation/perfusion heterogeneity in the

lungs, increase O_2 carrying capacity, and/or blood volume) over levels required to carry out maximal levels of physical activity. If these adjustments are successfully carried out, the resulting $\dot{V}O_2$ would be a simple summation of the individual O_2 increments (in addition to any extra O_2 requirements for elevated weight, and costs of ventilation) – an "additive" effect (Bennett and Hicks, 2001). On the other hand, if the requisite cardiopulmonary and hemodynamic adjustments are not (or cannot be) effectuated by the animal, O_2 delivery capacity is limited, and the performance of one functional state (e.g., maintenance of speed or endurance) is prioritized at the expense of the others (i.e., digestive processes are ramped-down, and/or embryos experience a period of hypoxia) – termed prioritization (Bennett and Hicks, 2001). Under a prioritization scenario, the resulting $\dot{V}O_2$ will not be different (or lower than) previously attained maximal values (i.e., for most species, during physical activity) (Bennett and Hicks, 2001). Figure 1.5 (from Bennett and Hicks, 2001) illustrates these two general outcomes.

A number of previous studies have investigated how the simultaneous conflicts for O_2 between digestion and activity are resolved, and the results from these studies are presented in Table 1.5. Overall, there is no stereotypical pattern of interaction that emerges. Squamates (i.e., *Python molurus* and *Varanus exanthematicus*) exhibit additive patterns of O_2 delivery, and the mechanism for matching O_2 supply with demand is an increase in O_2 extraction from ventilated air possibly via a reduction in R-L shunt fraction and/or decreased ventilation/perfusion heterogeneity (Secor et al., 2000; Bennett and Hicks, 2001). Similar patterns of additivity have been demonstrated for a number of species of fish (e.g., *Gadus morhua*, *Dicentrarchus labrax*, and *Carassius auratus*; Blaikie and Kerr, 1996; Altmires et al., 2008; Pang et al., 2011), although the cardiopulmonary mechanisms in these species were not investigated. In contrast, the anuran *Bufo marinus* (Anderson and Wang, 2003) and some species of fish (*Anoplopoma fimbria*,

Oncorhynchus tshawytscha, *Oncorhynchus mykiss*; Furnell, 1986; Aslop and Wood, 1997; Thorarensen and Farrell, 2006) exhibit a prioritization pattern of O_2 delivery.

A number of factors have been demonstrated to influence the pattern of interaction between digestion and physical activity, including: hypoxia, environmental temperature, acclimation temperature, and a physical activity training protocol (Li et al., 2010; Jourdan-Pineau et al., 2010; Pang et al., 2010; Pang et al., 2011). For example, *Dicentrarchus labrax* can match O_2 supply with tissue demand during post-prandial activity (i.e., additivity) under normoxic conditions, but when exposed to hypoxic water there is a prioritization pattern of O_2 delivery to the digestive processes, and activity performance is curtailed (Jourdan-Pineau et al., 2010). Overall, patterns of interaction between simultaneously occurring elevated metabolic states have been studied in a limited number of vertebrates (i.e., numerous fish, two squamate reptiles, and one species of anuran), and considerable variation exists in: 1) the activity protocols (e.g., swimming *versus* treadmill protocol), 2) meal sizes (i.e., levels range from 1 to 25% of body), and 3) the temperature treatments (i.e., 8 to 35ºC) among studies. Additional work across multiple vertebrate lineages, for different activity protocols, meal size treatments and for more functional states besides just activity and digestion (e.g., reproduction, physical injury, etc.) is required before a more concrete understanding of these patterns can be achieved.

In his seminal work, August Krogh proposed that "for many problems there is an animal on which it can be most conveniently studied", coined later by Hans Krebs as the Krogh Principle (Krogh, 1929; Krebs, 1975). Snakes represent an excellent "Krogh" model system for examinations of the integrated response to simultaneously occurring functional states because unlike most vertebrate, snakes exhibit large factorial increments in $\dot{V}O_2$ during both physical activity and digestion (i.e., ~2 to 12 fold above SMR) (Secor et al., 2000). Furthermore, during

reproduction snakes exhibit long-lasting (i.e., months) sub-maximal increments in $\dot{V}O_2$ (i.e., 1.3 to 2.9 fold above SMR). For my dissertation work, two actively foraging species of colubrid snakes were used: The African House Snake (*Lamprophis fuliginosus*) and the Checkered Garter Snake (*Thamnophis marcianus*). Both species have modest husbandry requirements, consume large meals (i.e., up to 30% in body mass), and exhibit robust reproductive output (e.g., *Lamprophis fuliginosus* undergoes up to six reproductive cycles per annum) (Haagner, 1987; Perry-Richardson et al., 1990; Ford, 2001). Moreover, females of both species continue to ingest large meals throughout much of the reproductive cycle (see Chapter 1, descriptive statistics).

Although maternal metabolic (i.e., $\dot{V}O_2$, RQ) and thermoregulatory patterns have been investigated in a number of snake species during reproduction (Table 1.1 and Table 1.2), there is considerable interspecific variation, and previous work has not examined *Lamprophis fuliginosus* or *Thamnophis marcianus*. Therefore, the first chapter of my dissertation tested whether metabolic parameters (i.e., $\dot{V}O_2$ and RQ) and thermoregulatory preferences (i.e., preferred body temperature, PBT) shifted at different phases of the reproductive cycle (i.e., vitellogenesis, embryogenesis) relative to the non-reproductive condition in these two species. More specifically, metabolic parameters were measured using flow through-through respirometry and PBT was measured by exposing females to a linear thermogradient following the ingestion of a rubber-coated data logger. Ultrasound imaging was used to correlate metabolic and PBT measurements with the underlying phase of the reproductive cycle.

The second (*Lamprophis fuliginosus*) and third (*Thamnophis marcianus*) chapters of my dissertation tested the hypothesis that males and females (non-reproductive and reproductive) would exhibit an additive pattern of O_2 delivery when confronted with the simultaneous functional demands of activity, digestion, and for reproductive females, reproduction. Although

SMR and peak metabolic increments during the digestion of a large meal (Table 1.3) and during physical activity (Table 1.4) have been described for snakes, measurements have not been carried out on adult *Lamprophis fuliginosus* or *Thamnophis marcianus*. Therefore, metabolic parameters (i.e., $\dot{V}O_2$ and RQ) were first measured under standard conditions (i.e., SMR), during physical activity (while fasting), and following the ingestion of a 10% (*Thamnophis marcianus*) or 20% (*Lamprophis fuliginosus*) body mass meal. Then, within 28 (*Thamnophis marcianus*) to 56 (*Lamprophis fuliginosus*) hours following ingestion, the activity treatment was repeated in post-prandial snakes to examine the interaction effects.

The Oxygen Transport Cascade

Structures:
- Skin and lungs
- Heart, Blood, and Circulation
- Tissues

CAPILLARIES

CAPILLARIES

Functions:
- Convection
- Diffusion
- Convection
- Diffusion
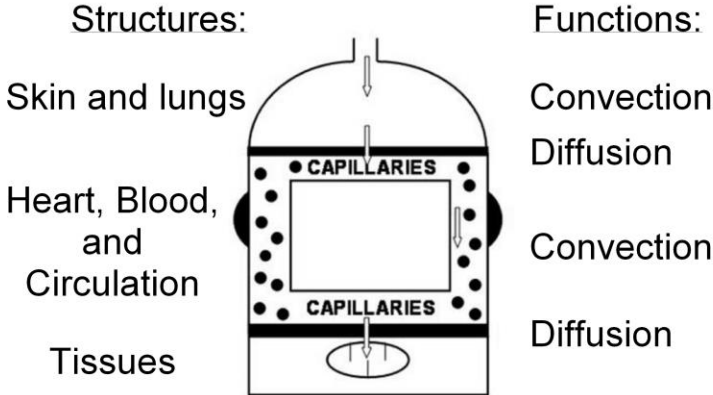
Figure 1.1. Redrawn from Wagner (2010). A depiction of the major structures of the oxygen transport cascade, and their functions, for a vertebrate with a closed circulatory system.
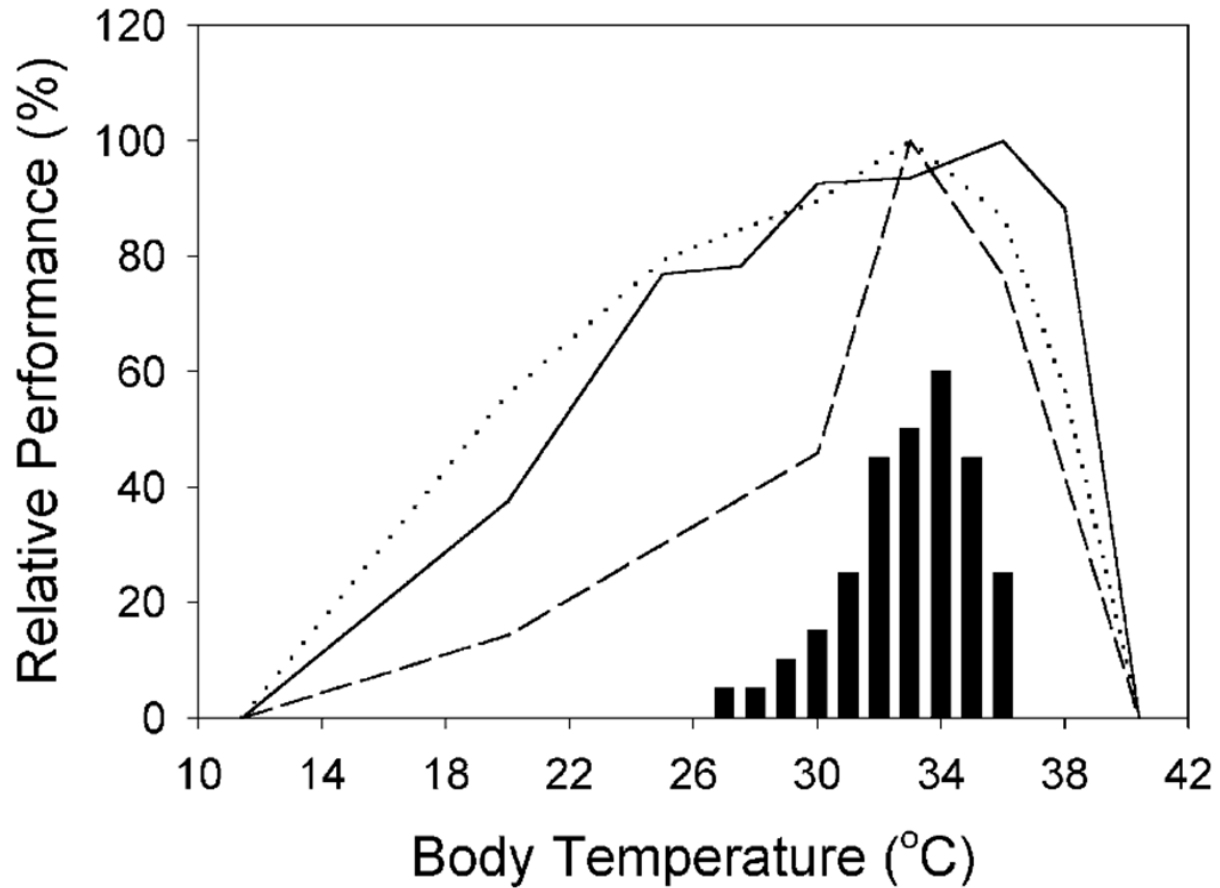
Figure 1.2. From Angilletta et al., (2002). The influence of body temperature on the performance (i.e., relative to maximal values attained at the preferred body temperature) of whole-animal functions (i.e., sprint speed – solid line; endurance – dotted line; and energy metabolism – dashed line) in *Sceloporus undulates*. The solid black bars emerging from the X-axis indicate the range of measured preferred body temperature (PBT) values for this population of lizards.
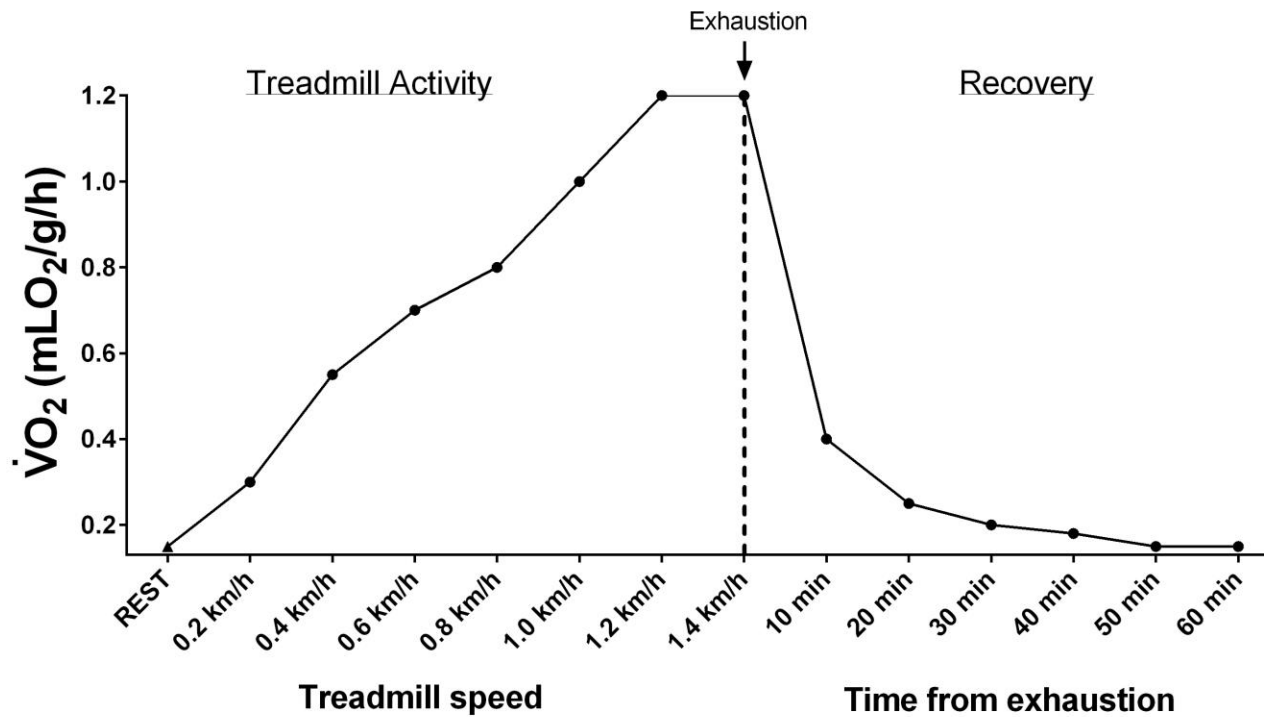
Figure 1.3. Redrawn using data from Gleeson et al. (1980) and Gleeson and Bennett (1982)'s work in lizards of the genus *Varanus*. A depiction of the influence of exercise (i.e., treadmill speed) intensity on $\dot{V}O_2$ until exhaustion. The dashed line indicates the point of exhaustion, and the ensuing portion of the figure demonstrates the excess post exercise oxygen consumption (EPOC).
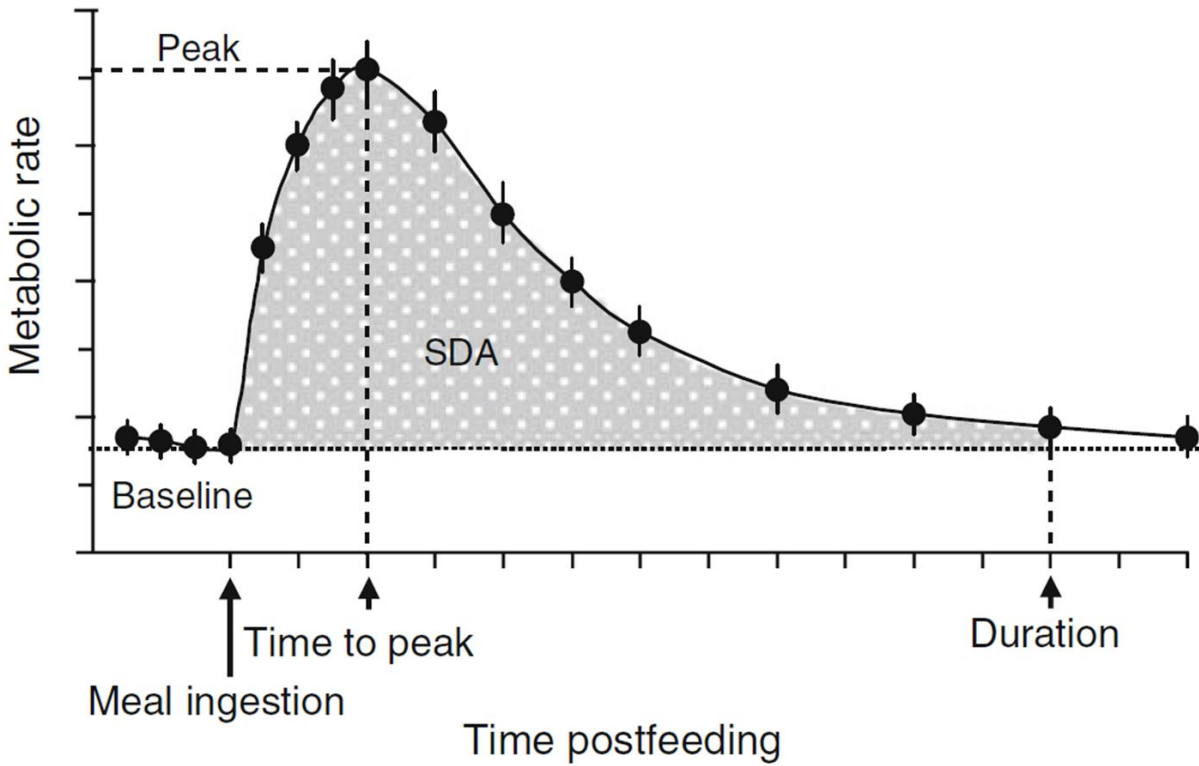
Figure 1.4. From Secor (2009). A hypothetical framework depicting metabolic rate (i.e., $\dot{V}O_2$) before (i.e., SMR), and then following ingestion of a large meal in carnivorous reptile. Each tick on the X axis represents a 12 hour interval. Dashed lines indicate the components of the SDA response (i.e., time to peak, peak increment, and duration).
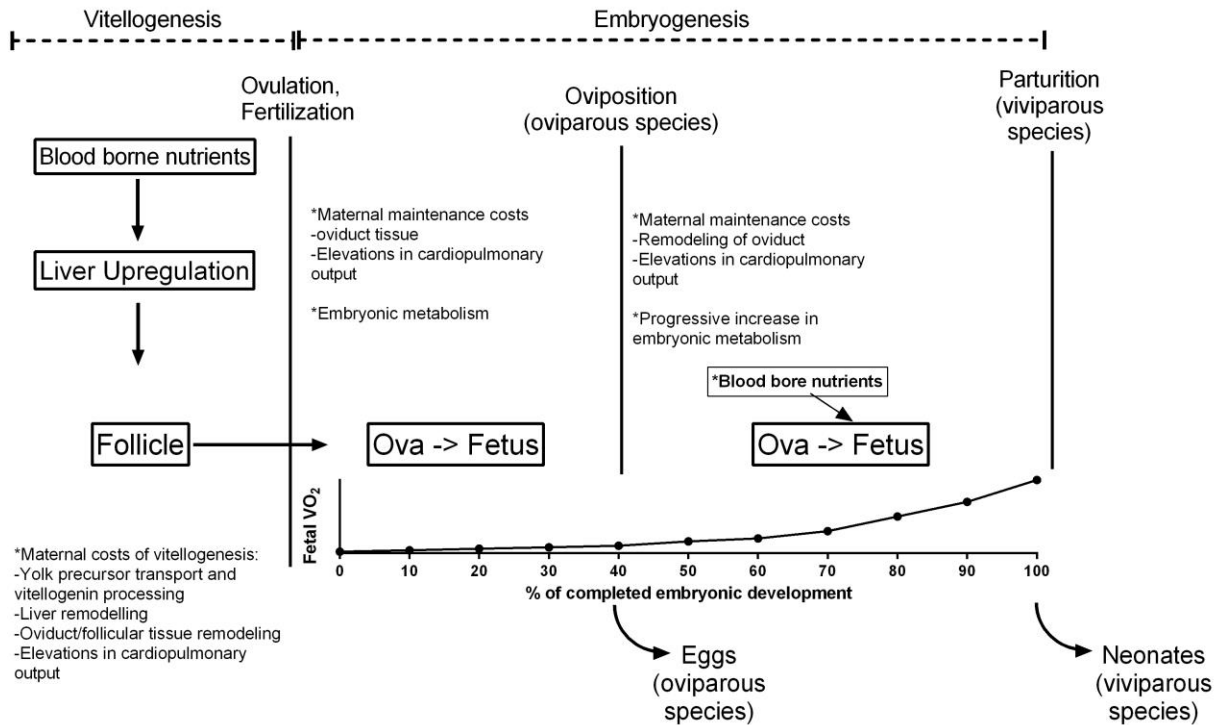
Figure 1.5. Adapted from Van Dyke and Beaupre (2011). A hypothetical framework depicting:
(1) the general phases of the reproductive cycle (top), (2) the major reproductive cycle events
(verticle bars), (3) the general flow of nutrients from the mother to reproductive processes (text
boxes are connected with arrows), and (4) the physiological processes that may serve to elevate
maternal $\dot{V}O_2$ (text with an asterisk, *) in squamate reptiles of both parity modes (i.e., oviparity
and viviparity). The graph below the embryogenic phase of the reproductive cycle indicates
embryonic metabolism as a function of the percent of completed embryonic development (based
on Thompson and Stewart, 2000).
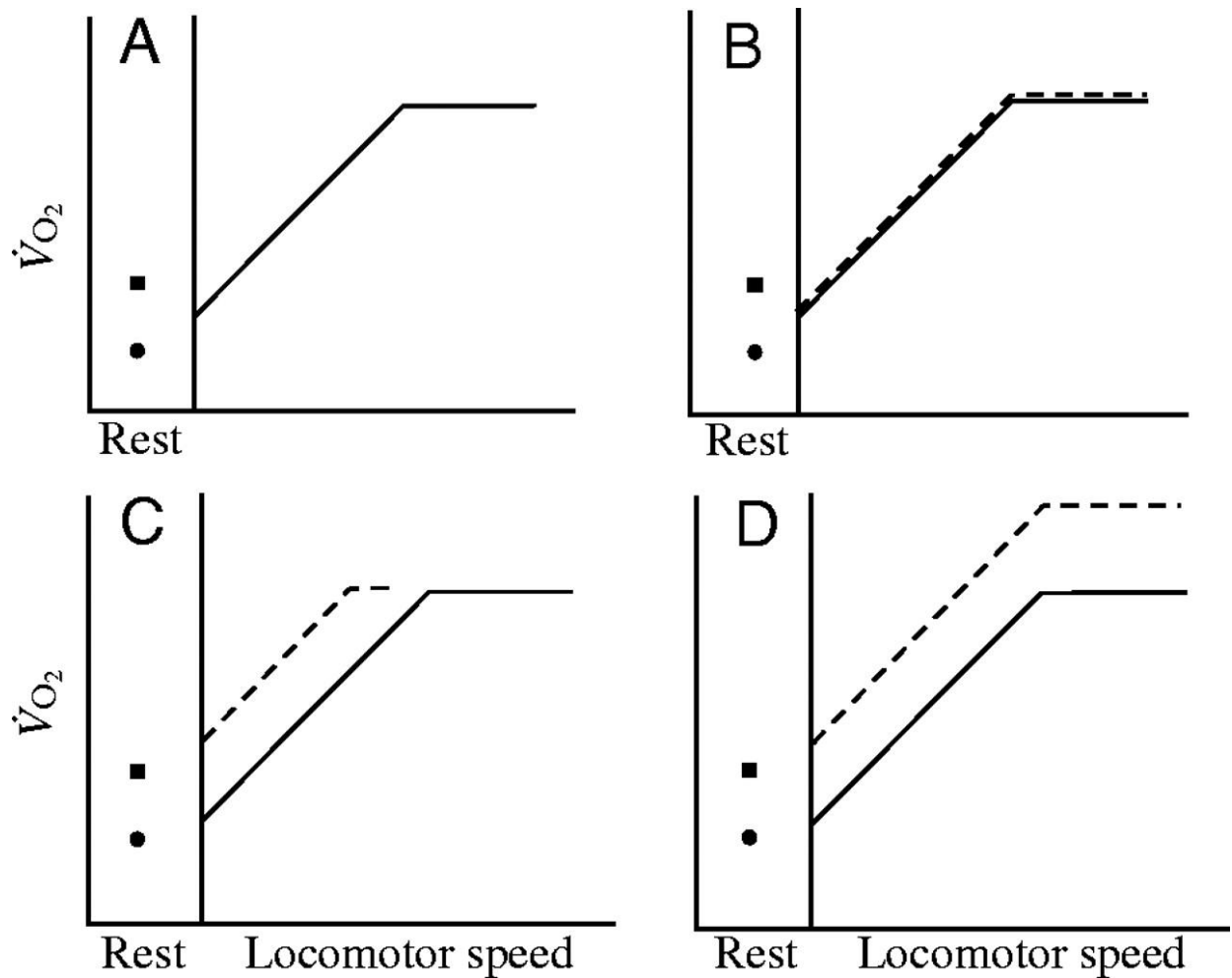
Figure 1.6. From Bennett and Hicks (2001). Hypothetical framework model demonstrating the $\dot{V}O_2$ increment associated with rest (circle, left portion of each graph) and digestion (square, left portion of each graph), and then $\dot{V}O_2$ response to a treadmill activity protocol while fasting (solid line). The dashed line (in B, C, and D) indicates the $\dot{V}O_2$ response to the same treadmill activity protocol in an animal that is digesting a meal (i.e., post-prandial activity). The outcome in (B) represents a prioritization towards activity (i.e., activity performance is maintained, and digestive processes are curtailed). The outcome in (C) represents a prioritization towards digestion (i.e., activity performance is sacrificed to maintain the digestive processes). The outcome in (D) represents additivity of the responses (i.e., both activity performance and the digestive metabolic increment are maintained simultaneously).

Table 1.1. Previously published factorial increments in maternal $\dot{V}O_2$ (relative to the non-reproductive condition) during the reproductive cycle phases (i.e., vitellogenesis and embryogenesis) in snakes.

Species and Parity Mode	Vitellogenesis	Embryogenesis	Reported Unit	Phase Verification Method	Reference
Agkistrodon contortrix (viviparous)	1.6 fold	1.4 fold	kJ/reproductive cycle phase	Ultrasound	Van Dyke and Beaupre, 2011
Boa constrictor (viviparous)	1.6 fold	1.5 fold	kJ/reproductive cycle phase	Ultrasound	Van Dyke and Beaupre, 2011
Eryx colubrinus (viviparous)	1.4 fold	1.6 fold	kJ/reproductive cycle phase	Ultrasound	Van Dyke and Beaupre, 2011
Nerodia sipedon (viviparous)	1.4 fold	1.6 fold	kJ/reproductive cycle phase	Ultrasound	Van Dyke and Beaupre, 2011
Thamnophis sirtalis (viviparous)	1.5 fold	1.5 fold	kJ/reproductive cycle phase	Ultrasound	Van Dyke and Beaupre, 2011
Storeria dekayi (viviparous)	~1.8 fold*	1.1 fold*	$mLO_2/g/h$	Not indicated	Clausen, 1936
Acanthophis praelongus (viviparous)	No difference	2.7 fold	mLO_2/h	Post-hoc, following parturition	Schultz et al., 2008
Crotalus atrox (viviparous)	1.4 fold	-	mLO_2/day	Ultrasound	Beaupre and Duvall, 1998
Python regius (oviparous)	-	1.3 fold	$mLO_2/kg/h$	Post-hoc, following oviposition	Ellis and Chappell, 1987
Thamnophis sirtalis sirtalis (viviparous)	-	2.9 fold	mLO_2/h	Post-hoc, following parturition	Birchard et al., 1984
Vipera aspis (viviparous)	No difference	No difference	mLO_2/h	Post-hoc, following parturition	Ladyman et al., 2003

*Indicates that no formal statistical tests were carried out.

Table 1.2. Previously published shifts in maternal preferred body temperature (PBT) values (relative to the non-reproductive condition) during the reproductive cycle phases (i.e., vitellogenesis or embryogenesis) in snakes.

Species and Parity Mode	Vitellogenesis	Embryogenesis	Operative Temp. Apparatus	Measurement Technique	Reference
Natrix natrix (oviparous)	2.2°C lower	-	Outdoor enclosure	Surgically implanted radiotelemeter	Issac and Gregory, 2004
Crotalus viridis oreganus (viviparous)	-	No difference	Laboratory thermal gradient	Surgically implanted radiotelemeter	Grier et al., 1989
Antaresia children (oviparous)	~2°C higher	~4°C higher	Laboratory thermal gradient	Surgically implanted data logger	Lourdais et al., 2008
Crotalus horridus (viviparous)	-	4.4°C higher	Field	Surgically implanted radiotelemeter	Gardner-Santana and Beaupre, 2009
Nerodia rhombifera (viviparous)	-	2.9°C higher	Laboratory thermal gradient	Cloacal thermocouple	Tu and Hutchison, 1994
Crotalus viridis (viviparous)	-	No difference	Outdoor enclosure	Surgically implanted radiotelemeter	Charland and Gregory, 1990
Elaphe obsoleta (oviparous)	No difference	-	Laboratory thermal gradient	Surgically implanted radiotelemeter	Blouin-Demers and Weatherhead, 2001
Malpolon monspessulan (oviparous)	3.3°C higher	-	Field	Assist-fed data logger	Blazquez, 1995
Acanthophis praelongus (viviparous)	-	No difference	Laboratory thermal gradient	Surgically implanted radiotelemeter	Webb et al., 2006

Table 1.3. Previously published factorial increments in $\dot{V}O_2$, and the time required to attain this level, associated with the ingestion of a 10% or 20% body mass meal in snakes.

Species	10% Body Mass*	20% Body Mass*	Time to Peak 10%	Time to Peak 20%	Meal Type	Temperature	Reference
Thamnophis elegans	3.6 fold	-	~20 h	-	Fish	30°C	Britt et al., 2006
Python molurus	5.1-5.6 fold	6.3-8.6 fold	24 h	34h	Rodent	30°C	McCue et al., 2005; Wang et al., 2003
Nerodia sipedon	3.2 fold	-	20 h	-	Fish	27°C	Sievert and Andreadis, 1999
Natrix maura	4.2 fold	-	24 h	-	Fish	25°C	Hailey and Davies, 1987
Lamprophis fuliginosus	3.2 fold	5.1 fold	24 h	24 h	Rodent	25°C	Roe et al., 2004
Crotalus durissus	-	3.7 fold	-		Rodent	30°C	Andrade et al., 1997
Crotalus atrox	3.9 fold	-	20 h	-	Rodent	30°C	McCue, 2007
Boa constrictor	3.8 fold	4.0 fold	16 h	23 h	Rodent	30°C	Toledo et al., 2003
Acanthophis praelongus	5.3 fold	-	48 h	-	Rodent	30°C	Christian et al., 2007

*Indicates ±2%

Table 1.4. Previously published peak factorial increments in $\dot{V}O_2$ associated with physical activity (while fasting) in snakes.

Species	Aerobic scope	Duration of Trial	Experimental Protocol	Temp	Reference
Coluber constrictor	9.0-9.5 fold	5 min; 120 min	Magnet jabs; Treadmill	35°C; 30°C	Ruben, 1976; Walton et al., 1990
Masticopus flagellum	9.5 fold	5 min	Magnet jabs	35°C	Ruben, 1976
Crotalus viridis	4.3 fold	5 min	Magnet jabs	35°C	Ruben, 1976
Lichanura roseofusca	1.9 fold	5 min	Magnet jabs	32°C	Ruben, 1976
Natrix rhombifera	~ 4 fold	10 min	Electrical stimulation	35°C	Gratz and Hutchison, 1977
Python regius	12 fold	15 min	Manual rotation	30°C	Ellis and Chappell, 1987
Crotalus durissus	4.5 fold	5 min	Manual rotation	30°C	Andrade et al., 1997
Python molurus	6.0 – 10.0 fold	0.04-0.05km/h	Treadmill	30°C	Secor and Diamond, 1995; Secor et al., 2000
Crotalus cerastes	3.8 fold	.05-.06km/h	Treadmill	30°C	Secor et al., 1992
Thamnophis butleri	7.9 fold	2 min	Manual rotation	25°C	Kamel and Gatten, 1983

Table 1.5. Previously published patterns of interaction between digestion and physical activity (i.e., during post-prandial physical activity trials) in vertebrates.

Species	Pattern of interaction	Activity Protocol	Meal Size (% body mass)	Temperature	Reference
Python molurus	Additive	Treadmill	25%	30°C	Secor et al., 2000
Varanus exanthematicus	Additive	Treadmill	20%	35°C	Bennett and Hicks, 2000
Bufo marinus	Prioritization	Manual rotation	8.5%	25°C	Anderson and Wang, 2003
Dicentrarchus labrax	Additive	Swim chamber	2.7%	22.5°C	Altmiras et al., 2008
Gadus morhua	Additive	Swim chamber	2.5%	8°C	Blaikie and Kerr, 1996
Peltebagrus vachelli	Additive/ prioritization to digestion[b]	Swim chamber	7 %	25°C	Li et al., 2010
Dicentrarchus labrax	Additive/ prioritization to digestion[c]	Swim chamber	3%	20°C	Jourdan-Pineau et al., 2010
Carassius auratus	Additive/prioritization to digestion[a]	Swim chamber	~3%	15°C, 25°C	Pang et al., 2011
Cyprinus carpio	Additive/ prioritization to digestion[a]	Swim chamber	~3%	15°C, 25°C	Pang et al., 2011
Spinibarbus sinensis	Additive/ prioritization to digestion[a]	Swim chamber	~3%	15°C, 25°C	Pang et al., 2011
Silurus meridionalis	Additive[d]	Swim chamber	~10-15%	15°C, 21°C, 27°C, and 33°C	Pang et al., 2010
Oncorhynchus mykiss	Prioritization to digestion	Swim chamber	1%	15°C	Aslop and Wood, 1997
Oncorhynchus tshawytscha	Prioritization to digestion	Swim chamber	2 %	9-10°C	Thorarensen and Farrell, 2006
Anoplopoma fimbria	Prioritization to activity	Swim chamber	5 %	8.5°C	Furnell, 1986

[a] Additive at 15°C and prioritization towards digestion at 25°C

[b] Additive for untrained individuals and prioritization towards digestion in trained individuals

[c] Additive for normoxia and prioritization in hypoxia

[d] $\dot{V}O_2$ values were additive, but activity performance declined

CHAPTER 1

THE INFLUENCE OF REPRODUCTION ON METABOLISM AND PREFERRED BODY TEMPERATURE IN THE OVIPAROUS *LAMPROPHIS FULIGINOSUS* AND THE VIVIPAROUS *THAMNOPHIS MARCIANUS*

INTRODUCTION

Viviparity (live bearing) as a mode of reproduction has arisen in many vertebrate lineages, including fish (Chondrichthyans and Teleosts), amphibians (Urodeles, Gymnopiona, and Anura), squamate reptiles, and therian mammals (Amoroso, 1968). Among these lineages, the squamate reptiles (snakes, lizards, and amphisbaens) have independently evolved viviparity from oviparity on over 100 separate occasions (20 % of currently extant species), which is more frequent than all other vertebrates (Blackburn, 1985; Blackburn, 1999). Furthermore, viviparity in squamates has evolved at low taxonomic levels (i.e., family, genus, and species), and in recent geological time periods (e.g., late Pleistocene), making squamates an ideal model system for comparative examinations of reproductive anatomy and physiology (Shine, 1983; Stewart, 1992; Blackburn, 2006).

In squamates, oviparity (egg laying) is considered the ancestral mode of reproduction, where nutrient requirements of the embryos are met exclusively by stored yolk (lecithotrophy), provisioned to the developing oocytes during the process of vitellogenesis. After vitellogenesis, ovulation occurs, and the developing embryos are retained within the maternal oviduct for varying lengths of time (approximately ¼ - ½ of embryonic development, depending on the species) (DeMarco, 1993; Shine, 1983; Blackburn, 1995; Andrews, 2004). Most viviparous species also undergo a phase of vitellogenesis prior to ovulation, but embryos are retained within

the oviduct to term, where gas exchange and varying degrees of nutrient provision (e.g., water, inorganic ions, and macronutrients) occur across modified chorioallantoic and oviduct membranes (Stewart, 1992; Blackburn, 1992; 1995; 2000).

The reproductive cycle lasts for months, and is generally characterized by a distinct phase of vitellogenesis, followed by ovulation, and then varying durations of embryonic retention (Dessauer and Fox, 1959; Bonnet et al., 1994; Garstka, et al., 1985). During this period, maternal metabolic demands and thermoregulatory preferences may vary considerably over time (e.g., Schultz et al., 2008; Robert and Thompson, 2000; Ladyman et al., 2003). However, few studies have correlated the observed patterns of metabolism and thermoregulatory preferences with the phase of the reproductive cycle (e.g., using dissection, DeMarco and Guillette, 1992; or ultrasound imaging, Van Dyke and Beaupre, 2011).

The present study was designed to (1) to obtain regular measures of metabolism (i.e., oxygen consumption, $\dot{V}O_2$) and preferred body temperature (PBT) on reproductive females of an oviparous and a viviparous species of snake, and (2) to correlate these measurements with the observed morphological features of the ovarian follicles. We chose the oviparous African Brown House Snake (*Lamprophis fuliginosus*) and the viviparous Checkered Garter Snake (*Thamnophis marcianus*), two colubrid snakes that are well known for their modest husbandry requirements and tractability for laboratory research (Haagner, 1987; Ford, 2001; Ford and Karges, 1987; Perry-Richardson et al., 1990). Based on previously published work, we predicted that both species would exhibit an increased metabolic rate during the vitellogenic and embryogenic phases of their reproductive cycles (e.g., Beaupre and Duvall, 1998; Schultz et al., 2008; Van Dyke and Beaupre, 2011); we also predicted that maternal thermoregulatory preferences would shift towards a higher values to accelerate the processes of yolk allocation and organ remodeling

during vitellogenesis, and to optimize development during embryogenesis (e.g., Lourdais et al., 2008; Tu and Hutchison, 1994).

MATERIALS AND METHODS

Husbandry

Adult female and adult male *Lamprophis fuliginosus* were purchased from commercial retailers in September 2010, and established as members of a captive breeding colony at the University of California, Irvine. All adult female (n=10) and adult male (n=5) *Lamprophis fuliginosus* in the current study were members of this colony. The Garter Snakes used in this study were part of a long term captive breeding colony established at the Ophidian Research Colony at the University of Texas at Tyler. Adult female *Thamnophis marcianus* (n=20) and adult males (n=14) were placed into a dark brumation chamber, held at 15°C, in May, 2012. In July, 2012, the animals were removed, and immediately shipped to University of California, Irvine (UCI). Individuals of both species were held in a large vivarium room maintained at 26±2°C, with a 12L:12D photoperiod.

Females of both species were housed individually within an opaque shallow rectangular plastic enclosure (50 x 34 x 14 cm), each of which contained a subsurface heating element (Sunbeam Products, Inc., Boca Raton, Florida, USA; or Zoo Med Laboratories, Inc., San Louis Obispo, California, USA) under approximately ⅓ of the enclosure. Enclosures were lined with newsprint bedding and contained two hide boxes: one located on the warm end, the other on the cool end, the latter of which contained moist sphagnum moss to facilitate oviposition/parturition. Water was provided *ad libitum* and animals were offered thawed rodents (approximating 5 - 10 % body mass), at least once weekly. For both species, body mass was obtained adjacent to all

experimental procedures to the nearest ± 1g (Ohaus SCF0A0., Parsippany, New Jersey, USA) and snout vent length (SVL) was measured for all animals (± 1 cm) at least once during the experimental period.

Experimental Timeline

Lamprophis fuliginosus. —Adult female *Lamprophis fuliginosus* that had recently oviposited a clutch of eggs were considered subjects in the present study. Females were offered meals *ad libitum*, and males were cycled among the females, multiple times weekly, for approximately two to four weeks. Following this re-feeding and mating period, females underwent experimental treatments, on average, once every 17 days (range $= 9 - 33$), until a subsequent oviposition event. Males remained with the females until follicles were observable. If females failed to develop vitellogenic follicles within two months following mating, they were removed from the study. For the females that completed a reproductive cycle, measurements were binned, *post hoc*, into reproductive phases based on: (1) the number of days prior to oviposition, and (2) the observed ovarian follicle morphology (via ultrasound imaging, detailed below). Based on this classification, measurements were binned as follows: pre-vitellogenic (PV; following mating and re-feeding, but no ovarian follicles detected), primary vitellogenesis (V1; 6-9 weeks prior to oviposition, small vitellogenic follicles), secondary vitellogenesis (V2, 3-6 weeks prior to oviposition, large vitellogenic follicles), and embryogenesis (E1; 0-3 weeks prior to oviposition). Measurements obtained one to five days following oviposition were considered to represent the baseline, non-reproductive (NR) condition. In this NR condition, females did not have any observable vitellogenic ovarian follicles, were fasting, and were not mating. The reproductive cycle period was defined as the time from detection of vitellogenic ovarian follicles (via

ultrasound imaging) until oviposition. A schematic diagram illustrating the above experimental timeline for *Lamprophis fuliginosus* is presented in Figure 2.1.

Thamnophis marcianus.—Within five days following removal from brumation, fasting and non-mating females underwent gas exchange measurements. Afterward, females were returned to their vivarium enclosures, paired with one male conspecific, daily, for approximately three weeks, and offered meals *ad libitum*. Following this mating and re-feeding period, females underwent experimental treatments, on average, once every 15 days (range = 14 – 17), until parturition, and then within two days following parturition. Females that failed to develop vitellogenic follicles and undergo parturition were removed from the study. Measurements were categorized into reproductive phases, based on the number of weeks prior to parturition, and the morphological features of the ovarian follicles (observed via ultrasound) as follows: Non-reproductive (NR; following emergence from brumation, prior to mating and re-feeding), primary vitellogenesis (V1; 8-10 weeks prior to parturition, small vitellogenic follicles), secondary vitellogenesis (V2; 6-8 weeks prior to parturition, large vitellogenic follicles), Embryogenic 1 (E1; 4-6 weeks pre-partum, amnion/allantois observable), Embryogenic 2 (E2; 2-4 weeks pre-partum, differentiated embryonic tissue), and Embryogenic 3 (E3; 0-2 weeks pre-partum, greatly enlarged embryonic tissue). The NR condition was considered the baseline, non-reproductive condition rather than the post-partum (PP) condition for three reasons: (1) Slightly enlarged vitellogenic ovarian follicles were observed in most females within two days following parturition, (2) all females in the present study immediately underwent a second reproductive cycle following the conclusion of this experiment, and 3) the $\dot{V}O_2$ and RQ values obtained in the NR condition were not significantly different from values obtained three months later, in a subsequent non-reproductive condition. The reproductive cycle was defined as the time period

following mating, when small vitellogenic follicles were first observed, until parturition. A schematic diagram illustrating the above experimental timeline for *Thamnophis marcianus* is presented in Figure 2.1.

Gas exchange measurements ($\dot{V}O_2$, $\dot{V}CO_2$)

Oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were measured using flow-through respirometry. Briefly, compressed atmospheric air was pushed into a mass flow controller (GF-3 Gas Mixing Flowmeter; Cameron Instruments Inc., Guelph, Ontario, Canada) which fed a stable current of air into a needle-valve manifold which separated the air stream into lines connected to nine separate metabolic chambers (250mL, 940mL, or 1555mL, depending on the size of the animal). The incurrent flow rate to each of the metabolic chambers was determined to the nearest ±1mL/min by a recently calibrated Aalborg mass flow meter (GFM17 AALBORG, Orangeburg, New York, USA). Flow meter accuracy was verified by using an upturned graduated cylinder in water at 29±1°C. The incurrent flow rates (range = 50 to 240 mL/minute, depending on the size of the animal) to each of the chambers were monitored using Cole Parmer rotameters (Cole-Parmer, Vernon Hills Illinois, USA), and checked multiple times daily for consistency. At least one of the nine chambers remained free from experimental animals and served as a baseline for referencing.

Excurrent air from each of the chambers flowed out through Tygon tubing connected to one of nine separate Cole Parmer three-way solenoid valves. The valves were selectively activated in a repeating fashion by an adjustable electronic timer (Industrial Timer Company, Centerbrook, Connecticut, USA). When activated, the excurrent air from just one of the nine chambers flowed into a sub-sampling line that terminated as a wide diameter Tygon tubing. An

18-guage needle, inserted into the wide-diameter Tygon tubing, pulled a subsample of the

excurrent air into a separate gas-analysis circuit at 20 to 50±1 mL/minute (depending on the

excurrent flow rate). The subsampling flow rate was controlled by a separate pump (Model R-1,

Applied Electrochemistry Technologies, Pittsburg, PA, USA) connected to a Brooks mass flow

controller (Brooks Instrument, Hatfield, PA, USA). By this method, each of the nine chambers

were selectively subsampled once every 80 minutes, for 6 to 11 minutes, repeatedly throughout

the duration of the experiment.

The gas analysis circuit consisted of the following items in this order: Applied

Electrochemistry Technologies (AEI) CD-3A CO_2 sensor/analyzer $\rightarrow$ soda lime cartridge

(remove CO_2) $\rightarrow$ drierite cartridge (remove H_2O) $\rightarrow$ Applied Electrochemistry Technologies

(AEI) S-3A O_2 sensor/analyzer. The analog outputs from the S-3A O_2 analyzer and the CD-3A

CO_2 analyzer were connected to a Biopac MP-100 hardware unit, downloaded to a laptop at

20MHz, and recorded using Acknowledge (Version 3.2.4) software (Biopac Inc., Santa Gotela,

CA, USA). $\dot{V}O_2$ was calculated using Lighton (2008)'s Equation 10.1, $\dot{V}CO_2$ using the Fick

equation, and RQ was calculated as the quotient of $\dot{V}CO_2$ and $\dot{V}O_2$.

The first four hours of all trials were discarded to account for recovery from handling

stress and acclimation to the metabolic chambers. The mean of the three lowest consecutive

measurements of $\dot{V}O_2$ (and corresponding $\dot{V}CO_2$) obtained at any point in the photocycle, over

the 24 to 48 hour measurement period, were chosen to represent the resting gas exchange rates.

By this procedure, the lowest values obtained over a four hour period were compared.

Gas exchange measurements were carried out at 25±1°C for *Lamprophis fuliginosus*, and

29±1°C for *Thamnophis marcianus*, the preferred body temperature values of these two species

(Zurovsky, et al., 1987; Lutterschmidt et al., 2002; Lutterschmidt & Hutchison, 2003; Rosen, 1991). All animals in the present study were measured in a post-absorptive condition because they had been fasting for a minimum period of four days (Roe et al 2004; Britt et al., 2006; Bessler et al, 2010). For both species, trials were also carried out in complete darkness (i.e., lights off and/or chambers covered with a black tarp), to minimize disruption from investigators entering or leaving the room.

Egg Clutch and Neonate Parameters

Within 48 hours following oviposition, we recorded clutch mass (± 1 g), and the number of viable (large and milky white), and non-viable (diminutive or brown/black) eggs deposited by (n= 5) female *Lamprophis fuliginosus* in this study. For *Thamnophis marcianus*, we recorded the number and mass of both live and stillborn neonates produced by females (n = 11 litters). In addition, clutch/litter $\dot{V}O_2$ (and corresponding $\dot{V}CO_2$ values) were measured within 48 hours post-oviposition (n=5 clutches) or post-partum (n = 10 litters), using either closed system respirometry (all *Lamprophis fuliginosus* clutches, and for litters containing four or fewer neonates, *Thamnophis marcianus*), or flow through respirometry (*Thamnophis marcianus* litters containing more than four viable neonates).

Our closed system respirometry design was as follows: O_2 and CO_2 values for ambient air (25±1°C, *Lamprophis fuliginosus*; 29±1°C, *Thamnophis marcianus*) were determined, afterwards each of the clutches or litters (four neonates or smaller), were placed into a respirometry chamber (470mL) containing this ambient air with approximately 0.5mL of water (to prevent desiccation). The chamber was sealed, and placed into an environmental room maintained at 25±1°C (*Lamprophis fuliginosus*) or 29±1°C (*Thamnophis marcianus*) in complete

darkness. The duration of the trial ranged from 3.5 to 24 hours, depending on the size of the clutch/number of neonates. Upon completion of the trial, the time was recorded, and air was pulled at 50±1mL/min (verified using an Aalborg mass flow meter) by a diaphragmatic pump, into the aforementioned gas analysis circuit. The lowest recorded expired O_2 and corresponding CO_2 values were considered to represent gas exchange values for the clutch/litter. The volume of the entire clutch/litter was determined to the nearest 1.0 mL, and this value was subtracted from the chamber volume. $\dot{V}O_2$ and $\dot{V}CO_2$ values were calculated using equations 4.18 and 4.21 from Lighton (2008), respectively. For all *Thamnophis marcianus* neonate trials, we verified that the neonates were either completely inactive, or minimally active (sluggish undulations) using an infrared bulb.

Preferred Body Temperature

Within 24 hours (*Thamnophis marcianus*) or 48 hours (*Lamprophis fuliginosus*) of the gas exchange trials, we carried out preferred body temperature (PBT) trials on adult females of both species using a thermogradient design similar to those described by Scott and Pettus (1979) and Lutterschmidt (1991)'s. In brief, two thermogradient (182 x 52 x 38 cm) boxes with plywood frames and steel bottoms were constructed and placed into a walk-in environmental chamber, maintained at 25±1°C (*Lamprophis fuliginosus*) or 29±1°C (*Thamnophis marcianus*) with a 12L:12D photoperiod (*Lamprophis fuliginosus*) or no photoperiod (*Thamnophis marcianus*). Each thermogradient box was separated in half with a plywood barrier (approximately 179 x 2 x 38 cm), creating two identical lanes (179 x 22 x 38 cm), thus permitting four animals to be measured simultaneously. Each of the four thermogradient lanes were fitted with a bottomless full-length hide box (178 x 11 x 9 cm) that covered half of the lane,

and consequently provided cover, while also exposed the animals to the temperature gradient of the steel floor.

The temperature of the steel bottom ranged, on average, from $14\pm1.0°$ to $45\pm2.0°C$ (slope, $Y = 0.4643X+10.26$) for *Lamprophis fuliginosus*, and $17\pm1°C$ to $40\pm1°C$ (slope, $Y=0.3578X+16.28$) for *Thamnophis marcianus*. The warm end of the thermogradient was maintained by conduction with subsurface heating elements (Sunbeam Products, Inc., Boca Raton, Florida, USA) controlled by potentiometers; the central portion was maintained by ambient air temperature; and the cold end was maintained by conduction with subsurface copper coils, perfused with a sub-zero ethanol solution generated by a refrigerated circulating water bath and cooler (Neslab RTE-8DD and Cryocool; Neslab Instruments Inc., NH, USA).

Rubber coated (Plasti Dip, PDI, Inc., Minnesota, USA) data loggers (Thermochron iButton, Maxim, Dallas, TX, USA) were programmed to record once every 60 minutes, and assist-fed to the females by gently massaging the device into the animal's stomach, following brief (approximately 30 second) exposure to gaseous isoflurane. After feeding, one female was placed into each of the four thermogradient lanes, in the ambient temperature zone, outside of the hide box, and remained undisturbed for the trial duration. Following the conclusion of the trial, the females were lightly sedated using gaseous isoflurane, and assist-fed approximately 1 to 2 mL of warm water through a syringe barrel. The data logger was then gently massaged up through the esophagus, and carefully removed from the animal's mouth.

Preferred body temperature trials lasted for 30 to 78 hours, and the first six hours of each trial were discounted to account for any residual effects handling stress and isoflurane exposure on maternal thermoregulatory preferences. In some cases, the female vomited the data logger. If

vomiting occurred sooner than 30 hours from the beginning of the trial, the data was discarded

and not considered for analysis. The average Tb values recorded over the trial period (average =

38 hours, *Lamprophis fuliginosus*; 40 hours, *Thamnophis marcianus*) were considered to

represent the individual's preferred body temperature (PBT).

Ultrasound Imaging

Ultrasonography can be used to accurately quantify reproductive characteristics in

squamate reptiles (Martinez-Torres et al., 2006; Gilman and Wolf, 2007; Stahlschmidt et al.,

2011; Van Dyke et al., 2012). In the present study, we used this technique in order to assess the

ovarian follicular morphological features, and to correlate these features with the results obtained

from the gas exchange and PBT measurements. For both species, ultrasound imaging occurred

within 48 hours of the PBT trials. Scans (approximately 10 to 30 minutes) were carried out on

mildly anesthetized (i.e., voluntary respiration occurred) females. Following sedation, a portion

of the female's caudal abdomen was placed into a bath of warm water, and 4.2 cm lateral

sections at variable adjustable depths (approximately 2 to 6 cm) were imaged using a 5.0 to 10.0

MHz linear array transducer (CTS 3300, Shantou Institute of Ultrasonic Instruments,

Guangdong, China). Each ovarian follicle was assigned and identification number and digitally

photographed using the software provided with the ultrasound machine. We estimated the

anterior-posterior axis length (width) and left-to-right axis length (height) of each follicle to the

nearest 1 mm using either the digital calipers provided with the ultrasound machine's software,

or ImageJ (U.S. National Institutes of Health; Bethesda, MA, USA). Sequential ultrasound

images and follicle measurements were obtained for nine out of the final thirteen *Thamnophis*

marcianus and five out of the six *Lamprophis fuliginosus* females. Images were obtained for the

remaining females of both species to confirm the presence of vitellogenic follicles during the V1

phase of the reproductive cycle. Ultrasound imaging was not carried out during the NR phase

for *Thamnophis marcianus* to avoid any possible influence of isoflurane exposure and physical

manipulation on mating success.

A number of females in our colony died during the course of our research. Necroscopies

were carried out to explore the gross morphological changes that occurred during the

reproductive cycle phases. These deaths occurred during the approximately non-reproductive

(NR, both species), secondary vitellogenesis (V2, both species), and embryogenic 3 (E3,

Thamnophis marcianus) reproductive conditions.

Statistical Analysis

We carried out a repeated measures non-parametric ANOVA (Steel's Method for

Multiple Comparisons versus Control Group) to evaluate the differences in $\dot{V}O_2$, RQ and PBT

between the reproductive cycle phases and the non-reproductive condition. Because PBT values

for *Thamnophis marcianus* during the non-reproductive (NR) condition were not obtained, we

carried out a separate non-parametric repeated measures ANOVA (Steel-Dwass Method for

Multiple Comparisons) to test whether differences in PBT values exist among all of the

reproductive cycle phases. Due to the variation in body mass (up to a 3 fold difference) among

the females of both species, and the confounding factor associated with the increased mass

during reproduction (up to a 1.6 fold increase) (Ellis and Chappel, 1987; DeMarco and Guillette,

1992; Angilletta & Sears, 2000) each female's $\dot{V}O_2$ values were converted into mass-specific

values (i.e., $mLO_2/g/h$), using non-reproductive body mass, for the statistical analyses.

Wilcoxon signed rank tests were carried out to evaluate whether female body mass values

increased during the reproductive cycle phases relative to the non-reproductive condition.

Analysis of variance and signed rank tests were carried out using JMP version 10.0 (SAS Institute, Inc., Cary, North Carolina, USA). We also carried out linear regressions to evaluate the relationship between the fold-difference in maternal $\dot{V}O_2$ between the embryogenic phase (E1, *Lamprophis fuliginosus;* E2 or E3, *Thamnophis marcianus*) of the reproductive cycle and the non-reproductive condition, relative to the number of viable eggs/neonates, and the total clutch/litter masses. Linear regression plots were produced by GraphPad Prism software (Version 6, GraphPad Software, La Jolla, CA, USA) and the slopes were evaluated using the software's linear regression analysis. Statistical significance was set at the p=0.05 level. All values are reported as mean ± S.E.M.

RESULTS

Descriptive Statistics

Overall, six out of the original ten *Lamprophis fuliginosus* females developed vitellogenic follicles and oviposited a clutch of eggs; and 13 of the original twenty *Thamnophis marcianus* females developed vitellogenic follicles and successfully underwent parturition. For *Lamprophis fuliginosus*, following the mating and re-feeding period, four out of the six females did not yet exhibit observable vitellogenic follicles. This pre-vitellogenic (PV) period lasted for two to eight weeks. There was no "pre-vitellogenic" period for *Thamnophis marcianus*. Females of both species continued to consume meals until five weeks (range = three to eight weeks) prior to oviposition (*Lamprophis fuliginosus*), or three weeks (range = one to five weeks) prior to parturition (*Thamnophis marcianus*).

Additional descriptive statistics are reported in Table 2.1. The average female body mass values that correspond to the reproductive phases in this study are reported in Figure 2.4C

(*Lamprophis fuliginosus*) and Figure 2.5C (*Thamnophis marcianus*). The fold-differences in body mass during the reproductive cycle phases, relative to the non-reproductive condition, are reported in Table 2.2.

Necroscopy and Ultrasonography

Ultrasound imaging was used correlate the morphological features of the ovarian follicles with the results obtained from gas exchange and PBT measurements. A series of sequential ultrasound images for one representative female of each species is presented in Figure 2.3. Necroscopy photographs are presented in Figure 2.2.

Vitellogenesis.— During primary vitellogenesis (V1, both species), follicles exhibited low echogenicity (dark color) relative to the surrounding tissues, and mean follicle size was 9mm x 9mm (l x w) for *Lamprophis fuliginosus* and 6mm x 8mm (l x w) for *Thamnophis maricanus.* During secondary vitellogenesis (V2, both species), follicles from *Thamnophis marcianus* exhibited higher echogenicity (lighter color) relative to the surrounding tissues, and nearly doubled in size to an average of 13mm x 14 mm (l x w). Follicle size in *Lamprophis fuliginosus* also exhibited substantial growth, to an average of 16mm x 11mm (l x w), but did consistently demonstrate higher echogenicity. For both species, the necroscopies of secondary vitellogenic females revealed large ovarian follicles and densely vascularized oviductal tissues that proceeded from the anterior colon to the posterior aspect of the gallbladder.

Embryogenesis.—During the embryogenic (E1) phase of the reproductive cycle in *Lamprophis fuliginosus*, mean follicle size increased from V2 values to 31mm x 13 mm (l x w) and follicles exhibited contents with higher echogenicity (lighter color). All females exhibited a clearly delineated and highly echogenic egg shell membrane surrounding the follicular contents. We

were not able to identify any clear signs of developing embryos (e.g., amnion or allantois; Van Dyke and Beaupre, 2011). It is possible that the presence of an egg shell membrane prevented this observation.

In *Thamnophis marcianus* mean follicle size increased to 14mm x 17mm (l x w) during embryogenesis 1 (E1), and a clearly observable low echogenic fluid filled space was observed, indicating the presence of the amnion and/or allantois (Van Dyke and Beaupre, 2011). During embryogenesis 2 (E2), mean follicle size continued to increase (17mm x 20mm) (l x w), and highly echogenic differentiated embryonic tissue became observable within the amnion. The follicular dimensions during the final measurement period (embryogenesis 3; E3), were not possible to ascertain, but follicles were noticeably larger and numerous coils of highly echogenic embryonic vertebrae were observable.

Oxygen Consumption ($\dot{V}O_2$) and Respiratory Quotient (RQ)

The mean $\dot{V}O_2$ and corresponding RQ values for *Lamprophis fuliginosus*, during each of the reproductive conditions are presented in Figure 2.4A and 2.4B. The mean female $\dot{V}O_2$ and corresponding RQ values during each of the reproductive conditions for *Thamnophis marcianus* are presented in Figure 2.5A and 2.5B. The fold-differences in $\dot{V}O_2$, during each of the reproductive cycle phases, relative to the non-reproductive condition are reported for both species in Table 2.3.

For *Lamprophis fuliginosus*, the clutch $\dot{V}O_2$ values (n = 5 clutches), measured within 48 hours following oviposition, represented 3.3 % (range = 1.5 to 4.6 %) of the female $\dot{V}O_2$ values measured in the embryogenic (E) phase of the reproductive cycle. The *Thamnophis marcianus* neonate $\dot{V}O_2$ values (n=10 litters), measured within 48 hours following parturition, represented

33 % (range = 2 to 60%) of the female $\dot{V}O_2$ values, measured during the embryogenic (E2, n=2; E3, n=8) phase of the reproductive cycle. The RQ values from the *Lamprophis fuliginosus* clutches were, on average, 0.92 (range = 0.58 to 1.74) (n=4) and for *Thamnophis marcianus*, were on average, 0.81 (range =0.72 to 0.96).

For *Lamprophis fuliginosus*, there was no significant correlation between the number of viable eggs (r^2=0.0035; p=0.9104) or total clutch mass (r^2=-0.2397; p=0.3243) and the fold-change in maternal $\dot{V}O_2$ during the embryogenic phase of the reproductive cycle. On the other hand, for *Thamnophis marcianus* there was a significant correlation (r^2=0.4553, p=0.0323) (n=10) between total litter mass and the fold-change in maternal $\dot{V}O_2$ during the embryogenesis, but no significant correlation was found for the number of live neonates (r^2 = 0.381, p= 0.0573).

Preferred Body Temperature

A representative Tb trace for a single female of each species, measured during each of the reproductive conditions, is presented in Figure 2.6A (*Lamprophis fuliginosus*) and 2.6C (*Thamnophis marcianus*). The mean PBT values for the population, associated with each of the reproductive conditions, are presented in Figure 2.6B (*Lamprophis fuliginosus*) and Figure 2.6D (*Thamnophis marcianus*). Our results for *Lamprophis fuliginosus* indicate that mean PBT values ranged from 24.1°C to 26.9°C, and no statistically significant differences (p>0.1696) exist among the PV, V1, V2, or E1 reproductive phases relative to the NR condition. For *Thamnophis marcianus*, mean PBT values ranged from 27.1°C and 28.3°C, and no significant differences (p>0.6908) occurred among the V1, V2, E1, E2, E3, or PP conditions.

DISCUSSION

Metabolism

Vitellogenesis.—In snakes, vitellogenesis can be broadly categorized into a primary phase, where follicles are small and contain mostly water, and a secondary phase, where follicles are considerably larger and contain proportionally more yolk (Dessauer and Fox, 1956; Van Dyke and Beaupre, 2011). Based on previously reported echogenicity characteristics in snakes, the V1 phase of reproduction for both species in the present study roughly corresponds with primary vitellogenesis, and the V2 phase with secondary vitellogenesis (Dessauer, and Fox, 1959; Van Dyke and Beaupre, 2011). Consistent with our predictions, female $\dot{V}O_2$ was significantly elevated (by 1.5 fold in *Lamprophis fuliginosus* and 2.0 fold in *Thamnophis marcianus*) during primary vitellogenesis, and remained significantly elevated by a factor of 1.8 to 2.0 fold during secondary vitellogenesis. We also found that in *Thamnophis marcianus* RQ values increased from 0.74 in the non-reproductive condition to 0.80-0.82 during vitellogenesis (Figure 2.5B), indicating a shift in metabolic substrate from lipids to proteins (Kleiber, 1961). On the other hand, *Lamprophis fuliginosus* did not exhibit any significant shift in RQ value, although values trended towards a shift from protein catabolism in the NR condition (i.e., RQ of 0.79) to lipids during vitellogenesis (i.e., RQ as low as 0.68) (Figure 2.4B) (Kleiber, 1961).

To our knowledge our study for *Lamprophis fuliginosus* is the first to have investigated maternal $\dot{V}O_2$ during the vitellogenic phase of the reproductive cycle in an oviparous species and virtually no studies have examined RQ values in reproductive squamate reptiles. The maternal factorial increments in $\dot{V}O_2$ during the vitellogenic phases of the reproductive cycle reported in the present study are comparable to values reported for a number of viviparous species measured

during vitellogenesis. More specifically, the viviparous *Agkistrodon contortrix*, *Boa constrictor*, *Eryx colubrinus*, *Nerodia sipedon*, *Thamnophis sirtalis* and *Crotalus atrox* exhibit factorial $\dot{V}O_2$ increments that range from approximately 1.4 to 1.5 fold above non-reproductive levels (Beaupre and Duvall, 1998; Van Dyke and Beaupre, 2011).

Like many squamates reptiles, both *Lamprophis fuliginosus* and *Thamnophis marcianus* are lecithotrophic, that is, the energy required for embryonic development is derived entirely from stored yolk (Stewart, 1992). Yolk is provisioned during the physiological process of vitellogenesis, which is initiated following mating (provided females have sufficient fuel reserves), where increased levels of circulating estradiol stimulate the liver to synthesize the vitellogenic precursor molecule vitellogenin, very low-density lipoproteins (VLDL), and varying quantities of vitamin and mineral binding proteins (Yaron and Widzer, 1978; Garstka, et al., 1985; Wallace, 1985; Bonnet et al., 1994; White, 1991). These macromolecules are then transported to ovarian follicles by the bloodstream, and incorporated via endocytosis (Wallace, 1985). During vitellogenesis, liver mass increases substantially (e.g., up to 2.2 fold in *Lacerta vivipara jacquin*), and much of this increase is associated with the proliferation of cellular organelles responsible for the storage and processing of proteins and lipids (Dessauer, and Fox, 1959; Wallace, 1985; Gavaud, 1986; Nilsson and Raberg, 2001; Santos et al., 2007). Simultaneously, in response to elevated levels of circulating progesterone and gonadotropins, the female oviduct and the ovarian follicles undergo extensive remodeling (Yaron and Widzer, 1978; Garstka, et al., 1985). Changes include the hypertrophy of epithelial tissue, development of shell glands, increased thickness of the oviductal connective tissue and associated musculature, and increased vascularity (Guillette and Jones, 1985; Masson and Guillette, 1987; Parker et al. 2010; Blackburn and Stewart, 2011).

Previous studies on mammals and fish have demonstrated that rates of whole animal protein synthesis correlates with metabolism (Houlihan, 1991; Garlick et al., 1976).   Although not directly measured in the present study, the synthesis and processing of vitellogenin and the net production and maintenance of liver, follicular, and oviductal tissues may result in substantially increased rates of whole-animal protein synthesis. If cardiac output increases during vitellogenesis in response to elevated metabolism, rates of protein turnover in the heart may also increase, and further contribute to the whole animal metabolic increment (Houlihan et al., 1988).

*Embryogenesis.—*The embryos present within freshly oviposited eggs from female *Lamprophis fuliginosus* in our population exhibit developmental characteristics consistent with stage 33 of Hubert and Dufaure (1968)'s developmental scheme (Boback et al., 2012). According to previous work, this represents approximately 30 to 40% of completed embryonic development, which would require approximately three to five weeks of development time within the oviduct (Andrews, 2004; Boback et al., 2012). Therefore, measurements obtained 0 to 3 weeks prior to oviposition (embryogenic, E1) in *Lamprophis fuliginosus* most likely represents values for females that have ovulated. For *Thamnophis marcianus*, ovulation occurred between the secondary vitellogenic (V2) and embryogenic 1 (E1) phases of reproduction, because female follicles exhibited a clearly observable low echogenic fluid filled space, which is consistent with previous reports on post-ovulatory snakes (Van Dyke and Beaupre, 2011).

Consistent with our predictions, maternal $\dot{V}O_2$ values for both species during early embryogenesis (E1) remain elevated by a factor of 1.5 to 1.6 above non-reproductive values. RQ values remain shifted towards protein catabolism (0.81 and 0.83) in *Thamnophis marcianus,* while RQ values were not different (i.e., 0.79 and 0.81) between the NR and E1 phases in

Lamprophis fuliginosus. The $\dot{V}O_2$ increments are consistent with previously published values in oviparous squamates measured before oviposition, which range from 1.3 fold in *Python regius* to 2.0 fold in *Sceloporus aeneus aeneus* and *Sceleporus undulates* (Guillette, 1982; Ellis and Chappell, 1987; Angiletta and Sears, 2000). On the other hand, previous work on five species of viviparous species did not exhibit any difference in maternal $\dot{V}O_2$ between the post-ovulatory and non-reproductive conditions (Van Dyke and Beaupre, 2011).

Approximately half-way through embryonic development (E2 phase), *Thamnophis marcianus* exhibited a factorial increment in $\dot{V}O_2$ of 2.6 fold, and within two weeks prior to parturition (E3 phase), the factorial increment was 3.2 fold above NR values. During both mid and late embryogenesis, female RQ values shifted back to approximately non-reproductive values (0.75 to 0.76), indicating lipids as the primary metabolic substrate. The pattern of progressively increased maternal $\dot{V}O_2$ from early to late embryogenesis has been reported for a number of viviparous squamates (DeMarco, 1993; Robert and Thompson, 2000; Schultz et al., 2008; Van Dyke and Beaupre, 2011). The 3.2 fold increment exhibited by *Thamnophis marcianus* within two weeks prior to parturition was similar to the 2.7 to 2.9 fold increases previously published for *Thamnophis sirtalis* and *Acanthophis praelongus* (Birchard et al., 1984; Schultz et al., 2008).

Following ovulation, plasma levels of vitellogenic biomolecules (i.e., vitellogenin, and VLDLs), trace nutrients (e.g., calcium, cholesterol, phosphorus), and the size of the maternal liver all rapidly return to pre-gestational values (Dessauer and Fox, 1959; Garstka, et al., 1985; Bonnet et al., 1994). Oviparous species retain embryos until the pharyngula/limb-bud stage of development (Blackburn, 1995; Andrews, 2004). At this point, embryonic metabolism is still relatively low and the majority of the mass of each follicle consists of metabolically inert

substances (i.e., water, yolk, and albumin) (DeMarco, 1993; Blackburn, 1995; Robert and Thompson, 2000). On the other hand, viviparous species such as *Thamnophis marcianus* retain embryos to term, where embryonic metabolism is expected to increase progressively throughout development, reaching peak values just prior to parturition (Packard et al., 1977; Robert and Thompson, 2000). Previous work indicates that over ~70% of follicular mass in late embryogenesis consists of actively metabolizing fetal tissues (Wang and Ji, 1997; Thompson and Russell, 1999).

Embryonic metabolism may represent a large component of the total metabolic demand prior to parturition in some species. For example, the viviparous *Agkistrodon contortrix*, *Boa constrictor*, *Eryx colubrinus*, *Nerodia sipedon*, *Thamnophis sirtalis* all exhibited neonate metabolic values that accounted for nearly 100% of pre-partum maternal $\dot{V}O_2$ (Robert and Thompson, 2000; Schultz et al., 2008; Van Dyke and Beaupre, 2011). In contrast, we estimate that embryonic metabolism accounts for just ~3% of the metabolic increment in *Lamprophis fuliginosus,* and ~33% of the increment in *Thamnophis marcianus.* The remaining metabolic increment is possibly derived from increased rates of protein synthesis associated with the maintenance of reproductive tissues, the production of oviductal structural adaptations, and chronically elevated cardiopulmonary output (Birchard et al., 1984).

Preferred Body Temperature

The preferred body temperature (PBT), defined as the mean body temperature selected by a reptile in a laboratory thermal gradient, is a commonly used index to describe thermoregulatory behavior in squamates (Huey, 1982; Huey and Kingsolver, 1989). Snakes exhibit wide-ranging variation in peripheral body temperature values (Gregory 1990; Roark and Dorcas 2000), but

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maintain relatively consistent perfusion of GI tract organs with blood, even at low environmental temperatures (Amiel et al., 2011). Therefore, we chose to assist-feed rubber coated data loggers to females in order to obtain core body temperature values for PBT measurements. Similar techniques have previously been employed in snakes (e.g., Ellis and Chappell, 1987; Blazquez, 1995).

Our results indicate that average maternal PBT values range from ~24 to 27°C in *Lamprophis fuliginosus* and ~27 to 28°C in *Thamnophis marcianus* (Figure 2.6B and 2.6D). These PBT values are similar to previously published PBT values for non-reproductive individuals in species (Zurovsky, et al., 1987; Lutterschmidt et al., 2002; Lutterschmidt & Hutchison, 2003; Rosen, 1991). Contrary to our predictions, there were no significant differences in PBT between the reproductive cycle phases relative to the non-reproductive condition (*Lamprophis fuliginosus*) or among the reproductive cycle phases (*Thamnophis marcianus*). Direct comparisons of maternal thermal preferences from the present study to previous work is problematic, as many previous studies do not clearly distinguish the phase of the reproductive cycle where measurements are determined or do not measure females throughout the reproductive cycle, and considerable variations exist in temperature measurement techniques (e.g., cloacal probe versus surgically implanted devices) and operative temperatures (i.e., field based versus laboratory based gradients).

Nevertheless, our results for *Lamprophis fuliginosus* and *Thamnophis marcianus* are most consistent with the lack of a shift in female thermal preferences in the viviparous lizards, *Oligosoma maccannii, Mabuya agilis, Mabuya macrorhyncha, and Mabuya frenata* (Vrcibradic and Rocha, 2004; Hare et al., 2009). On the other hand, the vast majority of previous studies on oviparous (Lourdais et al., 2008; Blouin-Demers and Weatherhead, 2001; Brana, 1993; Isaac and

Gregory, 2004; Luiselli and Akani, 2002) and viviparous species (Stewart, 1965; Garrick, 1974; Beuchat, 1986; Grier et al., 1989; Blazquez, 1995; Ladyman et al., 2003; Tu and Hutchison, 1994) indicate that squamates exhibit shifts in maternal PBT values during reproduction (i.e, either higher or lower PBT). Therefore, our results contribute to the growing trend in the literature which indicates that squamates may not exhibit a broadly consistent shift in maternal thermoregulatory patterns during reproduction. A similar lack of a broadly consistent shift in PBT values has also been described for the specific dynamic action (SDA) response to feeding in squamate reptiles (Tu and Hutchison, 1995; Wall and Shine, 2008). Ultimately, future work is required to elucidate whether these differences exist because of inherent species-specific variation (e.g., reproductive mode differences and/or variation in optimal temperatures associated with underlying reproductive physiological processes), discrepancies in experimental techniques (e.g., sampling time and frequency, or laboratory versus field studies), or a combination of both factors.

Conclusions

Reproduction represents a dramatic disruption to maternal homeostasis, and may result in substantial direct energetic costs and shifts in maternal thermoregulatory preferences. In both *Lamprophis fuliginosus* and *Thamnophis marcianus* reproduction lasts for months and is characterized by a distinct phase of vitellogenesis followed by ovulation and then embryogenesis. We found that female metabolic rate remains elevated throughout the reproductive cycle for both species, and that maternal thermoregulatory preference do not differ. Our work also represents one of the first to examine RQ values in reproductive females, and results indicate that *Thamnophis marcianus* exhibits significant shifts in metabolic substrate, while *Lamprophis fuliginosus* does not. We propose that a substantial majority of the direct

costs of reproduction are associated with increased rates of whole animal protein synthesis. Furthermore, the lack of a shift in maternal thermoregulatory preferences during vitellogenesis and embryonic development may indicate that the thermal optima for these processes (Dawson, 1975) are not different from maintaining the non-reproductive condition.

The metabolic increments reported in the present study (i.e., 1.5 to 3.2 fold) for *Lamprophis fuliginosus* and *Thamnophis marcianus* last for months, and these values rival or even substantially exceed increments reported for mammals and birds. For example, in birds, the metabolic increments associated with egg production range from approximately 1.2 to 1.3 fold above baseline values (Nilsson and Raberg, 2001; Vezina et al., 2003; Vezina and Williams, 2005). Likewise, both large (e.g., humans, ruminants) and small mammals (e.g., rodents) exhibit peak metabolic increments that range from approximately 1.3 to 3.0 fold during gestation (Brockway et al., 1963; Lof et al., 2005; Thompson, 1992; Urison and Buffenstein, 1995). Considering the similar reproductive metabolic increments between these species, and that mammals exhibit substantial cardiopulmonary and hemodynamic adjustments during pregnancy (e.g., increased cardiac output, cardiac hypertrophy, decreased peripheral vascular resistance, and increased blood volume) (Metcalfe and Parker, 1966; Hoversland, et al., 1974; Wolfenson, et al., 1982; Duvekot and Peeters, 1994; Wong et al., 2002), it is possible that similar adjustments also occur in snakes.

Experimental Timeline for *Lamprophis fuliginosus*

Previous
Oviposition
Event

Current
Oviposition
Event

Re-feeding and Mating Period (2-4 weeks)	Pre-Vitellogenic Period (PV) (n=4/6 females) (2-8 weeks)	Primary Vitellogenesis (V1) (3 weeks)	Secondary Vitellogenesis (V2) (3 weeks)	Embryogenesis 1 (E1) (3 weeks)	Non-Reproductive (NR) (0-1 week)

Brumation
Ends

Experimental Timeline for *Thamnophis marcianus*

Parturition

Non-Reproductive (NR) (within 1 week)	Re-feeding and Mating Period (3 weeks)	Primary Vitellogenesis (V1) (2 weeks)	Secondary Vitellogenesis (V2) (2 weeks)	Embryogenesis 1 (E1) (2 weeks)	Embryogenesis 2 (E2) (2 weeks)	Embryogenesis 3 (E3) (2 weeks)

Figure 2.1. Schematic illustrating the experimental timeline for *Lamprophis fuliginosus* and *Thamnophis marcianus*. Time flows from the left to the right, and the duration of time spent in each of the measurement periods is denoted within a parentheses.

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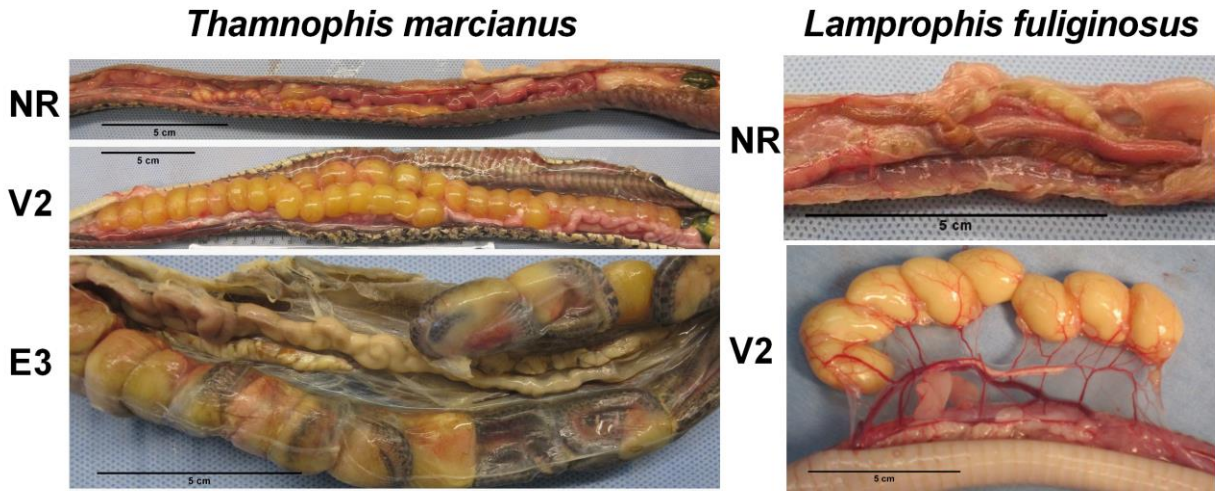
Figure 2.2. Photographs from necroscopies of a segment of the caudal abdomen of three female *Thamnophis marcianus* (left) and two female *Lamprophis fuliginosus* (right). *T. marcianus* images represent females during the non-reproductive (NR), secondary vitellogenic (V2), and embryogenic 3 (E3) reproductive conditions. *L. fuliginosus* images represent females during the non-reproductive (NR) and secondary vitellogenic (V2) reproductive conditions. For all photographs, skin and adipose tissues have been removed to expose the underlying organs. *T. marcianus* female (E3) was fixed in formalin prior to dissection. Scale bars were calibrated against known length values using Image J software.
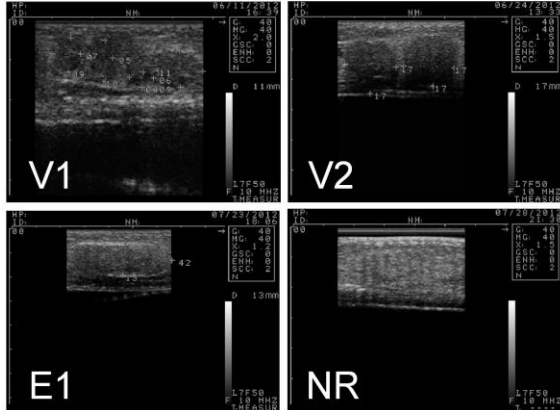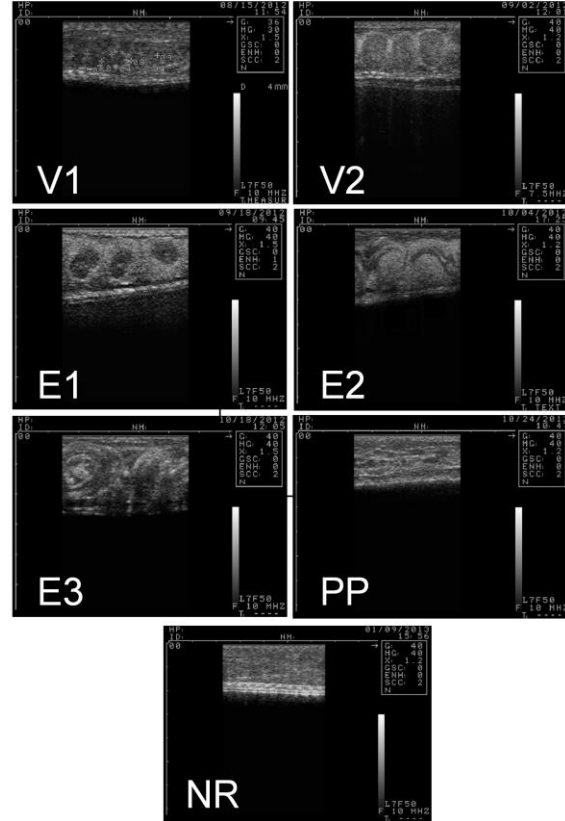
Figure 2.3. Ultrasound images of one representative female *Thamnophis marcianus* (right) and *Lamprophis fuliginosus* (left). Images are shown for primary vitellogenesis (V1), secondary vitellogenesis (V2), embryogenic 1 (E1), embryogenic 2 (E2), embryogenic 3 (E3), post-partum (PP), and a non-reproductive (NR) conditions for *Thamnophis marcianus*. For *Lamprophis fuliginosus*, images are shown for the primary vitellogenesis (V1), secondary vitellogenesis (V2), embryogenic 1 (E1), and non-reproductive (NR) reproductive conditions. All images were obtained using an adjustable 5-10MHz linear array transducer (CTS 3300, Shantou Institute of Ultrasonic Instruments, Guangdong, China), and saved using the system's built-in software. In some photographs, digital caliper measurements on the observable follicles are present, and the zoom level may differ among the images (e.g., 1.5X versus 1.2X).
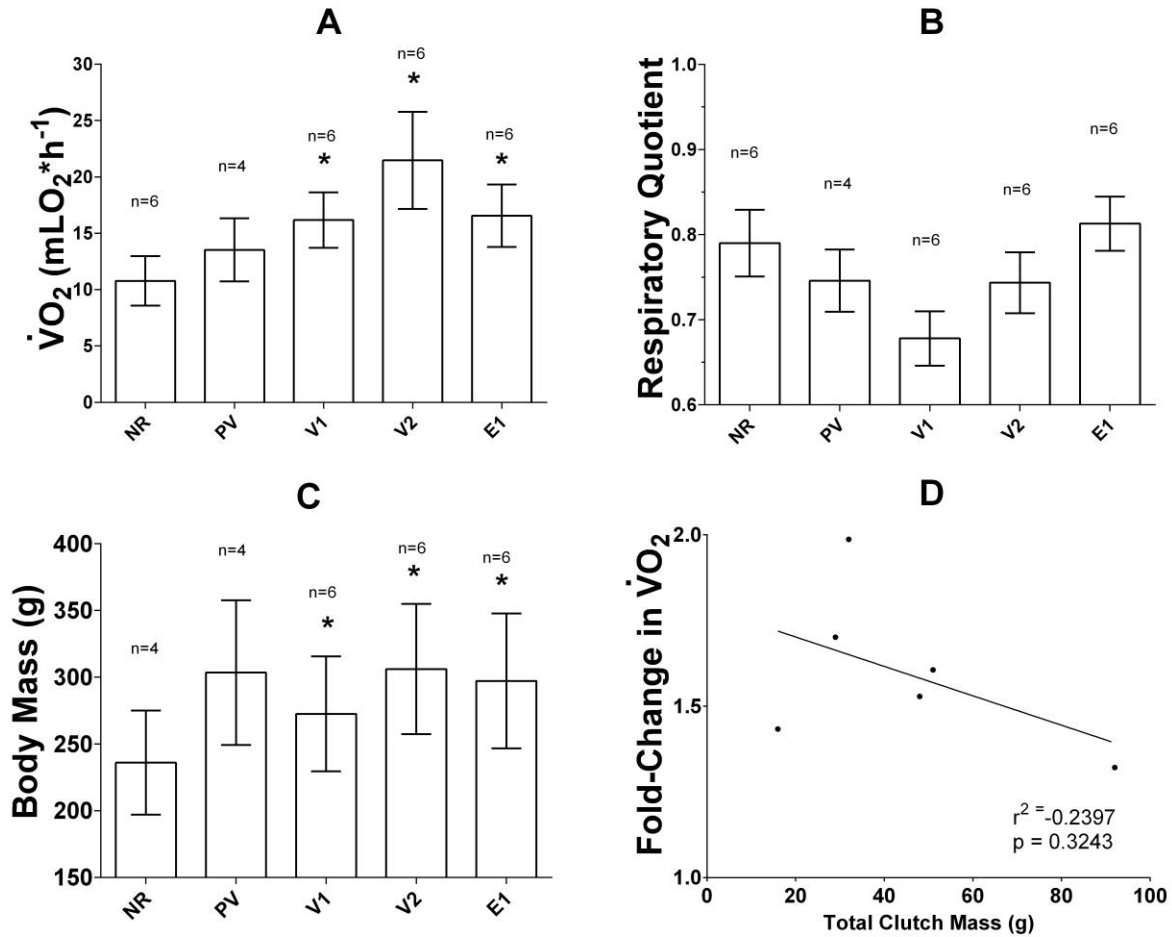
Figure 2.4. *Lamprophis fuliginosus*. Mean $\dot{V}O_2$ (A), RQ (B), and body mass (C) values for females measured during the non-reproductive (NR), pre-vitellogenic (PV), primary vitellogenesis (V1), secondary vitellogenesis (V2), and embryogenesis (E1). (D) Represents the correlation between the fold-change in pre-oviposition (i.e., embryogenic) maternal $\dot{V}O_2$ values relative to the NR condition, and the females' total clutch mass (g). An asterisk (*) represents a statistically significant difference ($p<0.05$) from the NR condition. Data for A, B, and C are presented as mean ± 1 S.E.M. Sample sizes are located above each of the values.
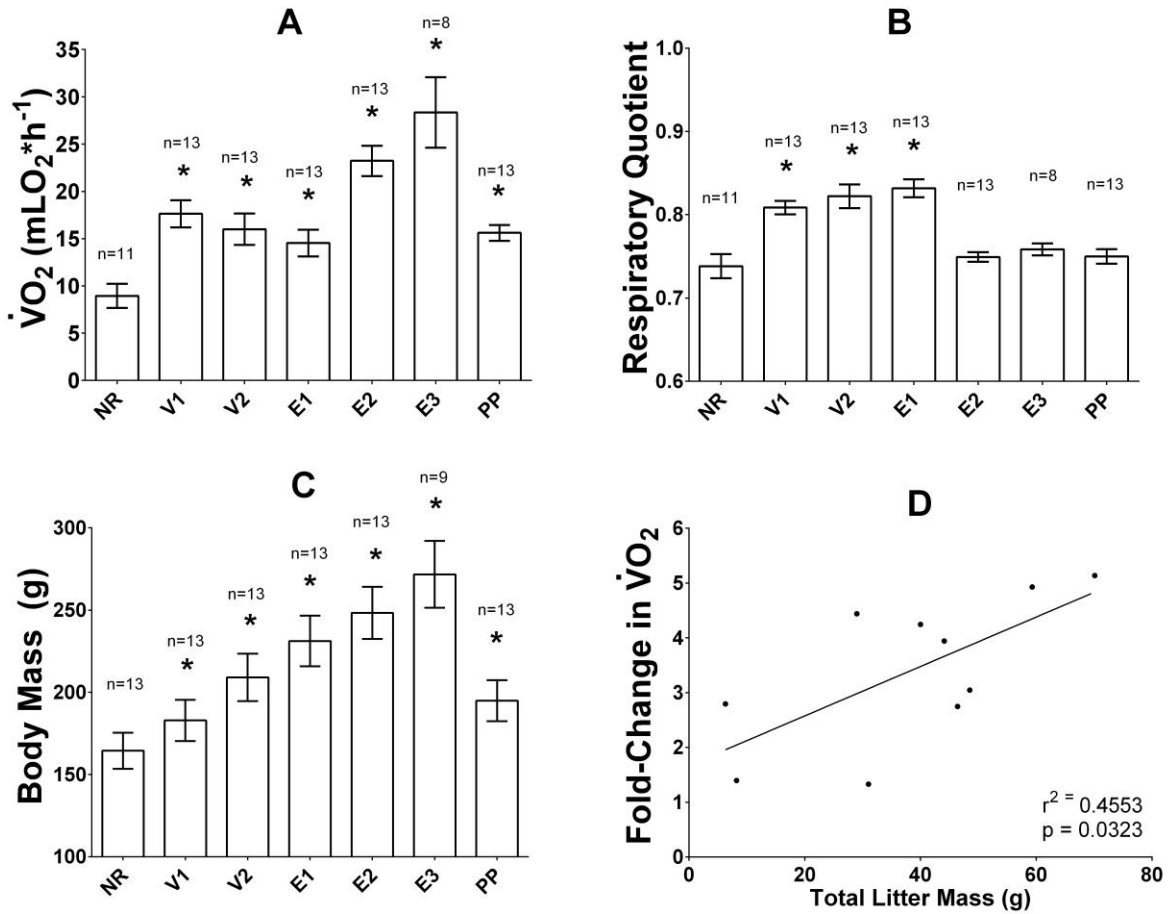
Figure 2.5. *Thamnophis marcianus.* Mean $\dot{V}O_2$ (A), RQ (B), and body mass (C) values obtained during the non-reproductive (NR), primary vitellogenesis (V1), secondary vitellogenesis (V2), embryogenic 1 (E1), embryogenic 2 (E2), embryogenic 3 (E3), and post-partum (PP) conditions. (D) Represents the correlation between the fold-change in pre-partum (i.e., embryogenic 2 or 3) maternal $\dot{V}O_2$ values relative to the NR condition, and the females' total clutch mass (g). An asterisk (*) represents a statistically significant difference ($p<0.05$) from the NR condition. Data for A, B, and C is presented as mean $\pm$ 1 S.E.M. Sample sizes are located above each of the values.
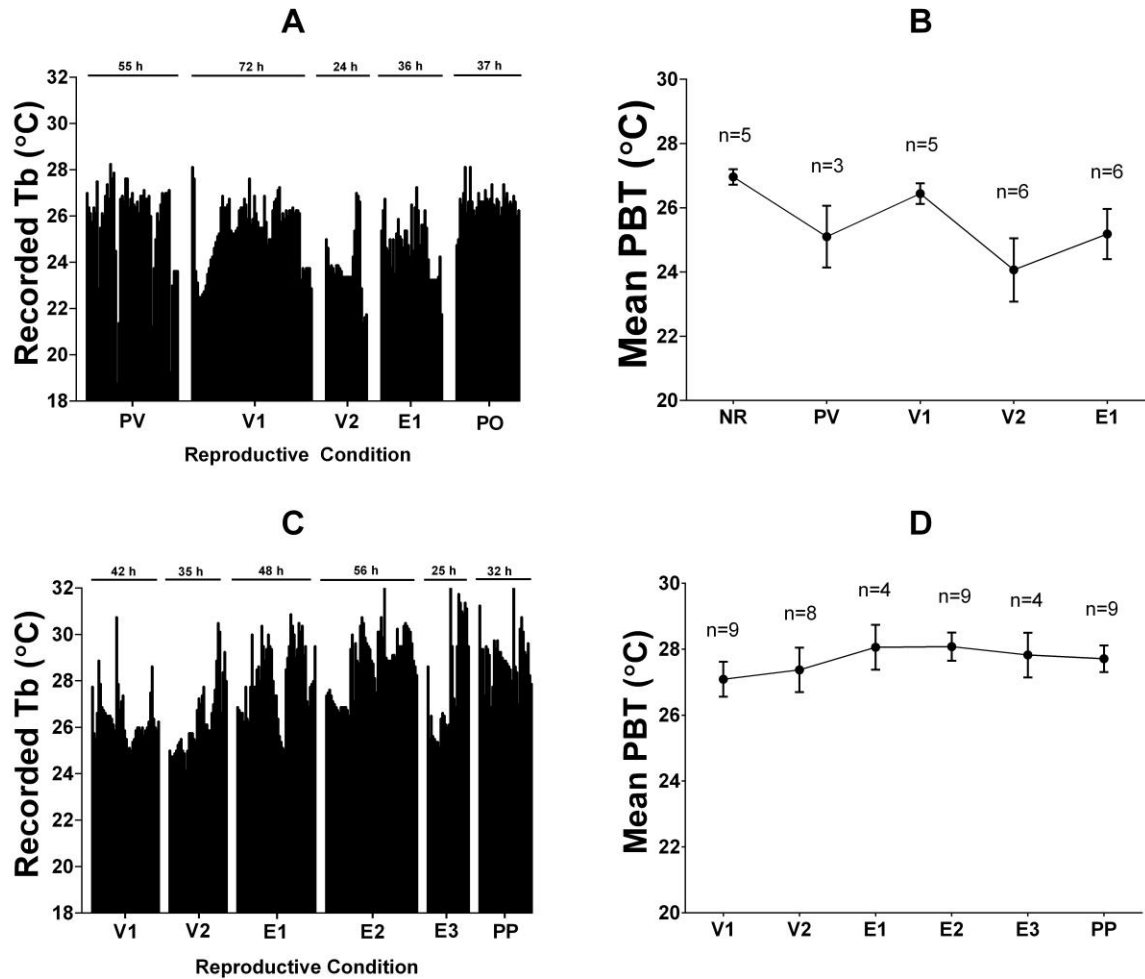
Figure 2.6. Body temperature (Tb) values for one representative female *Lamprophis fuliginosus* (A) and *Thamnophis marcianus* (B). Tb values were obtained once every 60 minutes for 24-72 hours (trial duration noted as black bar above each trace), during each of the reproductive phases and the non-reproductive (NR) condition. For each of the trials, the leftmost bar represents the first Tb value and the rightmost bar the final. The mean preferred body temperature (PBT), defined as the mean of all Tb values obtained for each trial, are presented for *Lamprophis fuliginosus* (B) and *Thamnophis marcianus* (D). PBT values are presented as mean 1 S.E.M. Sample sizes for each of the PBT categories are listed in the results section of this paper.

Table 2.1. Descriptive Statistics for *Lamprophis fuliginosus* and *Thamnophis marcianus.*

Species	Females (n)	Snout-Vent Length (SVL)	Reproductive Cycle Length (weeks)	Clutch/ Neonate Mass (g)	# of Eggs/ Neonates (n)	# Viable Eggs/ Neonates (n)	Relative Clutch Mass (RCM)
Lamprophis fuliginosus	6	93 (80-105)	8.5 (6.5-9.5)	45 (14-92)	9 (6-20)	5 (0-12)	0.14 (0.10-0.19)
Thamnophis marcianus	13	61 (52-71)	9.5 (9 -11)	35 (6-70)	13 (3-27)	12 (1-27)	0.13 (0.04-0.23)

Table 2.2. Fold-differences in body mass values obtained during the reproductive cycle phases relative to the NR condition for female *Lamprophis fuliginosus* and *Thamnophis marcianus.*

Species	PV	V1	V2	E1	E2	E3	PP
Lamprophis fuliginosus	ns, p=0.0625, n=4	1.1 fold, p=0.0156, n=6	1.3 fold, p=0.0156, n=6	1.2 fold, p=0.0156, n=6	N/A	N/A	N/A
Thamnophis marcianus	N/A	1.1 fold, p=0.0024, n=13	1.3 fold, p<0.0001, n=13	1.4 fold, p<0.0001, n=13	1.5 fold, p<0.0001, n=13	1.6 fold, p<0.0001, n=13	1.2 fold, p=0.0010, n=13

Table 2.3. Fold-difference in $\dot{V}O_2$ values obtained during the reproductive cycle phases relative to the NR condition for female *Lamprophis fuliginosus* and *Thamnophis marcianus.*

Species	PV	V1	V2	E1	E2	E3	PP
Lamprophis fuliginosus	ns, p=0.958, n=4	1.5 fold, p=0.0456, n=6	2.0 fold, p=0.0456, n=6	1.5 fold, p=0.0185, n=6	N/A	N/A	N/A
Thamnophis marcianus	N/A	2.0 fold, p=0.0012, n=13	1.8 fold, p=0.0093, n=13	1.6 fold, p=0.0063, n=13	2.6 fold, p=0.0003, n=13	3.2 fold, p=0.0025, n=8	1.7 fold, p=0.0023, n=13

Table 2.4. Results for respiratory quotient (RQ) values obtained during the reproductive cycle phases relative to the NR condition for female *Lamprophis fuliginosus* and *Thamnophis marcianus.*

Species	PV	V1	V2	E1	E2	E3	PP
Lamprophis fuliginosus	ns, p=0.8744, n=4	ns, p=0.2021, n=6	ns, p=0.8872, n=6	ns, p=0.9978, n=6	N/A	N/A	N/A
Thamnophis marcianus	N/A	p=0.0042, n=13	p=0.0051, n=13	p=0.0011, n=13	p=1.0000, n=13	p=0.9731, n=8	p=0.9999, n=13

CHAPTER 2

THE INTERACTION EFFECTS OF REPRODUCTION, DIGESTION, AND ACTIVITY IN THE OVIPAROUS SNAKE, *LAMPROPHIS FULIGINOSUS*

INTRODUCTION

In vertebrates, the oxygen demands resulting from elevated metabolic states are matched by the transport capacity of the cardiopulmonary system. Physiologists have traditionally studied the physiological response to elevated metabolic states in isolation (e.g., the physiological response to activity). However in nature, animals may carry out several metabolically demanding activities simultaneously and it is important to determine how such conflicting demands for oxygen are resolved (Jackson, 1987; Bennett and Hicks, 2001).

In some organisms, specifically carnivorous reptiles, the metabolic increments associated with digestion (specific dynamic action; SDA) can be equivalent to values measured during strenuous activity (Secor and Diamond, 1997; Secor et al., 2000). Consequently, during postprandial activity, the oxygen transport capacity of the cardiopulmonary system may be exceeded, and oxygen delivery may be prioritized either to digestive functions or to exercising muscles (Hicks and Bennett, 2004). Alternatively, if the oxygen transport capacity accommodates the simultaneous oxygen demands of both activity and digestion, then the response is additive (Hicks and Bennett, 2004). For example, in the monitor lizard, *Varanus exanthematicus*, the maximum $\dot{V}O_2$ during postprandial activity was higher that the $\dot{V}O_{2max}$ during fasting exercise, indicating an additive response (Bennett and Hicks, 2001).

In reptiles, reproduction is a less thoroughly studied physiological state that also elevates oxygen demand over resting values. Snakes exhibit viviparous (live-bearing) and oviparous (egg-laying) modes of reproduction, often within the same family (e.g., *Colubridae, Elapidae, Viperidae, Boidae;* Neill, 1964). Oviparity is considered the ancestral mode of reproduction, but many of the same physiological processes (i.e., vitellogenesis, organ remodeling and embryogenesis) that increase oxygen demand occur in both oviparous and viviparous species (Stewart, 1992; Blackburn, 1995). The patterns of oxygen transport in response to the simultaneous demands of digestion, activity and reproductive state is unknown. Therefore, we ask: Does the animal specifically prioritize between digestion, activity, or reproduction? Alternatively, is the cardiopulmonary system's capacity to deliver oxygen sufficient to meet the demands of all the elevated metabolic states, and hence additive (Hicks and Bennett, 2004)?

The goal of this study was to compare the metabolic demand from digestion, activity, and reproduction, both individually and when combined simultaneously in the African Brown House Snake (*Lamprophis fuliginosus*) (Broadley, 1983). This oviparous old-world colubrid, native throughout sub-Saharan Africa, regularly produces multiple clutches of eggs per annum, ingests large meals at frequent intervals, and as an active-foraging species has a large capacity for maximal energy production during activity (Fitch, 1970; Ruben, 1976; Broadley, 1983; Ford, 2001). Furthermore, *Lamprophis fuliginosus* does not require a hibernation cycle to initiate reproduction, can store sperm for long intervals of time between mating events, and has very modest husbandry requirements (Haagner, 1987; Ford and Seigel, 2006). This study expands upon the work of Hicks and Bennett (2004) and Secor et al., (2000) by examining the additivity and/or prioritization of metabolic responses in a squamate reptile during three physiological states. Based on results obtained in these previous studies, we hypothesized that *Lamprophis*

fuliginosus would also exhibit an additive $\dot{V}O_2$ response to digestion and activity, and that any $\dot{V}O_2$ increment associated with reproduction would sum in a similar manner.

MATERIALS AND METHODS

Study Animals and Procedures

Adult *Lamprophis fuliginosus* were purchased from commercial retailers in September, 2010 and housed in a large vivarium room at the University of California, Irvine maintained at $25\pm2°C$ with a 12L:12D photocycle. Animals were maintained individually in opaque plastic enclosures (50.2 x 34.3 x 14.2 cm), each with water *ad libitum* and a subsurface heating element (Sunbeam Products, Inc., Boca Raton, Florida, USA; or Zoo Med Laboratories, Inc., San Louis Obispo, California, USA). All animals were offered pre-killed mice *ad libitum* approximately once weekly. All procedures involving this species were approved by the University of California, Irvine Institutional Animal Care and Use Committee (IACUC protocol #2010-2966 and 2009-2906).

Reproductive condition was assessed using ultrasonography (Smith et al., 1989; Stahlschmidt et al., 2011; Van Dyke et al., 2012). To limit struggling, females were mildly sedated with gaseous isoflurane in a bell-jar prior to all scans. Following mild sedation, a portion of the animal's caudal abdomen was placed in a shallow bath of warm water, and brief (approximately 5-10 minute) scans were performed using a 5-10MHz linear array transducer (CTS 3300, Shantou Institute of Ultrasonic Instruments, Guangdong, China) as 3-5cm lateral sections (depending on the magnification) at varying adjustable depths (range = 2-4cm).

Females recovered from sedation within five minutes following exposure to gaseous isoflurane, and were observed to have regained normal behavior (i.e., alertness, behavioral thermoregulation, and feeding without regurgitation) within 12 hours of exposure to gaseous isoflurane. The presence of follicles was observable based on the soft-tissue echogenicity of the developing follicles and the boundary differences in echogenicity of the shell parchment versus the surrounding tissues (Figure 3.1) (Stahlschmidt, et al., 2011). Coelomic palpation was used to confirm visual analyses.

If no follicles were observable using ultrasonography, females were considered to be non-reproductive; but if enlarged ovarian follicles were observed, and the individual subsequently laid eggs, the female was considered to be reproductive. Once the reproductive female oviposited her clutch, we determined *post hoc*, the percentage of the reproductive cycle from which our measurements were made, and the reproductive cycle length was considered to be the time elapsed between previous and current oviposition events. For reproductive females, the mean reproductive cycle period was 76±6 days, and the measurements obtained for the reproductive experimental observations were obtained, on average, 22±13 days prior to oviposition - which indicates that females were measured either during secondary vitellogenesis or early embryonic development.

In many oviparous squamates, ovulation occurs shortly before oviposition and embryos of the freshly ovipoisted clutch present at stages 25-33 of Hubert and Dufare (1968)'s developmental scheme (Shine, 1983; Blackburn, 1995). In *Lamprophis fuliginosus*, freshly oviposited eggs contain embryos of stage 33 in this developmental scheme, but embryos represents less than .01 % of the pre-partum female's body mass (Boback et al., 2012; Jackson et

al., personal observations). Therefore, our measurements obtained, on average, nearly three weeks prior to oviposition were not likely influenced by embryonic metabolism.

Measurement of $\dot{V}O_2$ and $\dot{V}CO_2$

The preferred body temperature for *Lamprophis fuliginosus* has previously been determined to be $25 \pm 0.1°C$ (Zurovsky et al., 1987, Lutterschmidt et al., 2002, Lutterschmidt and Hutchison, 2003). Therefore, all gas exchange experiments were carried out in a large walk-in environmental chamber maintained at $25 \pm 1°C$. Body mass was obtained to the nearest $\pm 1g$ either before or after all metabolic trials. If possible, experiments were carried out in complete darkness, but some the trials were carried out under 12L:12D conditions due to simultaneously occurring experiments that required those conditions.

Oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) values in adult male and adult female *Lamprophis fuliginosus* were measured using flow-through respirometry. In brief, atmospheric air was pushed by a mass flow controller (Brooks Instruments, Model 5841; Emerson Electric Co., Hatfield, Pennsylvania, USA; or GF-3 Gas Mixing Flowmeter; Cameron Instruments Inc., Guelph, Ontario, Canada), and directed into up to eight metabolic chambers (940mL or 1555mL depending on the size of the animal). Incurrent flow rates were determined and then monitored at least once daily using mass flow meters (± 1 to 5mL/min) (GFM17 AALBORG, Orangeburg, New York, USA) and/or Cole Parmer rotameters (± 5mL/min) (Cole-Parmer, Vernon Hills Illinois, USA). At least one of the metabolic chambers remained free from experimental animals, and served as a baseline for referencing. Flow rates ranged from approximately 100-200mL/min, depending on the size of the animal, and the metabolic state under examination. Excurrent air from each of the metabolic chambers was then subsampled by

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a separate gas analysis circuit in one of two ways: (1) Excurrent air was carried away from each of the metabolic chambers by a separate line of Tygon tubing, which ended with a syringe barrel that facilitated subsampling. An 18 gauge needle was affixed to each of the syringe barrels, attached to a Cole Parmer three-way solenoid valve, and selectively drew a subsample of air into the gas analysis circuit. (2) Each of the excurrent Tygon tubing lines from the metabolic chambers fed directly into one of eight Cole Parmer three-way solenoid valves. Air through the solenoid exited through one of two ports, depending on whether or not the solenoid valve was activated. If the solenoid valve was not activated, excurrent air flowed freely into the room; if the solenoid line was activated, the chamber's excurrent air flowed through a separate port into a final line of Tygon tubing, into which an 18 gauge needle was inserted. Through the 18 gauge needle, a subsample of this excurrent air was pulled into the gas analysis circuit. By both methods, only one chamber was sampled at a time, in a repeating fashion, without changing the incurrent flow rate experienced by the animal. In both subsampling setups, the sampling time and frequency of measurements were determined by a motorized rotational multiplexer (Industrial Timer Company, Centerbrook, Connecticut, USA) which electronically activated the eight Cole Parmer three-way solenoid valves for approximately 4 to 10 minutes at a time, once every 80 minutes for the duration of the trial.

The gas analysis air circuit's flow rate was controlled by a diaphragmatic pump (Model R-1, Applied Electrochemistry Technologies, Pittsburg, PA, USA) attached to a Cole Parmer mass flow controller. The gas analysis equipment was arranged as follows: AEI CD-3A CO_2 sensor/analyzer $\rightarrow$ soda lime cartridge (remove CO_2) $\rightarrow$ drierite cartridge (remove H_2O) $\rightarrow$ Sable Systems FC-II oxygen analyzer or AEI S-3A oxygen sensor/analyzer. Analog outputs from the S-3A/FC-II and the CD-3A were connected to a Biopac MP-100 hardware system,

downloaded to a PC at 20 MHz, and quantified using Acknowledge (Version 3.2.4) software (Biopac Inc., Santa Gotela, CA, USA). The O_2 and CO_2 analyzers were calibrated regularly throughout the experimental period, and if signal drift occurred between calibrations, verified for consistency in signal linearity by inserting calibration gas with known O_2 and CO_2 concentrations. To calculate oxygen consumption ($\dot{V}O_2$), we used equation 10.1 from Lighton (2008); the Fick equation to calculate carbon dioxide production ($\dot{V}CO_2$); and respiratory quotient (RQ) was calculated as the quotient of $\dot{V}CO_2$ and $\dot{V}O_2$.

Standard Metabolic Rate (REST)

On the day of the experiment, snakes were removed from their enclosures and transported to a walk in environmental chamber, placed in opaque plastic metabolic chambers, and gas exchange was measured for 20 to 48 hours. Previous studies in snakes report that the attainment of a postabsorptive state may take as long as 14 days in some species (Secor and Diamond, 1995; Secor, 2009). Thus, we chose to fast all animals for 14 to 16 days prior to the determination of resting or "standard" metabolic rate. Although previous work in juvenile *Lamprophis fuliginosus* indicates that large differences exist between photophase and scotophase $\dot{V}O_2$ values (~2.1-2.7 fold difference; Roe et al. 2004), more recent work in adults indicates that these differences are either not present (reproductive females and males), or are small (1.15 fold, non-reproductive females; Appendix B, Figure 6.2). Thus, the three lowest consecutive $\dot{V}O_2$ (and corresponding $\dot{V}CO_2$) measurements, regardless of the phase of the photocycle, were considered to represent the resting or "standard" metabolic rate. We chose to eliminate the first four hours of each trial to permit recovery from handling stress, and acclimation to the new environment.

Fasting Activity (ACT)

Females (reproductive and non-reproductive) and males were fasted 17 to 19 days prior to the exhaustive activity trial. The animals were placed in a fixed-volume glass chamber (either 2.7L or 3.9L, depending on the size of the animal), which was sealed at the top with a screw-on cap containing incurrent and excurrent airlines. The airlines protruding into the glass container from the cap were fixed at various lengths to ensure proper air mixing. Incurrent air was pushed from a Brooks mass flow controller (Brooks Instruments 5841-A1A2QG) into the chamber, and excurrent air was forced out through an excurrent air line by the slight positive pressure of the system (less than $1mmH_2O$). Flow rates of air entering and leaving the chamber were constantly monitored via an AALBORG mass flow meter or a calibrated Cole Parmer rotameter. A subsample of the excurrent air stream was pulled through an 18-guage needle into the gas analysis circuit described above.

The exhaustive activity trial and peak $\dot{V}O_2$ was measured using an approach that has been previously described (Ruben, 1976; Gratz and Hutchison, 1977; Ellis and Chappell, 1987; Andrade et al., 1997). Briefly, a snake was placed in a metabolic chamber and the snake was forced to right itself from an investigator-induced rotation. The snake was repeatedly flipped (via rotation of the chamber), until the snake could no longer perform a righting response. The time to exhaustion (time from the first rotation until exhaustion: TTE) was recorded, and the snake was allowed to recover for 10 to 20 minutes. The highest FEO_2 values and the corresponding $FECO_2$ values during the entire trial were considered to represent peak gas exchange levels.

Post-prandial (DIG)

Females and males were fasted 20 to 23 days, and then fed a meal equivalent to 20% their body mass (pre-killed mice). The snake was transferred from its enclosure to a metabolic chamber in the constant temperature environmental chamber ($25\pm1°C$), and flow rates were adjusted to reflect an expected increase in $\dot{V}O_2$ and $\dot{V}CO_2$. Values were recorded approximately once every 80 minutes for 46-60 hours (females) and 45 hours (males). This time frame encompasses the duration required for $\dot{V}O_2$ values to begin to decline following the post-prandial peak (Appendix B, Figure 6.3). The mean of the three consecutively highest $\dot{V}O_2$ values (and corresponding RQ values) were chosen to represent peak post-prandial gas exchange values. The first four hours of measurements were discarded to account for recovery from handling stress and acclimation to the chamber.

Post-Prandial Activity (DIG+ACT)

To determine whether there was an interaction between digestion and exhaustive activity, both males and females (reproductive and non-reproductive) were fasted for 20 to 23 days, and fed a meal equivalent to 20% of their body mass (pre-killed mice). After 46 to 56 hours (females) and 47 to 49 hours (males) of digestion, the snake was placed into the glass exercise chamber (mentioned above), and subjected to the same exhaustive activity trial as previously described under the same conditions. Although post-prandial $\dot{V}O_2$ had begun to decline from peak levels by 46 to 56 hours post-ingestion (Appendix B, Figure 6.3), the decline is relatively minor and likely suffices to expose the snake to a near-maximal interaction between digestion and exhaustive activity.

Statistical Analysis

The data collected in this research occurred between May 2011 and September 2012. Over this time, the female *Lamprophis fuliginosus* in this study underwent multiple reproductive cycles, and consequently samples obtained for the experimental conditions of REST, DIG, ACT, and DIG+ACT did not always occur within the same reproductive cycle. Repeated measures (range = 1-5) were obtained from the same individual during more than one reproductive cycle for one or more of the experimental conditions, and these measurements were considered correlated. Thus, a linear mixed model (LMM) was used to evaluate the difference in $\dot{V}O_2$, $\dot{V}CO_2$, RQ, and time to exhaustion (TTE) between non-reproductive and reproductive states, among the four experimental conditions, and possible interactions between reproductive state and experimental condition. The repeated measures were modeled with a compound symmetry covariance structure in the LMM. Also due to the variation associated with body mass among individuals in our population (up to a 5-fold difference), and the confounding factor associated with the increased mass during reproduction by females (Ellis and Chappell, 1987; Angilletta and Sears, 2000), the lowest non-reproductive body mass value obtained for each individual during an experimental observation was included in the LMM as a covariate to adjust for the effect of body mass on the outcomes. For males, a separate LMM was applied for each outcome to evaluate the differences among the experimental conditions. The $\dot{V}O_2$ and $\dot{V}CO_2$ values were log-transformed to reduce variation and skewness of the data, and then the fold change was used to describe the differences between the four experimental conditions. *Post hoc* pairwise comparisons were performed if a significant experimental condition effect or a significant reproductive state and experimental condition interaction was observed. Tukey-Kramer's method was utilized for multiple comparison adjustment. Adjusted p-values are presented for

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the multiple comparison results. All analyses were performed with SAS 9.3 (Cary, NC) and the significance level was set at the 0.05 level.

RESULTS

Descriptive Statistics

There were a total of 17 females and 6 males on which multiple experimental observations were carried out. Females (reproductive and non-reproductive) had a median of 7 (ranged 2 to 12) total observations and males had a median of 4 (ranged 2 to 4) total observations. The average body mass of males obtained during observations was 79 ± 20g. For females, the average body mass obtained while non-reproductive was 247 ± 81g; for reproductive observations, average female body mass increased to 315 ± 98g (a factorial increment of 1.2).

Oxygen Consumption ($\dot{V}O_2$)

The results for average whole-animal $\dot{V}O_2$ values in females (reproductive and non-reproductive) for all four experimental conditions are reported in Figure 3.2A; $\dot{V}O_2$ values for males are reported in Figure 3.3A. The fold-change in $\dot{V}O_2$ during each of the three treatments over REST for females (non-reproductive and reproductive) and males are reported in Table 3.1.

For non-reproductive females, the peak $\dot{V}O_2$ during ACT was significantly higher than both DIG (adj. $p<0.0001$, 1.6 fold) and DIG+ACT (adj. $p=0.0041$, 1.5 fold) $\dot{V}O_2$ values. During reproduction, no differences were identified among DIG, ACT, and DIG+ACT values. When comparing between the reproductive and non-reproductive states, the $\dot{V}O_2$ from REST was significantly higher during reproduction compared to the non-reproductive state (adj. $p<0.0001$,

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1.4 fold). Furthermore, the $\dot{V}O_2$ from ACT in the non-reproductive state was significantly higher than while reproductive (adj. p=0.012, 1.3 fold).

For males, the $\dot{V}O_2$ values from ACT (adj. p<0.0002, 2.1 fold) and DIG+ACT (adj. p=0.021, 1.5 fold) were significantly higher than DIG. The $\dot{V}O_2$ from ACT (adj. p=0.014, 1.4 fold) was significantly larger than DIG+ACT.

Respiratory Quotient (RQ)

The results for respiratory quotient (RQ) associated with each of the four experimental conditions for females (reproductive and non-reproductive) are reported in Figure 3.2B; RQ results for males are reported in Figure 3.3B. The *post hoc* pairwise comparisons showed that females in both reproductive and non-reproductive states had RQ values from ACT, and DIG+ACT that were significantly larger (adj. p<0.0001) than from both REST and DIG values. No differences were observed in RQ values between ACT and DIG+ACT, nor between REST and DIG. For females, it was shown that the RQ from ACT in the reproductive state was significantly higher than the ACT values obtained during the non-reproductive state (adj. p = 0.0001). For males, significant differences were observed among all four experimental conditions (adj. p<0.003).

Time to Exhaustion (TTE)

The results for time to exhaustion (TTE) for ACT, and DIG+ACT for both males and females (reproductive and non-reproductive) are reported in Figure 3.4A and 3.4B. For females during the non-reproductive state, the time to exhaustion (TTE) was 11.4±3.2 minutes longer (adj. p=0.0062) during ACT compared to DIG+ACT (Figure 1E). There was no significant

difference between ACT and DIG+ACT during the reproductive state (adj. p=0.95), nor between reproductive and non-reproductive states for ACT or DIG+ACT (adj. p>0.38). For males, the TTE during ACT (n=6) was not significantly different from DIG+ACT (n=5) (p=0.056) (Figure 1F).

DISCUSSION

Under natural conditions animals respond to a variety of metabolic challenges, often simultaneously, and the integrated response to these challenges is not well understood (Jackson, 1987; Anderson and Wang, 2003). Digestion, activity, and reproduction all independently elevate oxygen consumption, and therefore it is likely that an interaction effect (additivity or prioritization) occurs when an animal's cardiopulmonary system is simultaneously challenged with multiple metabolic demands (e.g. digestion and activity) (Hicks and Bennett, 2004). Our results support the hypothesis for a prioritization response to post-prandial activity (ACT+DIG) because $\dot{V}O_2$ increments were either unchanged or lower than those elicited during fasting activity (ACT). Reproductive females also exhibit a prioritization response during fasting activity (ACT) and digestion (DIG) (i.e., unchanged $\dot{V}O_2$ increments from the non-reproductive state). This contrasts with previous work on squamates, which exhibit an additive response to post-prandial activity (DIG+ACT) (Secor et al., 2000; Bennett and Hicks, 2001). Our results are more consistent with work on amphibians and some species of fish which either do not change their maximum $\dot{V}O_2$ and/or performance during DIG+ACT, or demonstrate a lower peak $\dot{V}O_2$ and/or performance (Beamish, 1974; Aslop and Wood, 1997; Anderson and Wang, 2003; Thorarenson and Farrell, 2006; Fu et al., 2009).

Reproduction

The 1.4 fold increment exhibited by reproductive *Lamprophis fuliginosus* likely represent the maternal costs of allocating yolk to developing follicles (i.e., vitellogenesis) and extensive remodeling of the maternal reproductive tract and the liver (Dessauer and Fox, 1959; Masson and Guillette, 1987; Van Dyke and Beaupre, 2011). Similar metabolic increments have also been demonstrated by the oviparous *Python regius* (Ellis and Chappell, 1987) and by multiple viviparous species, measured during the vitellogenic phase of reproduction (Beaupre and Duvall, 1998; Van Dyke and Beaupre, 2011).

During vitellogenesis, the maternal liver up-regulates the synthesis of the yolk precursor molecule vitellogenin, very low density lipoproteins (VLDL), and a number of vitamin/mineral binding proteins, which are then transported by the bloodstream to the developing ovarian follicles, and incorporated into the cells via receptor mediated endocytosis (Wallace, 1985; White, 1991). During vitellogenesis, the maternal liver increases in mass (over 2-fold in some species; Dessauer and Fox, 1959; Gavaud, 1986), and much of this increase may be associated with the storage of protein, and the proliferation of organelles responsible for protein and lipid processing (Dessauer and Fox, 1959; Hahn, 1967; Gavaud, 1986; Santos et al., 2007). Simultaneously, follicular and oviduct tissues undergo substantial growth and remodeling, with concomitant angiogenesis (Masson and Guillette, 1987; Blackburn, 1998; Parker et al., 2010). Together, these processes may increase the rate of whole-animal protein synthesis, an energetically demanding process which has been demonstrated to correlate linearly with increments in $\dot{V}O_2$ (Houlihan, 1991).

Digestion (DIG)

Our results indicate that the ingestion of a meal equivalent to 20% of body mass was accompanied by a large increment in $\dot{V}O_2$ over standard values: approximately 3 fold in males, 4 fold in reproductive females, and 6 fold in non-reproductive females (Figure 3.2A and 3.3A). In the present study, the lower digestive aerobic scope by reproductive females is principally due to an elevated baseline, not because of a lower absolute $\dot{V}O_2$, suggesting that either reproduction and digestion are not additive, or that the magnitude of the metabolic increments of reproduction (1.4 fold) to digestion (~6 fold) is relatively too small to observe any statistically significant difference. Our results are consistent with previously published $\dot{V}O_2$ factorial increments in snakes (following 20% body mass meals), which range from 3.7 fold in *Crotalus durissus* to 8.6 fold in *Python molurus* (Andrade et al., 1997; Hopkins et al., 2004; Toledo et al., 2003; Wang et al., 2003; Roe et al., 2004).

Although the post-prandial $\dot{V}O_2$ increment in reptiles following a period of prolonged fasting has been observed for over half of a century (Benedict, 1932), only more recently have the mechanisms associated with this process become elucidated. Proposed mechanisms include the energetically demanding processes associated with digestion (e.g., gastric acid secretion, up-regulation of intestinal nutrient transporters), and the increased rate of protein synthesis in the liver, stomach, intestine, lung, kidney, and heart tissues (McMillan and Houlihan, 1988; Houlihan, 1991; Secor and Diamond, 1995; Andrade et al., 1997; Overgaard et al., 1999; McCue, 2006; Secor, 2009). Indeed, previous research on snakes demonstrate that all of the aforementioned organs exhibit significant hypertrophy within 48 hours post-ingestion, a change

which can be correlated with an increased rate of protein synthesis, and hence oxygen

consumption (Houlihan, 1991; Secor and Diamond, 1995; Anderson et al., 2005).

To our knowledge, this is one of the first studies that has attempted to measure the

metabolic interactions between reproduction and digestion in an ectothermic vertebrate. The

physiological processes associated with reproduction (i.e. vitellogenesis and organ remodeling)

and digestion may compete for gas exchange by the cardiopulmonary system and for nutrient

processing capacity from the liver (specifically protein and lipid processing). However, no

differences were observed to manifest in maternal $\dot{V}O_2$ increments in the present study. It is

possible that the relatively low increment associated with reproduction (1.4 fold) was not

sufficient to elicit an interaction effect in maternal $\dot{V}O_2$ values; however previous work in

reproductive *Thamnophis sirtalis sirtalis* and *Acanthophis praelongus* indicate that reproductive

females exhibit $\dot{V}O_2$ increments as large as 2.7-2.9 fold (Birchard et al., 1984; Schultz et al.,

2008). It is therefore possible that similar experiments carried out in these viviparous species

may reveal significant interaction effects between digestion and reproduction.

Fasting Activity (ACT)

Our values for aerobic scope during exhaustive activity for non-reproductive (~9.5 fold)

and reproductive (5 fold) females, and our values for males (~6 fold) all fall within the ranges

previously published for snakes. For example, *Python regius, Python molurus, Coluber*

constrictor, Crotalus viridis, and *Mastocophis flagellum* demonstrate aerobic scopes between 5-

12 fold resting values (Ruben, 1976; Chappel and Ellis, 1987; Secor et al., 2000). In this study,

we hypothesized that reproductive females would exhibit an additive response, that is, the

metabolic increment associated with reproduction would be additive with the aerobic scope of

exhaustive activity. However, our results do not support this hypothesis, instead, peak $\dot{V}O_2$ is 30% lower, and RQ is increases from 1.3 to 1.7; interestingly, reproductive females do not change their time to exhaustion.

During reproduction, the presence of developing follicles increases body mass, which may increase the drag that the animal must overcome to propel itself, and thus increase the force necessary to perform bodily movements (Shine, 2003; Moon and Gans, 1998). Furthermore, in some oviparous species, protein catabolism during reproduction results in considerable epaxial musculature loss, and subsequent reductions in strength (Lourdais et al., 2004, 2013). These factors, in addition to other competing and/or confounding physiological processes associated with reproduction, may result in a greater overall reliance on anaerobic metabolism in order to produce force, hence a more profound metabolic acidosis which manifests as higher RQ values (Gleeson and Bennett, 1982) and a lower aerobic scope. However, the effects of reproduction were not sufficient to decrease endurance time (TTE), which is contrary to previous studies in snakes (Seigel et al., 1987; Brodie, 1989) and lizards (Shine, 1980; Cooper et al., 1990; Sinervo et al., 1991; Shine, 2003), where reproduction results in 20 to 50% decrements for both endurance and speed.

Our results also indicate possible sex-specific differences in activity parameters in *Lampmrophis fuliginosus*. Males exhibit a lower aerobic scope (~6 fold versus 9.6 fold) and rely more heavily on anaerobic metabolism (RQ of 1.7 versus 1.3) than non-reproductive females. Previous work on squamate reptiles have either not compared males with females (e.g., Seigel et al., 1987; Brodie, 1989; Cooper et al., 1990; Shine, 2003), or did not indicate any differences in locomotory performance between non-reproductive females and males (Shine 1980). Relatively little published work describes the natural history of *Lamprophis fuliginosus* (Broadley, 1983;

Haagner, 1987), making it difficult to extract ecological causations associated with our results; however, as an actively-foraging income breeder (Bonnet, 1998) it is possible that males and females differ in life history characteristics (i.e., increased female foraging demand associated with multiple clutches per annum). Consequently there may be higher selection pressure on females to increase activity performance parameters than for males.

Post-Prandial Activity (DIG+ACT)

To study digestion and activity, a particularly useful lineage of organisms are squamate reptiles, particularly the snakes. With the exception of some sit-and-wait foraging species (e.g., *Python molurus;* Secor and Diamond 1997), the oxygen demand during digestion peaks at values that ranges from 3-8 fold resting values, and this increment varies with meal composition, size of the meal, and body temperature (McCue, 2006; Secor, 2009). Likewise, the oxygen demand during unsustainable activity in snakes produces aerobic scopes that range from 2-12 fold resting values (Ruben, 1976; Gratz and Hutchison, 1977; Ellis and Chappell, 1987; Andrade et al., 1997). Despite the similarity in peak aerobic scopes, the physiological processes associated with digestion and activity differ in a variety of properties, including their active tissue location (active muscle *versus* gastrointestinal tract), metabolic pattern (catabolic *versus* anabolic), acid-base status (acidosis *versus* alkalosis), and time course (minutes *versus* days) (Hicks et al. 2000).

The present study's data clearly support a prioritization pattern of O_2 delivery for males and non-reproductive females, that is, post-prandial activity results in lower (i.e., up to 40% lower) aerobic scopes, and unchanged (males) or lower (i.e., by ~40%; non-reproductive females) activity performance. This is the first study to investigate the possible interactions between activity, digestion, and reproduction in a squamate reptile. Contrary to our predictions,

post-prandially active reproductive females exhibit unchanged peak $\dot{V}O_2$ values (i.e., ACT and ACT+DIG values were similar both within and among reproductive conditions), and activity performance was maintained – also indicating a prioritization pattern of O_2 delivery.

In contrast to our results, previous studies on squamate reptiles have demonstrated an additive $\dot{V}O_2$ response to the combined challenges of digestion and activity. This additive response was hypothesized to occur because of increased tissue O_2 extraction, evidenced by an elevated arterial-venous O_2 difference (Secor et al., 2000; Hicks and Bennett, 2001). In *Python molurus* this additive $\dot{V}O_2$ response occurs in spite of a 47 to 81 % decrease in blood flow to the GI tract (Secor and White, 2010). Our results are more similar to the prioritization response exhibited by a number of fish species, for example, Rainbow Trout (*Oncorhynchus mykiss*), Chinook Salmon (*Oncorhynchus tshawytscha*), and Largemouth Bass (*Micropterus salmonoides*) all demonstrate a prioritization response to post-prandial activity, which manifests as a decrement in activity performance and or/aerobic scope of similar magnitudes as those for *Lamprophis fuliginosus* in the present study (Aslop and Wood 1997; Thorarensan and Farrell 2006). The decreased TTE exhibited by *Lamprophis fuliginosus* are also similar to the decrements in speed and endurance exhibited by the post-prandially active (non-reproductive) Garter Snakes *Thamnophis elegans* and *Thamnophis marcianus* (Garland and Arnold, 1983; Ford and Shuttlesworth, 1986).

While the mechanisms responsible for the prioritization response presented in the current study are unknown, results indicate that for non-reproductive females there may be a shared prioritization between activity and digestive processes (i.e., activity performance is sacrificed to maintain digestion), while for males and reproductive females, priority for O_2 delivery may be

accorded to actively contracting skeletal musculature and digestive processes curtailed. The ingestion of a meal resulted in a 20% increase in body mass for males and non-reproductive females, and further elevated reproductive female body mass up to 40% above non-reproductive levels. An elevation in body mass will increase the metabolic cost of physical activity (by 10% for each 10% increase in body weight; Taylor et al., 1980), but *Lamprophis fuliginosus* did not exhibit such a change. RQ values did increase in males (and trended towards an increase in females); possibly indicating that some of this additional energy requirement was met by elevated levels of anaerobic glycolysis (Gleeson and Bennett, 1982).

The presence of a large meal within the stomach, and for females, the enlarged ovarian follicles may have compressed the visceral organs, resulting in lung compression and reduced venous return, thereby limiting O_2 and CO_2 exchange by the lungs and/or cardiac output (Munns et al., 2004; Munns, 2013). These factors may explain the lack of change/decrement in $\dot{V}O_2$ during post-prandial activity in all groups in the present study. Under this framework, reproductive females should have experienced proportionally larger decrements in both aerobic scope and in TTE. However our results indicate that the differences were minor, and therefore it is possible that reproductive females possess mechanisms that circumscribe some of these constraints. For example, it is possible that under these circumstances, additional blood flow is redistributed away from the GI tract to the axial musculature (the pattern exhibited by *Python molurus* and *Dicentrarchus labrax;* Altmiras et al. 2008; Secor and White 2010) or there is a decrease in the R-L shunt fraction and/or reduced ventilation/perfusion heterogeneity in the lungs, resulting in increased O_2 extraction from ventilated air (hence elevated arterial PO_2 and a steeper O_2 diffusion gradient at the tissues) (Secor et al., 2000; Bennett and Hicks, 2001), serving to both maintain $\dot{V}O_2$ and prevent a decrement in activity performance.

Considering that this species continues to consume meals throughout gestation and that survival during reproduction is more important than any other period (Shine, 1980; Brodie, 1989) it is possible that this species has evolved safeguards to enhance cardiopulmonary performance and sustain locomotor performance when challenged with the simultaneous demands of reproduction, digestion, and activity. However, additional experiments designed to systematically examine ecological patterns of reproductive *Lamprophis fuliginosus*, and laboratory based studies focusing on cardiopulmonary parameters, such as arterial and venous PO_2, minute ventilation, heart rate, and systemic blood flow distribution are required before causal mechanisms can be elucidated.
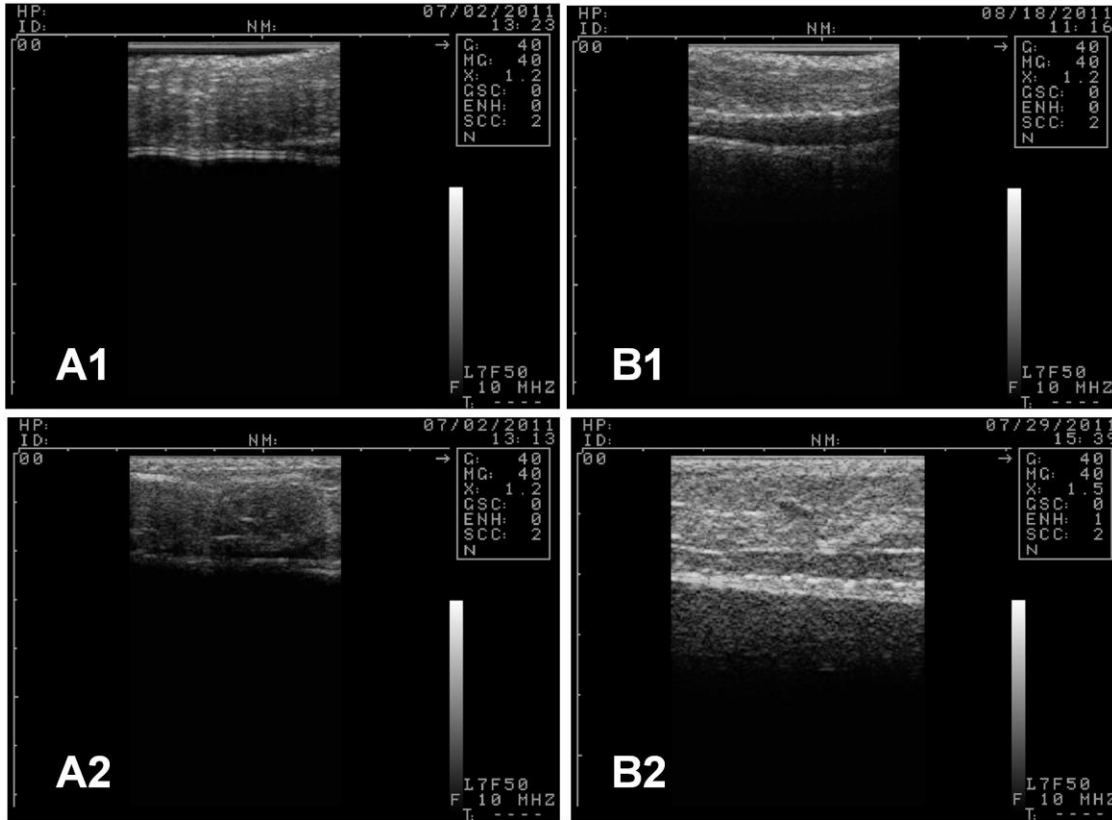
Figure 3.1. Representative ultrasound images of two *Lamprophis fuliginosus* females measured while reproductive (A1 and A2), and non-reproductive (B1 and B2). Images represent the lateral aspect of one or more ovarian follicles (A1 and A2) or a lateral aspect the posterior coelemic cavity (B1 and B2). Images were obtained using a 5 to 10MHz linear array transducer (SIUI 3300) at a zoom level of 1.2 X (approximately 5cm section) or 1.5 X (approximately 4 cm section) at varying adjustable depths (range = 2 to 3cm).

Figure 3.2. Female whole animal $\dot{V}O_2$ (A) and respiratory quotient (RQ) (B) values for each of the experimental treatments. The reproductive condition is indicated by the pattern key. Acronyms: REST = rest, DIG = post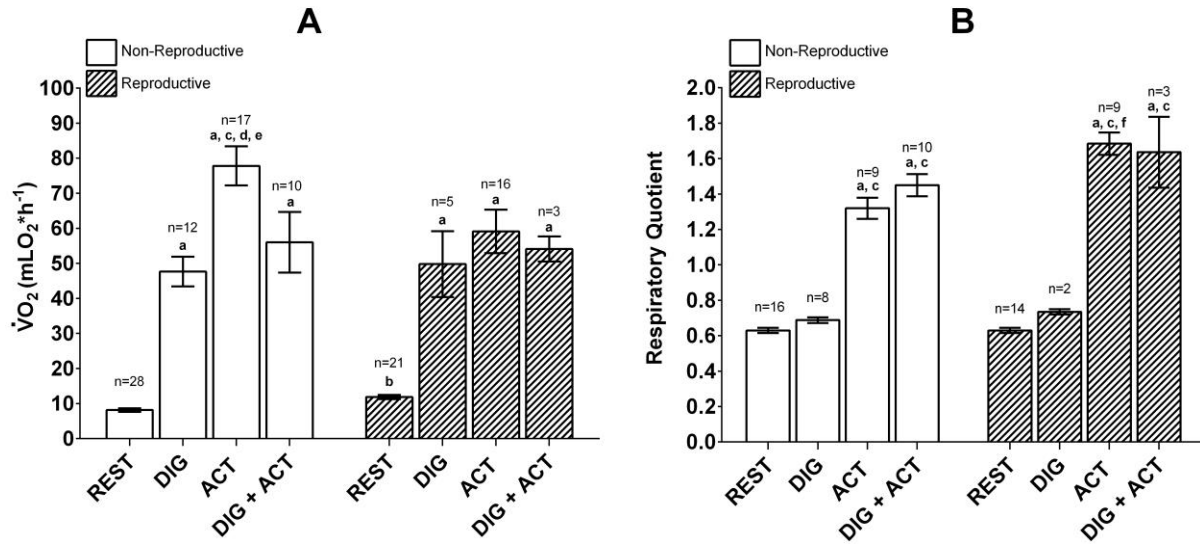-prandial, ACT = fasting exhaustive activity, DIG+ACT = post-prandial exhaustive activity. Lowercase letters above the represent a statistically significant difference: (a) = significantly larger than REST values within the reproductive condition; (b) = significantly larger than non-reproductive REST values, (c) = significantly larger than DIG values within the reproductive condition; (d) = significantly larger than ACT+DIG values within the reproductive condition; (e) = significantly larger than reproductive ACT values; (f) = significantly larger than non-reproductive ACT values. Sample size for each treatment located above the values. All values represent mean ± S.E.M.

A

B
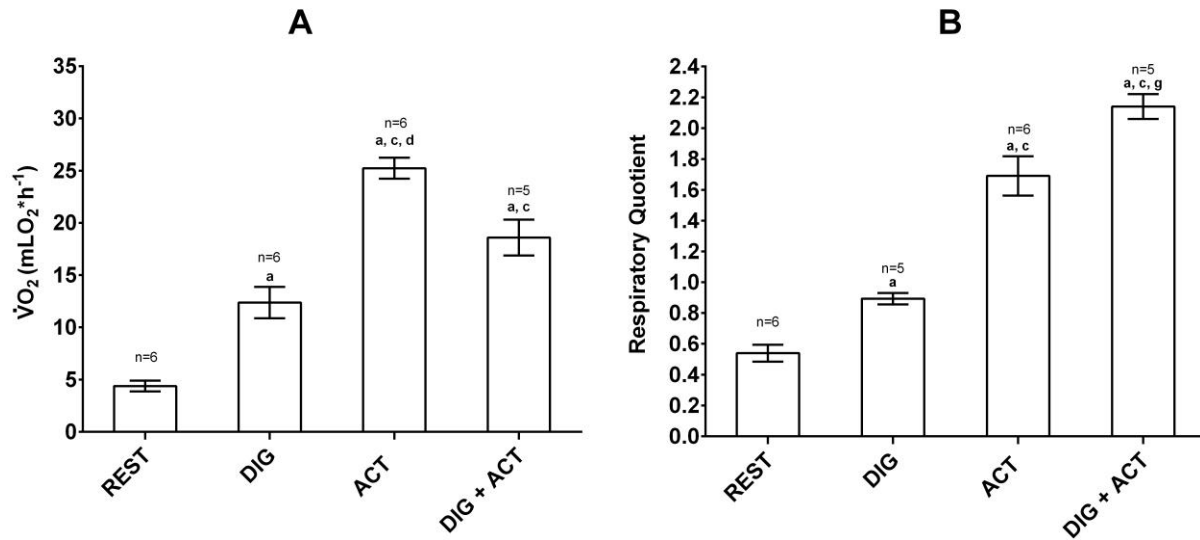
Figure 3.3. Male whole animal $\dot{V}O_2$ (A) and respiratory quotient (RQ) (B) values for each of the experimental treatments. Acronyms: REST = rest, DIG = post-prandial, ACT = fasting exhaustive activity, DIG+ACT = post-prandial exhaustive activity. Lowercase letters above the represent a statistically significant difference: (a) = significantly larger than REST values within the reproductive condition; (c) = significantly larger than DIG values within the reproductive condition; (d) = significantly larger than ACT+DIG values within the reproductive condition; (g) = significantly larger than ACT values. Sample size for each treatment located above the values. All values represent mean ± S.E.M.
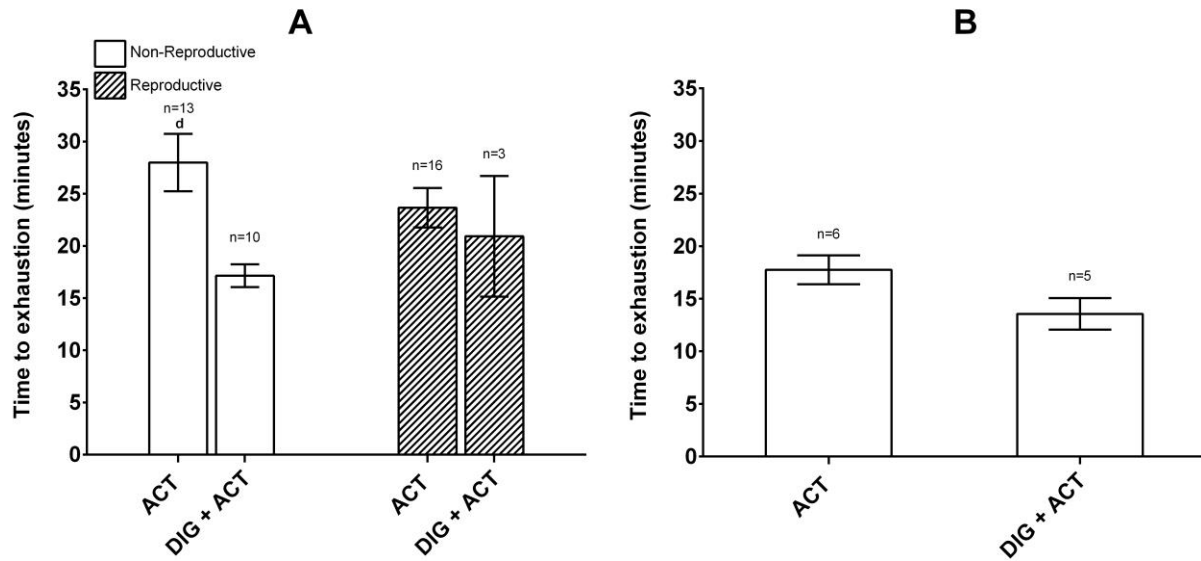
Figure 3.4. Time to exhaustion (TTE) values for females (A) and males (B) associated with fasting exhaustive activity (ACT) and post-prandial exhaustive activity (DIG+ACT). Lowercase letters above the represent a statistically significant difference: (d) = significantly larger than ACT+DIG values within the reproductive condition. Sample size for each treatment located above the values. All values represent mean $\pm$ S.E.M.

Table 3.1. Fold-change in $\dot{V}O_2$ during each of the three treatments over REST values for females (non-reproductive and reproductive) and males. All values are significantly elevated over REST ($p > 0.0001$).

Group	Fasting activity (ACT)	Digestion (DIG)	Post-Prandial Activity (ACT+DIG)
Non-reproductive Females	9.6	6.1	6.6
Reproductive Females	5.0	4.1	4.7
Males	5.9	2.8	4.3

CHAPTER 3

THE INTERACTION EFFECTS OF REPRODUCTION, DIGESTION, AND ACTIVITY IN THE VIVIPAROUS SNAKE, *THAMNOPHIS MARCIANUS*

INTRODUCTION

Studies on mammals and birds have shown that maximum levels of oxygen consumption (i.e., $\dot{V}O_{2max}$) are attained during intense physical activity (Jones, 1994; Bishop, 1999). On the other hand, some species of squamate reptiles (i.e., snakes and lizards) which consume large and protein rich meals at infrequent intervals exhibit factorial increments in $\dot{V}O_2$ during the specific dynamic action (i.e., SDA; Rubner, 1902) response that can approach values associated with physical activity (Hicks and Bennett, 2004; Secor, 2009). In squamates, reproduction represents another physiological state that may result in long lasting (i.e., months) sub-maximal aerobic scopes (Beaupre and Duvall, 1998; Schultz et al., 2008; Van Dyke and Beaupre, 2011). Females may continue to forage and consume meals throughout the reproductive cycle (Cooper et al., 1990), and it is important to examine how the simultaneous oxygen demands from reproduction, digestion, and activity are resolved (Jackson, 1987).

Bennett and Hicks (2001) proposed two general outcomes for $\dot{V}O_2$ during simultaneously occurring elevated metabolic states: 1) If the cardiopulmonary system's maximum capacity for O_2 transport is sufficient, performance for either demand is not sacrificed, and the resulting aerobic scope is a simple summation – termed additivity. Alternatively, 2) the cardiopulmonary system does not have additional capacity for O_2 transport, the performance of one state is scaled down at the expense of the other, and the resultant aerobic scope is unchanged – termed

prioritization. Intermediate effects are also possible, for example, shared prioritization between the competing functional demands (Bennett and Hicks, 2001).

The principle goal of the present study was to expand upon Bennett and Hicks's (2001) work by examining not only the interaction effects between activity and digestion, but also reproduction. The Checkered Garter Snake (*Thamnophis marcianus*), a viviparous (live-bearing) species native to southwestern North America (Ford and Karges, 1987) was used. Garter snakes undergo a reproductive cycle that can be divided into two distinct physiological phases: vitellogenesis (i.e., yolk allocation) and embryogenesis (i.e., embryonic development) (Desseur and Fox 1959; Garstka et al., 1985; Ford and Karges 1987), but it is not well understood how the phase of the reproductive cycle may influence the metabolic costs of digestion and activity. *Thamnophis marcianus* is particularly well suited for such examinations because females continue to consume large meals throughout much of the reproductive cycle, and as an actively foraging species, exhibiting a high propensity for physical activity (Ford and Shuttlesworth 1986; Seigel et al. 1987).

Based on previous work, we predicted that reproduction (i.e., vitellogenesis and embryogenesis), digestion, and activity would all result in significant metabolic increments; and during reproduction, the metabolic increment would be largest during embryogenesis (Birchard et al., 1984; Schultz et al., 2008). We also predicted that the cardiopulmonary system's capacity for oxygen transport would be sufficient to meet the demands of these three elevated metabolic states simultaneously (i.e., an additive response) because cardiac output, minute ventilation, and/or arterial O_2 extraction/carrying capacity would increase commensurate with tissue O_2 demand (Secor et al. 2000; Bennett and Hicks 2001).

MATERIALS AND METHODS

Animal Husbandry

A captive bred population of adult male (n=14) and female (n=20) Checkered Garter Snakes (*Thamnophis marcianus*) was obtained from the Ophidian Research Colony (University of Texas at Tyler) in July 2012. Snakes were maintained in a large vivarium at the University of California, Irvine at $27\pm2°C$ with a 12L:12D photocycle. Snakes were housed in individual enclosures (50 x 34 x 14 cm, females; 33 x 23 x 13 cm, males) lined with newsprint bedding and each enclosure was equipped with 1) a subsurface heating element, 2) two appropriately sized hide-boxes, and 3) a water dish. To facilitate fertilization, males were cycled among the females multiple times weekly. Between experimental treatments snakes were offered thawed mice approximating 5-10% body mass, multiple times weekly.

Experimental treatments (detailed below) were carried out on females in the vitellogenic (V), embryogenic (E), and non-reproductive (NR) conditions. The same experimental treatments were also carried out on males. For females, the phase of the reproductive cycle was assessed using ultrasonography (e.g., Stahlschmidt, et al., 2011; Van Dyke and Beaupre, 2011; Van Dyke et al., 2012). Briefly, a portion of the female's caudal abdomen was placed into a warm water bath and 4.2cm lateral sections were imaged using a 5.0-10.0 mHZ linear array transducer (CTS 3300, Shantou Institute of Ultrasonic Instruments, Guangdong, China). A representative scan from each individual was then photographed using the built-in software from the ultrasound machine, and saved to a portable USB drive.

The vitellogenic phase of the reproductive cycle (6-7 weeks prior to parturition) was identified based on the presence of large, highly echogenic ovarian follicles (Figure 4.1A) (Van

Dyke and Beaupre, 2011). The embryogenic phase of the reproductive cycle (3-4 weeks prior to parturition) was identified by 1) the presence of a low echogenic amnion/allantois fluid-filled space and, 2) the presence of highly echogenic embryonic somatic tissue within the ovarian follicles (Figure 4.1B) (Van Dyke and Beaupre, 2011). The baseline, non-reproductive state (1 to 2 weeks following parturition) was verified by the lack of vitellogenic follicles (Figure 4.1C), and follow-up scans (2 to 4 weeks later) confirmed that a subsequent reproductive cycle was not initiated.

Resting Metabolic Rate (REST)

On the day of the experiment, snakes were removed from their vivarium enclosures and transported to a walk-in environmental chamber, maintained at $29\pm1°C$, the preferred body temperature of *Thamnophis maricanus* (Rosen, 1991). Snakes were weighed to the nearest 0.1g (Mettler Toledo model SB8001, Columbus, OH, USA) and placed into individual metabolic chambers. Each chamber was covered by a thick black tarp to minimize disruption by investigators.

Oxygen consumption (i.e., $\dot{V}O_2$) and carbon dioxide production (i.e., $\dot{V}CO_2$) were measured using flow-through respirometry. Briefly, atmospheric air was pushed by a mass flow controller (GF-3 Gas Mixing Flowmeter; Cameron Instruments Inc., Guelph, Ontario, Canada) into the metabolic chambers, and the incurrent flow rate to each chamber was determined by an Aalborg mass flow meter ($\pm1mL/min$) (GFM17 AALBORG, Orangeburg, New York, USA). Incurrent flow rates ranged from 50 to 240mL/min. At least one chamber remained free from snakes and served as a baseline for referencing.

A sub-sample of the excurrent air from each of the metabolic chambers was pulled into a gas analysis circuit at 20-50mL/min (depending on the excurrent flow rate) by a Brooks mass flow controller (Brooks Instrument, Hatfield, PA, USA), once every 80 minutes, for 6 to 11 minutes at a time. The gas analysis circuit consisted of the following equipment in this order: Applied Electrochemistry Technologies (AEI) CD-3A CO_2 sensor/analyzer → soda lime cartridge (remove CO_2) → drierite cartridge (remove H_2O) → Applied Electrochemistry Technologies (AEI) S-3A O_2 sensor/analyzer. Analyzers were calibrated repeatedly throughout the experimental period. If baseline drift occurred during a trial, calibration gas with known O_2 and CO_2 values were used to confirm linearity of the signal. $\dot{V}O_2$ was calculated using Equation 10.1 from Lighton (2008), $\dot{V}CO_2$ using the Fick equation, and RQ as the quotient of $\dot{V}CO_2$ and $\dot{V}O_2$.

For REST treatments, gas exchange measurements were obtained once every 80 minutes for 6 to 11 minutes at a time, for a total of 8 to 25 hours (females), and 16 to 38 hours (males). The mean of the three lowest consecutive $\dot{V}O_2$ (and corresponding RQ) values was chosen to represent resting metabolic rate (RMR). To ensure that the snakes were in a post-absorptive state prior to measurements, females were fasted for at least seven days, and males for at least 13 days (Britt et al., 2006; Bessler et al., 2010).

Fasting Exhaustive Activity (ACT) and Recovery

Following the REST trial, one snake at a time was placed into a fixed volume (2.7 L, males; or 3.9 L, females) glass metabolic activity-chamber. The activity-chamber was sealed with a screw-on cap, into which two incurrent and two excurrent air lines of varying lengths and diameters (to ensure proper air mixing) were inserted. The incurrent flow rate was set by a

Brooks mass flow controller, and then monitored constantly using an Aalborg mass flow meter. Incurrent flow rates to the activity chamber ranged from 120 to 250 mL/min (depending on the size of the animal). A subsample of the excurrent airstream was pulled through an 18-guage syringe at 50mL/min into the above mentioned gas-analysis circuit.

We used an exhaustion protocol similar in design to previous work on snakes (Ruben 1976; Gratz and Hutchison 1977; Ellis and Chappell 1987; Andrade et al. 1997). Briefly, the snake was continually forced to right itself from an investigator induced rotation. The regimen was maintained until the animal could no longer perform the righting response. The elapsed time between the first rotation and the point of exhaustion was considered to represent the time to exhaustion (TTE). The lowest FEO_2 and corresponding $FECO_2$ values were chosen to represent peak gas exchange levels.

Following the trial, each snake was removed from the activity chamber and placed back into the above mentioned flow-through respirometry metabolic chambers. Recovery time was considered to be the amount of time required before $\dot{V}O_2$ returned to within 10% of REST values, and estimated by obtaining gas exchange values once every 80 minutes, for five to 18 hours. If $\dot{V}O_2$ did not return to within 10% of REST values by the end of the recovery trial, data was not considered for analysis.

Digestion (DIG)

Following recovery, snakes were removed from the metabolic chambers, placed into their normal enclosures, and offered water *ad libitum*. Within one day, snakes were offered a meal equivalent to 10% of their body mass in thawed rodent, and within four hours following voluntary ingestion, returned to the flow-through respirometry metabolic chambers for post-

prandial gas exchange measurements. Gas exchange values were obtained once every 80

minutes (6-11 minutes at a time) for up to one day following the time of ingestion, a time frame

sufficient to expose peak values following ingestion (Britt et al. 2006; Bessler et al. 2010). The

mean of the three highest consecutive $\dot{V}O_2$ (and corresponding $\dot{V}CO_2$) values obtained during

this trial period was considered to represent peak post-prandial gas exchange levels.

Post-Prandial Exhaustive Activity (ACT+DIG)

Following the achievement of peak post-prandial gas exchange levels (i.e., 24 to 28 hours

following ingestion), we repeated the above mentioned activity trial. Although gas exchange

values had begun to decline by 24 to 28 hours following ingestion, the decline is relatively minor

(Britt et al. 2006; Bessler et al. 2010). Therefore, the ACT+DIG trials in the present study likely

suffices to expose snakes to near maximum interactions between the demands of activity and

digestion.

All procedures involving this species were approved by the University of California,

Irvine Institutional Animal Care and Use Committee (IACUC protocol #2010-2966 and 2009-

2906).

Statistical Analysis

To account for the variation in body mass among males (range = 40 to 74g) and females

(range = 125 to 269g) we carried out ANCOVAs to test for an effect of mass on $\dot{V}O_2$, RQ, and

TTE. For females, a significant fraction of the reproductive body mass increment (up to 1.3 fold

in this study) is associated with the mass of the ovarian follicles, which contain mostly water and

metabolically inert biomolecules throughout much of the reproductive cycle (Ellis and Chappel,

1987; DeMarco and Guillette, 1992; Angilletta and Sears, 2000). To control for this potentially confounding factor, non-reproductive body mass values were used in the analysis for females.

For both females and males, oxygen consumption ($\dot{V}O_2$) did vary significantly with body mass (p<0.0001, females and males), however respiratory quotient (RQ) (p=0.4635, females; p=0.8007, males), and time to exhaustion (TTE) (p=0.0558, females; p=0.8416, males) did not. Therefore, for females, two-way analysis of covariance (ANCOVA) was used to evaluate the difference in $\dot{V}O_2$, and a two-way analysis of variance (ANOVA) was used to evaluate the differences in RQ, and TTE between reproductive states (non-reproductive, NR; vitellogenic, V; and embryogenic, E) among the four experimental treatments (REST, DIG, ACT, and ACT+DIG) and for an interaction between reproductive state and experimental treatment. For males, a separate one-way ANCOVA was used to evaluate the differences in $\dot{V}O_2$, and ANOVA was used to evaluate the differences in RQ and TTE between experimental treatments (REST, DIG, ACT, and ACT+DIG). A separate ANOVA was also used to compare time to recovery from ACT between the reproductive conditions in females. *Post-hoc* pairwise comparisons were performed if a significant experimental treatment effect (females, males) or a significant reproductive state and experimental treatment interaction (females) was observed. Tukey-Kramer's method was utilized for multiple comparison adjustment. Analysis of variance and analysis of covariance tests were carried out using JMP version 10.0 (SAS Institute, Inc., Cary, North Carolina, USA). The level of statistical significance was set at the p=0.05 level. Values are reported as mean ± 1 S.E.M.

RESULTS

$\dot{V}O_2$

The average $\dot{V}O_2$ values associated with REST, ACT, DIG, and ACT+DIG for females (all reproductive conditions) are presented in Figure 4.2A. The factorial scopes (i.e., fold-change in $\dot{V}O_2$ relative to REST) are reported in Table 4.1.

In the non-reproductive condition, the $\dot{V}O_2$ values associated with ACT and ACT+DIG were significantly larger than DIG ($p<0.0001$). During vitellogenesis, the $\dot{V}O_2$ values associated with ACT+DIG were significantly larger than both ACT (1.3 fold, $p=0.0039$) and DIG (1.3 fold, $p=0.0325$). During embryogenesis, the $\dot{V}O_2$ values associated with ACT+DIG were significantly elevated over DIG (1.4 fold, $p=0.0016$) values.

Relative to the non-reproductive (NR) condition, we found that REST $\dot{V}O_2$ was significantly elevated by 1.7 fold during vitellogenesis ($p=0.0014$), and 1.6 fold during embryogenesis ($p=0.0065$). We also found that DIG $\dot{V}O_2$ was significantly elevated by 1.5 fold during vitellogenesis ($p<0.0001$) and 1.3 fold during embryogenesis ($p<0.0001$).

The average $\dot{V}O_2$ values associated with REST, ACT, DIG, and ACT+DIG for males are presented in Figure 4.3A. The factorial scopes are reported in Table 4.1. For males, the $\dot{V}O_2$ values associated with both ACT ($p<0.0001$) and ACT+DIG ($p<0.0001$) were significantly larger than values elicited during DIG.

RQ

The results for respiratory quotient (RQ) for females (V, E, and NR states) are presented in Figure 4.2B; RQ results for males are presented in Figure 4.3B. Our results indicate that females in the V, E, and NR reproductive conditions all had RQ values from ACT and

ACT+DIG that were significantly larger (p<0.0001) than both REST and DIG. We also found

that RQ values during ACT+DIG were significantly elevated over both ACT (p<0.0080) and

DIG (p<0.0001) for all reproductive conditions. For males, the RQ values from ACT and

DIG+ACT treatments were significantly larger than REST and DIG (p<0.0001) values.

Time to Exhaustion

The results for time to exhaustion (TTE) for females (all reproductive conditions) and

males during ACT and ACT+DIG treatments are presented in Figure 4.4A and 4.4B,

respectively. Our results indicate that for females, TTE for ACT was significantly shorter during

vitellogenesis (by 14 minutes, p=0.0192) than when non-reproductive (NR). There were no

significant differences in TTE between ACT and ACT+DIG treatments for females (p>0.3526)

or males (p>0.9300).

Recovery from ACT

Following exhaustion from fasting act (ACT), our results indicate that females returned

to within 10% of RMR by an average of 6 (range = 3-9) hours during vitellogenesis, 9 (range =

5-15) hours during embryogenesis, and 10 (range 4-17) hours in the non-reproductive state. The

duration of recovery between the vitellogenic and non-reproductive conditions was significantly

different (p=0.030). Males returned to within 10% of RMR by an average of 5 (range = 3-9)

hours. Upon recovery, RQ values for both females (all reproductive conditions) and males

ranged from .75-.79, and were not significantly different from RMR values (p>0.5259).

DISCUSSION

This work provides one of the first quantitative examinations of the interaction effects between reproduction, digestion, and activity in a viviparous squamate. Our results indicate that *Thamnophis marcianus* exhibits substantial metabolic increments during both digestion (~4 to 5 fold) and activity (~9 fold), and that a smaller metabolic increment occurs during reproduction (~1.6 to 1.7 fold). Contrary to our predictions, males and non-reproductive females exhibit a prioritization pattern of oxygen delivery during ACT+DIG because $\dot{V}O_2$ values are similar with those elicited by ACT. Interestingly, females exhibit a more-than-additive pattern of oxygen delivery during DIG (i.e., summation of the $\dot{V}O_2$ increments, and an extra 10 to 20mLO$_2$/h auxiliary cost), but a prioritization response to ACT and ACT+DIG (i.e., similar $\dot{V}O_2$ increments to these same treatments during the non-reproductive condition). Our results mostly contrast with the previous work in post-prandially active squamate reptiles (Secor et al., 2000; Bennett and Hicks, 2001) and are more consistent with prioritization responses exhibited by species of anurans and fish (Aslop and Wood 1997; Anderson and Wang 2003; Thorarensen and Farrell 2006).

Reproduction

Reproductive REST measurements were obtained during secondary vitellogenesis (Figure 4.1A) and approximately midway through embryonic development (Figure 4.1B) (Desseur and Fox, 1959; Van Dyke and Beaupre, 2011). Consistent with our prediction, the vitellogenic and embryogenic phases of the reproductive cycle were associated with significant increments in maternal $\dot{V}O_2$ (i.e. 1.6 to 1.7 fold), however there was no significant difference between the two phases of the reproductive cycle (Figure 4.2A). We did not find any significant

differences in RQ values between the reproductive conditions (Figure 4.2B). The metabolic increments reported in the present study are consistent with previously published values for multiple species of viviparous snakes measured during the vitellogenic (i.e., ~1.4 to 1.6 fold increments) (Beaupre and Duvall, 1998; Van Dyke and Beaupre, 2011) and embryogenic (~1.5 to 2.9 fold increments) (Birchard et al., 1984; Schultz et al., 2008; Van Dyke and Beaupre, 2011) phases of the reproductive cycle.

The metabolic increment associated with the vitellogenesis may be caused by increased rates of whole-animal protein synthesis (Garlick et al., 1976; Houlihan, 1991) due to biomolecule synthesis by the liver (i.e., vitellogenin, VLDLs and vitamin and trace mineral binding proteins; Wallace, 1985; White, 1991) and organ remodeling (i.e., oviduct and follicular tissues) (Masson and Guillette, 1987; Blackburn, 1998). Following vitellogenesis, the mature ova are fertilized and embryonic development begins. After ovulation, the vitellogenic processes are rapidly down-regulated to pre-gestational levels (Dessauer and Fox, 1959; Garstka, et al., 1985; Bonnet et al., 1994). Thereafter, the oviduct continues to undergo extensive remodeling to provide the developing embryos with an appropriate environment for gas exchange, and in some species, to facilitate the maternal-fetal transport of varying quantities of trace nutrients (Masson and Guillette, 1987; Parker et al., 2010; Blackburn and Stewart, 2011; Van Dyke and Beaupre, 2012). Similar to patterns observed in the eggs of oviparous species, embryonic metabolism increases progressively to term, from negligible levels during early embryonic development, to substantially higher values prior to parturition (Packard et al., 1977; Robert and Thompson 2000; Schultz et al., 2008). Current work (Chapter 1) indicates that *Thamnophis maricanus* ovulates at least six weeks prior to parturition. Thus, the embryogenic measurements in the present study (3-4 weeks prior to parturition) were obtained approximately mid-way through development -

where embryonic metabolism is still relatively low (Wang and Ji, 1997; Thompson and Russell, 1999). Therefore, we propose that much of the 1.6 fold metabolic increment during embryogenesis is associated with continued remodeling and maintenance costs of the oviduct, rather than embryonic metabolism, as would be expected during the latter stages of development.

Digestion

In *Thamnophis marcianus,* $\dot{V}O_2$ began to increase within hours following ingestion, and peak values occurred within 24 hours, increasing to 4.5 to 5.0 fold above REST (Figure 4.2A and 4.3A). There were no significant differences in RQ values between REST and DIG (Figure 4.2B and 4.3B). The temporal patterns of increased $\dot{V}O_2$ and the peak factorial increments exhibited by male and non-reproductive female *Thamnophis marcianus* are consistent with previously published values for snakes following a 10% body mass meal. More specifically, in *Thamnophis elegans*, *Lamprophis fuliginosus*, and *Nerodia sipedon*, peak post-prandial $\dot{V}O_2$ values are attained within one day following ingestion, and values range from ~3 fold in *Thamnophis elegans* and *Nerodia spedon* to ~5 fold in *Acanthophis praelongus* and *Python molurus* (Sievert and Andreadis, 1999; Roe et al., 2004; McCue et al., 2005; Britt et al., 2006; Christian et al., 2007).

During reproduction, females also reached peak levels of $\dot{V}O_2$ that were ~4.0 to 5.0 fold above REST within 24 hours following ingestion. The apparently unchanged peak factorial levels are due to the 1.6 to 1.7 fold reproductive REST increments, and absolute DIG $\dot{V}O_2$ values are actually 30 to 50% larger during the vitellogenic and embryogenic phases of the reproductive cycle. The sum of the reproductive REST increments and the NR DIG increment (i.e., ~54 to 55mLO$_2$/h) is substantially lower than the reproductive DIG increments (i.e., 64 to 74mLO$_2$/h).

Therefore, our results indicate that an additional energetic cost (~10 to 20mLO$_2$/h) associated with digestion exists for reproductive females.

The physiological mechanisms responsible for the large SDA response in snakes are understood to be related to increased organ mass and function (e.g., large increments in cardiopulmonary output, and intestinal brush-border protein transport capacity) and substantially increased rates of whole-animal protein synthesis (Houlihan, 1991; Hicks and Bennett, 2004; Secor, 2009). During reproduction, these processes are likely maintained, but to our knowledge this study represents one of the first examinations of the interaction effects of digestion and reproduction. Although our data cannot elucidate the specific mechanisms associated with the 30-50% increase in $\dot{V}O_2$, and only examined the first 24 hours of the SDA response, previous work indicates that the presence of enlarged ovarian follicles within the posterior abdomen compresses the maternal organs (Munns and Daniels, 2007; Gilman et al., 2013), resulting in substantially elevated costs of ventilation (e.g., 3 fold increase in *Tiliqua rugosa*) (Munns, 2013). It is possible that the mass/volume combination of the ovarian follicles with the 10% body mass meal was sufficient to produce a similar effect in *Thamnophis marcianus.*

Activity

Fasting exhaustive activity elicited a ~9 fold increment in $\dot{V}O_2$ for non-reproductive females and males (Figure 4.2A and 4.3A), lead to exhaustion after 27 (males) to 37 (females) minutes (Figure 4.4A and 4.4B), and RQ values of ~1.2 (Figure 4.2B and 4.3B). The aerobic scopes exhibited by male and female *Thamnophis marcianus* in the present study fall within the ~2 to 12 fold range described for multiple species of snakes (e.g., Ruben 1976; Chappel and Ellis 1987; Andrade et al. 1997; Secor et al. 2000). RQ values above 1.0 often occur in reptiles, and

reflect the large reliance on anaerobic glycolysis for supplementary ATP production during

unsustainable activity (Gleeson and Bennett, 1982).

Following exhaustion, *Thamnophis marcianus* required 5 to 10 hours to return to resting

$\dot{V}O_2$ levels, a time frame considerably longer than the approximately 2 to 3 hour recovery period

reported for the snake *Natrix rhombifera,* multiple species of lizards, and for crocodilians (Gratz

and Hutchison, 1977; Gleeson, 1982; Gleeson and Dalessio, 1989; Hartzler, et al. 2006).

However, the trial duration required to elicit exhaustion for *Thamnophis marcianus* was

substantially longer than the five to fifteen minute protocols used in previous studies (Ruben,

1976; Chappel and Ellis, 1987; Andrade et al. 1997; Secor et al., 2000). Previous work has

demonstrated that recovery time is directly related to the duration of the activity protocol, and

this discrepancy may explain the long recovery times in *Thamnophis marcianus* (Gleeson and

Hancock, 2002). Based on previous work in reptiles, it is likely that much of the excess post

exercise oxygen consumption (EPOC) is attributable to lactate oxidation and glycogenesis,

phosphocreatine repletion, and ATP repletion (Gratz and Hutchison, 1977; Gleeson, 1991;

Gleeson and Hancock, 2002).

Contrary to our predictions, reproductive females exhibited aerobic scopes and RQ values

that were not significantly different from values obtained in the non-reproductive (NR) state, and

reached exhaustion up to 14 minutes sooner (i.e., reduced performance), indicating a

prioritization pattern of oxygen delivery (Figure 4.2A, 4.3A, and 4.4A). The decrement in

activity performance during vitellogenesis reported in the present study is consistent with

previous work in this species (*Thamnophis marcianus*) (Seigel et al. 1987), and a number of

species of lizards (Shine 1980; Cooper et al. 1990; Sinervo et al. 1991; Shine 2003). We are not

aware of previous work that has examined how the competing oxygen demands from

reproduction and activity are resolved, but it is possible that the metabolic processes associated with reproduction are temporarily suspended during physical activity, or that the small signal to noise ratio of the reproductive metabolic increments (1.6 to 1.7 fold) relative to activity (~9 fold) made it difficult to detect an interaction effect.

ACT+DIG

Contrary to our predictions, males and females (all reproductive conditions) exhibit a prioritization pattern of oxygen delivery during ACT+DIG, that is, aerobic scopes were similar to values attained during ACT (Figure 4.2A and 4.3A). The prioritization pattern of $\dot{V}O_2$ exhibited by *Thamnophis marcianus* in the present study contrasts with previous work in *Varanus exanthematicus* and *Python molurus* (Secor et al., 2000; Bennett and Hicks, 2001), but is consistent with work in Rainbow Trout (*Oncorhynchus mykiss*), Chinook Salmon (*Oncorhynchus tshawytscha*), Sablefish (*Anoplopoma fimbria*) and Cane Toads (*Bufo marinus*) (Anderson and Wang, 2003; Furnell, 1987; Aslop and Wood 1997; Thorarensan and Farrell 2006).

During ACT+DIG, males and non-reproductive females must locomote with the 10% body mass meal, and reproductive females are further encumbered by a 20 to 30% body mass increment associated with the enlarged ovarian follicles. In vertebrates, additional weight during locomotion results in a linear increase in the force required for muscular contraction, and therefore increased metabolic cost (i.e., a ~10% increase in $\dot{V}O_2$ for each additional 10% in body weight) (Taylor et al., 1980). For reproductive females, the combined mass and volume constraints associated with the ingested meal and enlarged ovarian follicles may further result in elevated costs of ventilation (Munns, 2013). Therefore, if the cardiopulmonary system has

sufficient capacity for delivering O_2 to all metabolically active organ beds simultaneously (hence, additivity), the individual $\dot{V}O_2$ increments from digestion and activity would sum together, and the increased costs of performing locomotion and ventilation would result in a still larger increase in $\dot{V}O_2$ during ACT+DIG (i.e., an ~15 fold increase in $\dot{V}O_2$ over REST). This was not the case in the present study. Instead, the similar aerobic scopes exhibited by *Thamnophis marcianus* during ACT and ACT+DIG may indicate that this species' cardiopulmonary system has a limited capacity for oxygen transport (peaks at ~9 to 10 fold above REST). Alternatively, sufficient capacity for oxygen delivery exists, but the presence of a large meal within the stomach, and in reproductive females, ovarian follicles, may alter ventilation patterns (i.e., reduce maximal levels of O_2 and CO_2 exchange in the lungs) and decrease venous return (i.e., reduced maximal levels of cardiac output) (Munns et al., 2004; Munns and Daniels, 2007).

During ACT+DIG in the present study, all groups were capable of maintaining activity performance (TTE) in spite of the additional 10 to 40% body mass load (Figure 4.4A and 4.4B). Our data cannot discern why *Thamnophis marcianus* is capable of overcoming this burden without any noticeable decrement in activity performance, but it is possible that most of the metabolic processes associated with digestion and reproduction are temporarily suspended during exhaustive activity, and blood supply to the GI tract and oviduct are preferentially redistributed to the active skeletal muscles, thus prioritizing activity performance (Furnell, 1987; Thorarensen and Farrell 2006). Moreover, a decrease in R-L shunt fraction in reptiles may result in elevated O_2 extraction from ventilated air and thus increased O_2 diffusion gradient at the tissues (Secor et al., 2000; Bennett and Hicks, 2001). A similar effect may occur with a decrement in ventilation/perfusion heterogeneity at the lungs (Bennett and Hicks, 2001). Such

cardiopulmonary adjustments may provide *Thamnophis marcianus* with sufficient O_2 delivery capacity to simply maintain $\dot{V}O_2$ levels and activity performance. The shift in RQ from 1.2 to 1.5 may reflect the additional muscular work required to perform locomotion with the additional body mass burden that cannot be met by aerobic metabolism, thus resulting in a more pronounced metabolic acidosis (Gleeson and Bennett 1982).

Conclusions

Under natural conditions, animals may perform more than one metabolically demanding function simultaneously. Laboratory studies on the patterns of physiological prioritization that occur under these conditions represent an important area of investigation if conclusions are to be extrapolated to predicting performance outcomes in the natural environment (Jackson, 1987; Anderson and Wang, 2003). In the present study, we examined how the simultaneous oxygen demands associated with reproduction, digestion, and activity are resolved, and our results mostly indicate a prioritization pattern of oxygen delivery towards locomotion at the expense of digestive and perhaps reproductive functions. We hope that future work examines the specific cardiopulmonary mechanisms associated with the prioritization response described in the present study. More specifically, measurements of the components of cardiac output and minute ventilation, in addition to measurements of PO_2 and PCO_2 within the systemic and venous circulation would be useful for identifying possible limitations in oxygen transport (Secor et al., 2000; Bennett and Hicks, 2001). Furthermore, measurements of mesenteric artery and hepatic portal blood flow would be helpful for identifying possible shifts in blood flow distribution during post-prandial activity (Secor and White, 2010).
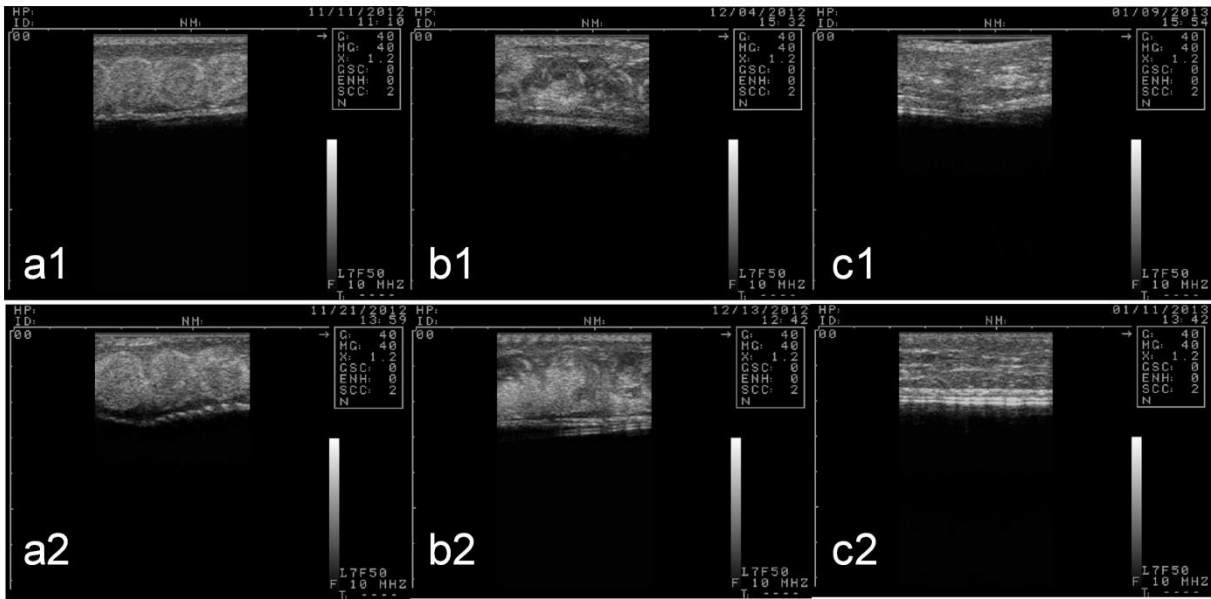
Figure 4.1. Ultrasound images of two representative female *Thamnophis marcianus,* imaged during the vitellogenic (V) (a1 and a2), embryogenic (E) (b1 and b2), and NR (c1 and c2) reproductive conditions. Images were obtained using a 5 to 10 MHz linear array transducer (CTS 3300, Shantou Institute of Ultrasonic Instruments, Guangdong, China), and represent approximately 4.5 cm sections (with a depth of approximately 2 to 3 cm) of a portion of the posterior coelemic abdomen.
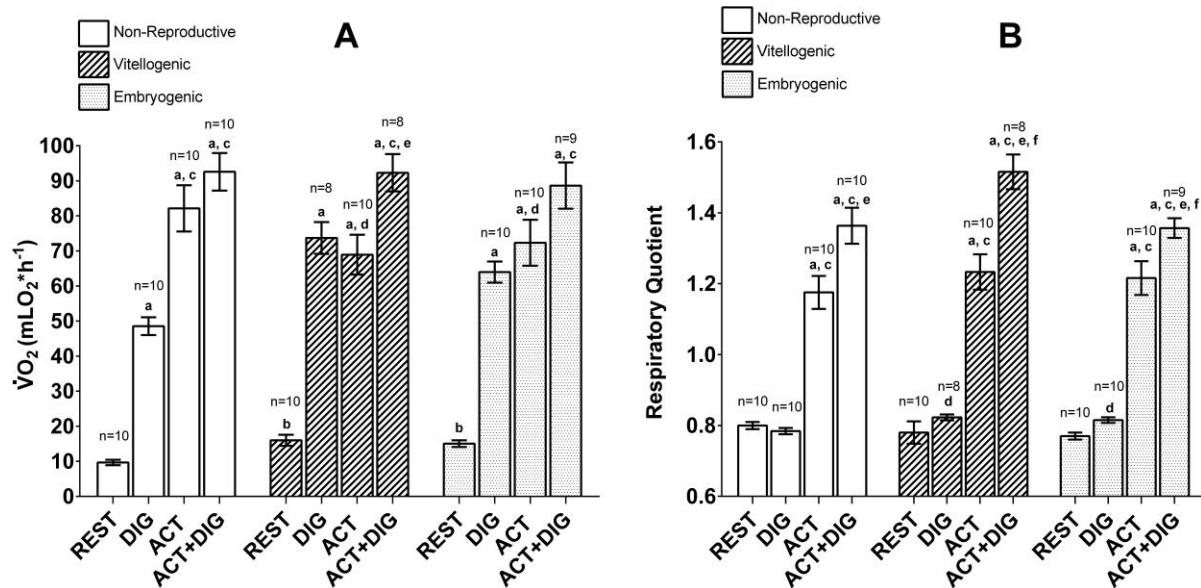
Figure 4.2. Female whole animal $\dot{V}O_2$ (A) and respiratory quotient (RQ) (B) values for each experimental treatment (REST = rest, DIG = post-prandial, ACT = fasting exhaustive activity, and ACT+DIG = post-prandial exhaustive activity). Reproductive condition is indicated by the patterns noted in the key in the upper left corner. Lowercase letters above the values represent a statistically significant difference: (a) = significantly larger than REST values within the reproductive condition; (b) = significantly larger than non-reproductive REST values, (c) = significantly larger than DIG values within the reproductive condition; (d) = significantly larger than NR DIG values; (e) = significantly larger than ACT values within reproductive condition; (f) = significantly larger than NR ACT+DIG values. Sample sizes are located above each value. All values represent mean ± S.E.M.
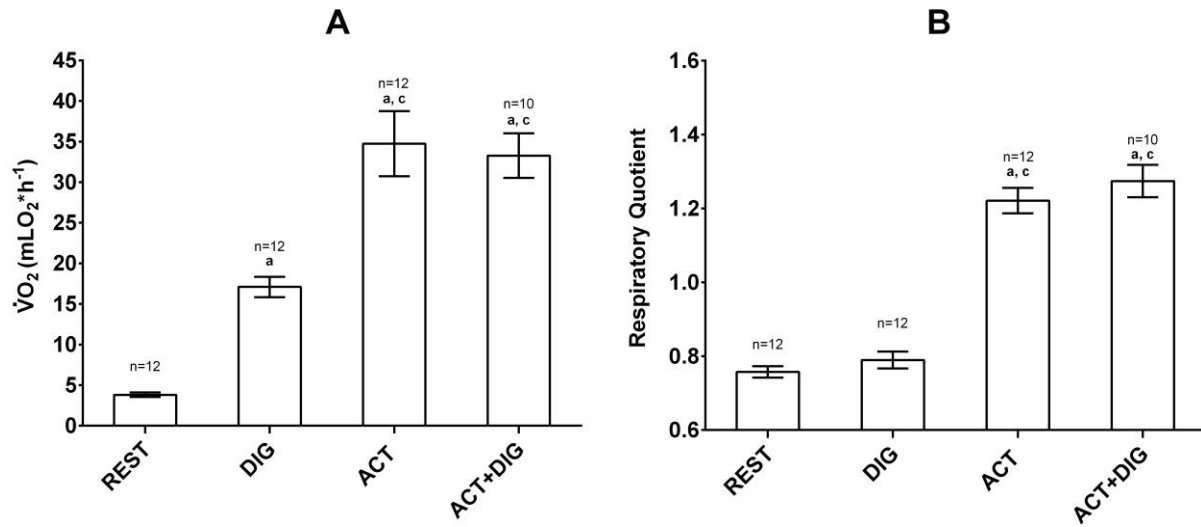
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Figure 4.3. Male whole animal $\dot{V}O_2$ (A) and respiratory quotient (RQ) (B) values for each experimental treatment (REST = rest, DIG = post-prandial, ACT = fasting exhaustive activity, and ACT+DIG = post-prandial exhaustive activity). Lowercase letters above the values represent a statistically significant difference: (a) = significantly larger than REST values within the reproductive condition; (c) = significantly larger than DIG values within the reproductive condition. Sample sizes are located above each value. All values represent mean ± S.E.M.
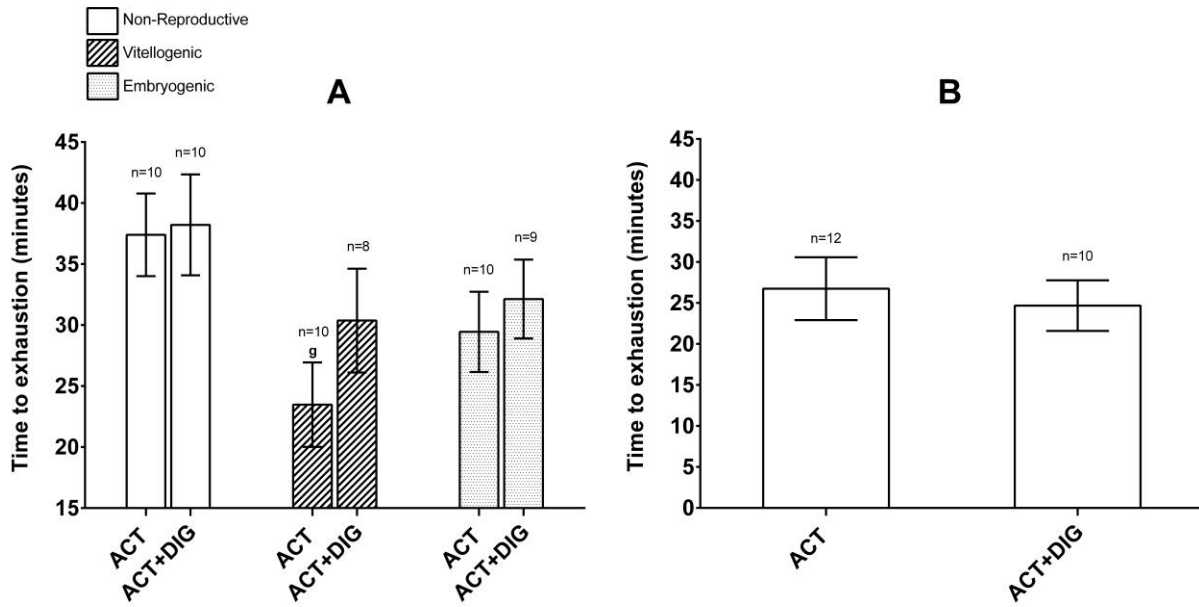
Figure 4.4. Female (A) and male (B) time to exhaustion (TTE) values associated with fasting exhaustive activity (ACT) and post-prandial activity (ACT+DIG). Female reproductive condition is indicated by the key in the upper left portion of the graph (A). Lowercase letters above the value represents a statistically significant difference: (g) = significantly lower than non-reproductive ACT values. Sample sizes are located above each value. All values represent mean $\pm$ S.E.M.

Table 4.1. Fold-change in $\dot{V}O_2$ during each of the three treatments over REST values for females (NR, V, and E conditions) and males. All values are significantly elevated over REST (p>0.0001).

Group	Fasting activity (ACT)	Digestion (DIG)	Post-prandial activity (ACT+DIG)
Non-reproductive (NR) Females	9.0	5.2	10.1
Vitellogenic (V) Females	4.7	5.1	6.4
Embryogenic (E) Females	5.0	4.3	6.3
Males	9.3	4.5	8.9

Chapter 4

CONCLUSIONS

Reproduction

The first chapter of my dissertation tested two hypotheses: 1) That reproductive females of both species would exhibit increments in $\dot{V}O_2$ during the vitellogenic and embryogenic phases of the reproductive cycle, and 2) that PBT would shift towards higher values to optimize the underlying physiological processes associated with vitellogenesis, organ remodeling, and embryonic development. Consistent with predictions, *Lamprophis fuliginosus* and *Thamnophis marcianus* exhibited significant factorial increments in $\dot{V}O_2$ during the vitellogenic (i.e., 1.5 to 2.0 fold) and embryogenic (i.e., 1.5 to 3.2 fold) phases of the reproductive cycle - patterns that are consistent with previously published values for snakes (see Table 1.1). Fetal metabolism accounted for, at most, 3% (*Lamprophis fuliginosus*) and 33% (*Thamnophis marcianus*) of maternal values, indicating that a majority of direct energetic cost of reproduction was related to maternal maintenance processes (i.e., elevated rates of whole-animal protein synthesis, increased cardiopulmonary output), rather than fetal gas exchange requirements.

In contrast to predictions, female PBT values during both phases of the reproductive cycle (i.e., 24 to 26ºC in *Lamprophis fuliginosus* and 27 to 28ºC in *Thamnophis marcianus*) were not significantly different from those selected by non-reproductive/post-partum females (i.e., 27ºC by *Lamprophis fuliginosus* and 28ºC by *Thamnophis marcianus*). The lack of shift in PBT during reproduction has also been demonstrated for other species of snakes (Table 1.1). These findings may indicate that the optimal temperatures for vitellogenic and embryogenic processes do not differ from the non-reproductive state.

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Physical Activity

The second and third chapters of my dissertation examined peak factorial increments in $\dot{V}O_2$ (and RQ) and performance (i.e., time to exhaustion, TTE) during physical activity (while fasting) in adult male and female *Lamprophis fuliginosus* and *Thamnophis marcianus* – two previously unexamined species. Similar to previous work (e.g., Ellis and Chappell, 1987), a manual rotation activity protocol was utilized. Results indicate that snakes reached exhaustion by 17 and 37 minutes, attained peak $\dot{V}O_2$ increments of 5.9 to 9.6 above SMR, and exhibited RQ values of 1.2 to 1.7. These peak $\dot{V}O_2$ increments are broadly consistent with previously published values for snakes (Table 1.4). RQ values above 1.0 reflect a large reliance on anaerobic metabolism to fuel activity performance (Ruben, 1976; Gratz and Hutchinson, 1977). Like other reptiles, maximal levels of activity also resulted in long recovery times (i.e., 5-10 hours) in *Thamnophis marcianus* (Gleeson, 1982; Gleeson and Hancock, 2002).

Digestion

The second and third chapters of my dissertation examined temporal patterns and peak factorial increments in $\dot{V}O_2$ following the ingestion of a 10% or 20% body mass meal in *Lamprophis fuliginosus* and *Thamnophis marcianus* . Results indicate that in the hours following ingestion, $\dot{V}O_2$ progressively increased for 24 (*Thamnophis marcianus*) to 48 (*Lamprophis fuliginosus*) hours, reaching peak increments of ~3 to 6 fold above SMR. Times to peak, and the factorial increments, are consistent with previously published values for snakes (Table 1.3).

Interaction of Elevated Metabolic States

Chapters two and three of my dissertation tested the hypothesis that *Thamnophis marcianus* and *Lamprophis fuliginosus* would exhibit an additive pattern of O_2 delivery (i.e., a summation of $\dot{V}O_2$ increments and the maintenance of activity performance) during post-prandial activity. Interaction effects were examined for the following combinations of functional states: 1) Physical activity and digestion (males and non-reproductive females), 2) reproduction and physical activity, 3) reproduction and digestion, and 4) reproduction, physical activity, and digestion.

*Males and Non-Reproductive Females.—*Contrary to predictions, post-prandial activity resulted in peak factorial increments in $\dot{V}O_2$ that were not significantly different (i.e., 9 to 10 fold above SMR; *Thamnophis marcianus*), or up to 40% lower (i.e., 4 to 7 fold above SMR; *Lamprophis fuliginosus*) than activity (while fasting). Activity performance (i.e., time to exhaustion, TTE) was typically maintained (with the exception of non-reproductive *Lamprophis fuliginosus* females). These results generally indicate a prioritization pattern of O_2 delivery towards active skeletal muscles. Increased body weight did not result in a 10 to 20% increase in $\dot{V}O_2$ (Taylor et al., 1980), but there was a pattern for elevated RQ in all groups (e.g., increase from 1.7 to 2.1 in male *Lamprophis fuliginosus*); possibly indicating that activity performance was maintained by catabolizing additional glycogen stores (Gleeson and Bennett, 1982).

*Reproductive Females.—*Contrary to predictions, the reproductive $\dot{V}O_2$ increment (i.e., 1.4 fold in *Lamprophis fuliginosus* and 1.6-1.7 fold in *Thamnophis marcianus*) was not maintained during physical activity (while fasting). Instead, peak factorial increments in $\dot{V}O_2$ during activity (while fasting) were either 30% lower (*Lamprophis fuliginosus*) or unchanged

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(*Thamnophis marcianus*) from non-reproducitve (NR) levels, while activity performance declined in one species (*Lamprophis fuliginosus*), but not the other (*Thamnophis marcianus*). These results indicate a shared prioritization of O_2 delivery between skeletal musculature and reproductive processes for *Thamnophis marcianus* and a prioritization of O_2 delivery towards actively contracting muscle tissues for *Lamprophis fuliginosus*. $\dot{V}O_2$ did not increase by 20 to 30% (Taylor et al., 1980) in spite of the added body weight, but there was a trend towards increased RQ (e.g., *Lamprophis fuliginosus* increases RQ from 1.3 to 1.7), possibly indicating a heavier reliance upon anaerobic glycolysis for energy production (Gleeson and Bennett, 1982).

The reproductive $\dot{V}O_2$ increment was not maintained during digestion in *Lamprophis fuliginosus* (i.e., peak $\dot{V}O_2$ was similar to NR levels) while *Thamnophis marcianus* not only maintained the reproductive increment, but also exhibited a 30 to 50% increase in peak digestive $\dot{V}O_2$ levels. Thus, results for *Lamprophis fuliginosus* indicate a prioritization pattern of O_2 delivery, while those for *Thamnophis marcianus* indicate a more-than-additive pattern of O_2 delivery. This auxiliary cost of digestion in *Thamnophis marcianus* may be related to elevations in the cost of ventilation as a consequence of lung compression (Munns, 2013). A similar pattern would be expected for *Lamprophis fuliginosus*, considering the larger meal size, but this was not the case. One possibility is that *Lamprophis fuliginosus* possesses a more flexible body cavity than *Thamnophis marcianus*, permitting females to circumscribe the lung expansion constraints associated with the ingested meal (Gilman et al., 2013).

Under the predicted additivity scenario, reproductive females subjected to post-prandial activity would exhibit a summation of the reproductive (i.e., 1.4 to 1.7 fold), digestive (i.e., 5 to 6 fold), and physical activity (i.e., 8.5 to 9.6 fold) O_2 demands. Reproductive and post-prandially

active females were 40% heavier, potentially elevating $\dot{V}O_2$ during physical activity by another 40% (Taylor et al., 1980). Therefore, under the additive scenario, the resulting factorial increment in $\dot{V}O_2$ would be approximately 15 fold (*Thamnophis marcianus*) and 18 fold (*Lamprophis fuliginosus*) above SMR levels and activity performance would not decline.

This was not the case for *Lamprophis fuliginosus* or *Thamnophis marcianus.* Instead, females of both species exhibited $\dot{V}O_2$ increments that were ~7 to 10 fold above SMR levels, activity performance (i.e., TTE) was maintained, and RQ values tended to increase (e.g., from 1.2 to 1.5 in *Thamnophis marcianus*), possibly indicating two trends: 1) Priority of O_2 delivery was accorded to actively contracting skeletal muscles, and 2) some of the weight-related increase in energy demand for locomotion was met by increasing levels of anaerobic glycolysis (Bennett and Hicks, 2001; Gleeson and Bennett, 1982). The compression of visceral organs by both the ovarian follicles and the ingested meal did not limit activity performance, nor did this factor prevent these two species from attaining levels of $\dot{V}O_2$ that were similar to those elicited by physical activity (while fasting) in the NR state. Some of the same cardiopulmonary adjustments that *Varanus exanthematicus* and *Python molurus* effectuated (i.e., reduced ventilation/perfusion heterogeneity in the lungs, redistribution of blood from the GI tract to actively contracting skeletal muscles, and/or decreased R-L shunt fraction) to increase $\dot{V}O_2$ and maintain performance (Bennett and Hicks, 2001; Secor et al., 2000; Secor and White, 2010) may have been effectuated by *Lamprophis fuliginosus* and *Thamnophis marcianus* to simply maintain $\dot{V}O_2$ and activity performance.

Future Directions

The first chapter of my dissertation work proposes that a majority of the direct energetic cost associated with reproduction was related to elevated rates of protein synthesis. Future work can test this hypothesis through administration of the protein synthesis inhibitor, cyclohexamine, and repeating the measurements described in Chapter 1. During gestation, mammals experience substantial cardiopulmonary and hemodynamic adjustments (e.g., increased cardiac output, ventricular hypertrophy, increased blood volume, decreased systemic blood pressure) in response to chronically elevated O_2 demand (e.g., Metcalfe and Parker, 1966). Considering the similarity in both duration (i.e., months) and magnitude of this response between snakes and mammals (i.e., 1.3 to 3.0 fold above baseline) (Thompson, 1992), similar adjustments may also occur in the former group (Birchard et al., 1984), but very few studies have attempted such measurements.

In the second and third chapters of my dissertation, males and females (non-reproductive and reproductive) generally exhibit a prioritization pattern of O_2 delivery during post-prandial activity. Additional work that repeats the experiments described in these chapters while measuring cardiopulmonary parameters (e.g., heart rate, stroke volume, breathing frequency, tidal volume, venous and arterial blood gas levels) and regional blood flow distribution patterns (e.g., superior and inferior mesenteric arterial blood flow) would help to identify the limiting factors in O_2 transport. Although additional body weight is proposed to increase energy demand during activity (i.e., by 10% for each 10% increment in body weight), this relationship has been confirmed only for mammals (Taylor et al., 1980), while such tests have not been carried out in reptiles. The consequences of visceral organ compression (i.e., increased cost of ventilation, decreased venous return) have been examined in the lizard *Tiliqua rugosa* during reproduction (Munns, 2013) and for *Varanus exanthematicus* following the injection of saline (Munns et al.,

2004). Additional measurements of the cost of ventilation, central venous/arterial blood

pressure, and venous return, in both reproductive and digesting snakes, would help to elucidate

whether or not these factors contributed to the prioritization pattern of O_2 delivery exhibited by

Thamnophis marcianus and *Lamprophis fuliginosus*.

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Appendix A

OPERATIVE TEMPERATURE MAP FOR CHAPTER 1 AND

HEMATOCRIT VALUES FOR *LAMPROPHIS FULIGINOSUS*

MATERIALS AND METHODS

Operative Temperature Map

The operative temperature values of the steel bottom for each of the four thermogradient lanes described in Chapter 1 were mapped using Omega thermocouples (Omega Engineering Inc., CT, USA) and/or an Extech infared thermometer (Extech Instruments, Corp., NH, USA), with a minimum precision of ± 1.5°C. Each lane was divided into 70 one-inch rows, each row consisting of 4 - 9 one-inch columns (approximately 1-2 inches per measurement), from left to right. The surface temperature of each of the 4 - 9 columns, per row, was obtained for each of the four lanes, once prior to the initiation of the experiments (December, *Lamprophis fuliginosus*; August, *Thamnophis marcianus*), and then once following the conclusion of the trials (September, *Lamprophis fuliginosus*; November, *Thamnophis marcianus*).

Hematocrit (Lamprophis fuliginosus)

Following the ultrasound scans in Chapter 1 for *Lamprophis fuliginosus*, animals were fully anesthetized with gaseous isoflurane (approximately 5-15 minute duration), and a 0.1 - 0.5mL sample of blood was drawn via cardiocentesis. The animal was rotated dorsally, the heart was palpated, isolated, and then stabilized using the investigator's thumb and index finger. A pre-heparinized 18 gauge needle was angled cranially and inserted between the ventral scutes

into the caudal aspect of the heart. After blood collection, the needle was withdrawn, and digital

pressure was maintained for at least 1 minute. Following this procedure, the animal was returned

to its enclosure, at $25 \pm 2°C$ with access to a heating element, and was monitored for the next 3-6

hours. Normal behavior (i.e., reflexes, locomotion, thermoregulation, and feeding) resumed

within 24 hours following recovery from anesthesia, and we did not observe any adverse effects

(i.e., infection, failure to thrive, etc). Hematocrit fraction was determined (three replicates) using

heparinized micro-capillary tubes rotated at 10,000 RPM for 3-5 minutes using a Zipocrit

portable centrifuge (LW Scientific, Inc., GA, USA).

Statistical Analysis

We carried out non-parametric repeated measures ANOVA (Steel Dwass Method) to

compare hematocrit values between the reproductive cycle periods. Mean hematocrit results are

presented in Figure 5.2. Statistical significance was set at the $p = 0.05$ level. Results are

presented as mean ± 1 S.E.M. The statistical test was performed using JMP Software (SAS

Institute, Inc., Cary, North Carolina, USA).

RESULTS

Operative Temperature Map

A schematic diagram, with the average operative temperature values for the

thermogradient lanes (separated into rows), for each of the two species, are presented in Figure

5.1.

Hematocrit

       The average hematocrit values obtained *for Lamprophis fuliginosus* during each of the

reproductive cycle quarters are presented in Figure 5.2. Our results indicate that no significant

differences exist among the HCT values for the reproductive cycle quarters, although a trend

towards lower values between the V1 and E1 reproductive cycle phases may exist (HCT

decrease from 23 to 19; $p = 0.1287$). These results provide indirect evidence that reproduction in

Lamprophis fuliginosus results in an unchanged maternal blood volume, manifesting as

unchanged hematocrit values. However, the statistical power of the repeated measures ANOVA

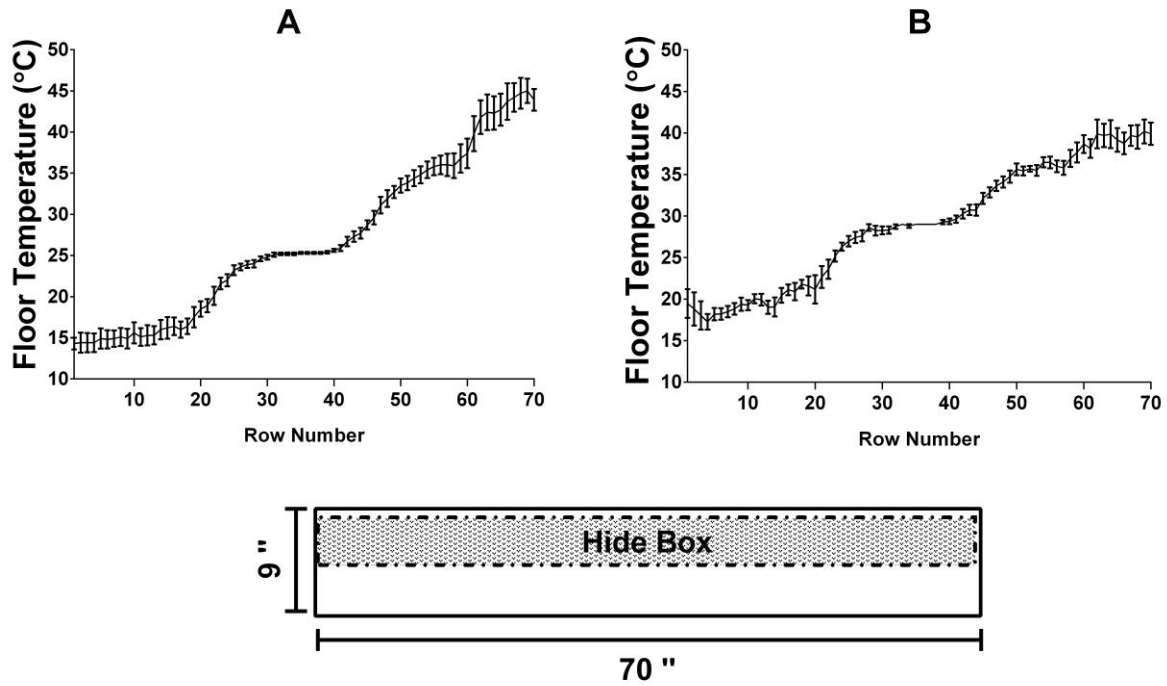is not large, and the sample sizes are low.

Figure 5.1. The mean operative temperature values of the thermal gradient lanes used to estimate preferred body temperature (PBT) for (A) *Lamprophis fuliginosus* and (B*) Thamnophis marcianus*. Each of the four thermal gradient lanes was divided into 70 one inch rows. For each row, four to eight floor temperature values (±1.5°C), measured from left to right, were obtained at the beginning and conclusion of the study. The mean ± S.E.M. floor temperature values for each row are presented. Below: Schematic illustrating the dimensions and features of a single thermogradient lane.
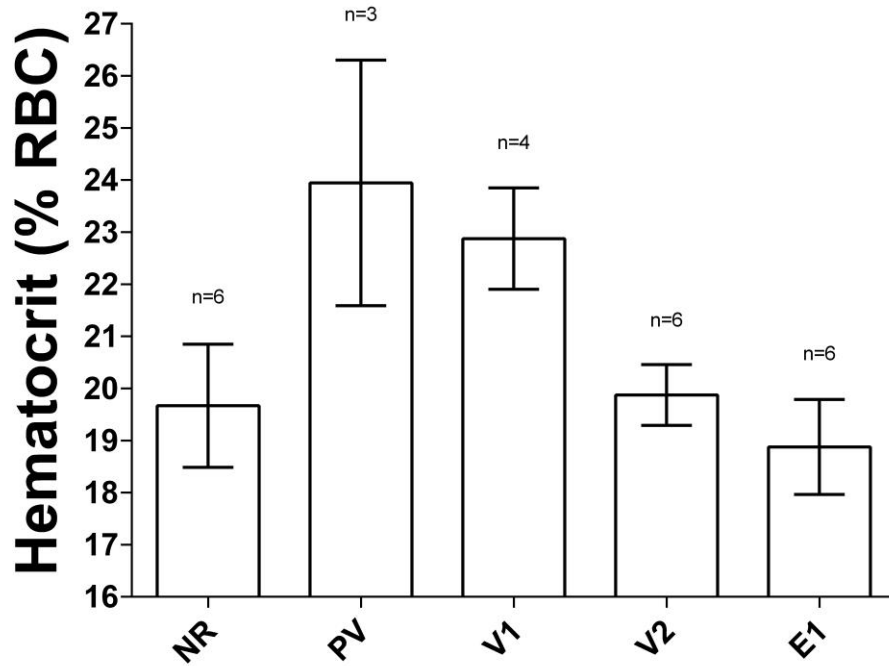
Figure 5.2. The mean hematocrit values for *Lamprophis fuliginosus* obtained during each of the reproductive cycle phases. Acronyms: PV=pre-vitellogenic, V1=primary vitellogenesis, V2=secondary vitellogenesis, E1=embryogenesis, NR=non-reproductive. All values represent mean ± 1 S.E.M.

APPENDIX B

DESCRIPTIVE INFORMATION, SDA CURVES, AND CIRCADIAN

METABOLIC PATTERNS IN *LAMPROPHIS FULIGINOSUS*

INTRODUCTION

Although non-reproductive circadian metabolic patterns in juveniles and circadian activity patterns in adults have previously been examined for *Lamprophis fuliginosus*, the circadian metabolic patterns between reproductive and non-reproductive individuals warrants further investigation (Lutterschmidt et al., 2002; Roe et al., 2004). Similarly, although the SDA curve for juveniles of this species have been previously described (Roe et al., 2004), the patterns in adult males and females have not. Therefore, we examined the circadian metabolic patterns in adult males and females, and examined whether or not reproduction significantly influenced these patterns. Moreover, we examined the SDA curve for adult males and non-reproductive females following the ingestion of a 20% body mass meal.

MATERIALS AND METHODS

Circadian Metabolic Patterns

All Dark.— We performed a test for $\dot{V}O_2$ and $\dot{V}CO_2$ patterns in constant-darkness (Circadian: All Dark) for seven non-reproductive females on 29 December 2010. Females were fasted for 21 days, and thick plastic bags were used to cover the metabolic cambers for the duration of four days. During this period our goal was to determine whether the lack of a photoperiod influenced $\dot{V}O_2$ and $\dot{V}CO_2$ values during the periods of scotophase and photophase in non-reproductive

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adult female *Lamprophis fuliginosus*. Metabolic chamber lines were sub-sampled once per 80

minutes, for an average duration of eight minutes. For this experimental period, 35

measurements made for photophase, and 32 measurements for scotophase over the trial duration

of four days. The first four hours of measurements were discarded and the average value for all

scotophase (07:00 - 18:59) and photophase (19:00 - 06:59) measurements for each non-

reproductive female was then used in pairwise comparisons.

12L:12D.—During the period of November 2011 to March 2012 (Circadian: 12L:12D) we

sampled eight females and six males, both sexes fasting for 14-16 days, and females were

measured once while reproductive, and once while non-reproductive. Trial length for females

was 120-168 hours, and 96-168 hours for males. We did not cover the chambers (chambers had

opaque sides which permitted the entry of diffuse light), and set the environmental walk-in

chamber to a 12:12 photophase/scotophase cycle (ON=7:00PM, OFF=7:00AM±15 minutes).

Investigators were careful to minimize disturbance throughout this experimental period. The

average value for all scotophase (07:00 - 18:59) and photophase (19:00 - 06:59) measurements

for each male, and female was used for pairwise comparisons. The number of measurements

that contributed to these mean values are as follows: Males (scotophase = 44±4 and photophase

= 42 ± 2); non-reproductive females (scotophase = 45±2 and photophase = 49±2); reproductive

females (scotophase = 45±2 and photophase = 49±3). Only one replicate for each group (male,

non-reproductive female, and reproductive female) was obtained.

SDA Curve

Seven months after the final data point for Chapter 2's data, we performed a trial

designed to describe the full SDA curve for (n=8) adult non-reproductive females and (n=5)

adult male *Lamprophis fuliginosus*. Females and males were fasted 25 to 26 days, and then transferred from their enclosure to a metabolic chamber (see Methods, Chapter 2) located within a walk-in environmental chamber maintained at $25 \pm 1°C$. To establish a baseline value, the snakes remained undisturbed for 21 to 31 hours at rest, and the excurrent air streams were repeatedly sampled approximately once every 80 to 200 minutes, repetitively, using the second subsampling design described in the Materials and Methods section of Chapter 2. The first four hours were discarded (as previously described), and the average of the three lowest consecutive measurements was considered the standard metabolic rate (SMR) value.

Within 24 hours after measuring baseline gas exchange values, the snakes were fed a meal equivalent to 20% of their body mass in pre-killed mice (fasting time of 26 to 28 days), and then placed back into the metabolic chambers within 1 hour following ingestion. A wire mesh was placed on the bottom of the chambers to provide a small distance between the animal and the bottom of the enclosure to prevent contact with digestive excrement, and excrement was removed once every 12 hours after the first 72 hours of measurements for the remainder of the trial. Excurrent air streams were sampled at the same rate (mentioned above) for a total of 145 hours. The first 4 hours were discarded to account for handling stress and acclimation to the chamber.

Statistical Tests

Circadian Metabolic Patterns.— We carried out two statistical tests for dependent variables in this experiment: analysis of variance (ANOVA) to test for differences in morphometric variables (mass and length) and gas exchange variables ($\dot{V}O_2$, $\dot{V}CO_2$, and RQ) between males and females; we used pairwise mean comparisons to compare average values for the same male

or female before and after treatment (male = scotophase *versus* photophase, and photophase *versus* scotophase).

*SDA Curve.—*To evaluate the duration of the SDA curve in male and female *Lamprophis fuliginosus*, we carried out analysis of variance (ANOVA) to test the differences between SMR and $\dot{V}O_2$ levels obtained during the approximately 24h, 48h, 72h, 96h, 120h, and 144h points following ingestion. Statistical significance was set at $p = 0.05$. Results are presented as mean ± 1 S.E.M. or mean ± 1 S.D. All statistical analyses were performed using JMP Software (SAS Institute, Inc., Cary, North Carolina, USA).

RESULTS

Descriptive Statistics

Our morphometric results confirm previously published sexual dimorphic traits in this species (Broadley, 1983; Ford, 2001). Female snout vent length (SVL) was 1.46 fold ($p<0.0001$) larger than males, and non-reproductive females were 2.9 fold ($p=0.0003$) heavier than males (Table 6.1). Pairwise comparisons between females in the non-reproductive and reproductive states reveal that there is a statistically significant increase in body mass by a factor of 1.2 ($p< 0.0001$) during reproduction.

In a subset of females during a seven month period, we measured both clutch statistics and body mass values. The body mass of a representative individual (Figure 6.1) demonstrates an overall increase throughout the gestation period, with peaks adjacent to meals, and declines between meals and then directly following oviposition. The relative clutch mass (RCM) in a subset of individuals ($n = 9$) was calculated as the ratio of the clutch mass to that of the pre-

partum female. On average, RCM in *Lamprophis fuliginosus* was 24% of the reproductive

female's body mass (Table 6.2).

Circadian Cycle

All dark.—When non-reproductive females are exposed to constant dark (Figure 6.2A) there is a

mean factorial $\dot{V}O_2$ increase of 1.18 during scotophase compared to photophase (p=0.0140).

Mean $\dot{V}CO_2$ values during scotophase are 1.26 fold higher than those during photophase

(p=0.0231). The mean respiratory quotient values during scotophase are 0.40 and 0.36 during

photophase.

12L:12D.—Non-reproductive females demonstrate a statistically significant 1.15 fold increment

(p=0.024) in $\dot{V}O_2$ during scotophase compared to photophase; and a statistically significant

(p=0.026) 1.39 fold increment in $\dot{V}CO_2$ during scotophase compared to photophase (Figure

6.2B). However, the increase for $\dot{V}O_2$ and $\dot{V}CO_2$ are proportional, resulting in no significant

difference (p=0.644) in respiratory quotient between scotophase (0.59) and photophase (0.59).

When the same females are measured during reproduction, the differences in $\dot{V}O_2$ and $\dot{V}CO_2$ for

photophase and scotophase are not statistically different (Figure 6.2C). On average, scotophase

$\dot{V}O_2$ values are 1.05 fold larger than photophase values (p=0.116), and scotophase $\dot{V}CO_2$ change

by a similarly non-significant (p=0.0974) factor of 1.05 over the values from photophase. The

increment in $\dot{V}O_2$ and $\dot{V}CO_2$ are also proportional during reproduction, with a resulting RQ

value of 0.58 for scotophase and 0.58 for photophase (p=0.618).

Finally, in males the scotophase $\dot{V}O_2$ values are 1.28 fold larger, and $\dot{V}CO_2$ values are

1.27 fold larger than photophase values, but these increments are not statistically significant

($\dot{V}O_2$ p=0.0588, $\dot{V}CO_2$ p=0.0764) (Figure 6.2D). Moreover, male respiratory quotient values remain statistically similar (p=0.645) during scotophase (0.53) and photophase (0.53).

SDA Curve. — The SDA curves for males and females (non-reproductive) are reported in Figure 6.3. Females (n=8) reached peak $\dot{V}O_2$ by an average of 35±6 hours following ingestion, and by 144 hours post-ingestion the lowest $\dot{V}O_2$ values were still elevated above SMR (p=0.0009) by 2 fold (Figure 6.3A). Patterns in RQ were present: average RQ values were 0.78 at SMR, declined within the first 5-10 hours post-ingestion to an average value of 0.69, and then varied between 0.73 and 0.83 for the remainder of the experimental period (Figure 6.3B).

Males (n=5) reached peak $\dot{V}O_2$ by an average of 42±19 hours post-ingestion, and $\dot{V}O_2$ remained elevated until 120 hours following ingestion (p=0.0055) (Figure 6.3C). Similar to females, patterns in RQ were present: average RQ values were 0.80 at SMR, and within the first 5-10 hours post-ingestion, dropped to 0.69, and then then varied between 0.72 and 0.87 for the remainder of the trial (Figure 6.3D).

DISCUSSION

Circadian Metabolic Patterns

Previous research in juvenile *Lamprophis fuliginosus* demonstrated a nearly two fold increase in oxygen uptake during scotophase (Roe et al., 2004). Our values for non-reproductive females are substantially lower, and males did not demonstrate a statistically significant difference between photophase and scotophase. The 15% increase in oxygen uptake during scotophase (the active phase for this species) in non-reproductive females is similar to the values

found in *Thamnophis elegans* and *Natrix rhombifera* (Gratz and Hutchinson, 1977; Hicks and Riedesel, 1983).

Reproductive female *Lamprophis fuliginosus* in our study did not exhibit significant variation between photophase and scotophase. A similar study in reproductive and non-reproductive snakes described a similar pattern in *Crotalus atrox* (Beaupre and Duvall, 1998). The presence of elevated scotophase $\dot{V}O_2$ in the presence of constant darkness indicates an intrinsic circadian metabolic rhythm. In previous studies, endogenous oscillations have also been reported for snakes (Hicks and Riedesel, 1983; Blem and Killeen, 1993; Roe et al., 2004).

Respiratory Quotient

The respiratory quotient values associated with resting values in the current paper are below the theoretical RQ lower limit estimate of 0.71 (Kleiber, 1961). Previous studies which measured both $\dot{V}O_2$ and $\dot{V}CO_2$ simultaneously, report low RQ values. For example, in squamates (Benedict, 1932; Roth, 1942; Clark, 1953; Roe et al., 2005) RQ values are below the expected 0.71 minimum value. Ectothermic vertebrates in general have demonstrated similar findings, for example crocodilians demonstrate lower than 0.71 RQs (Grigg, 1978; Hicks and White, 1991), and this has also been described in fish (Kutty, 1972). The low RQ values described in this paper may result from non-pulmonary CO_2 loss (e.g. nitrogen excretion in the urine; Grigg, 1978).

SDA Curve

In the present study, peak digestive $\dot{V}O_2$ values for non-reproductive female and male *Lamprophis fuliginosus* occur between 35-42 hours post-ingestion (Figure 6.2A and 6.2C). This

duration is considerably longer than previously published values for juvenile *Lamprophis fuliginosus* (24 hours; Roe et al. 2004), but consistent with values from the boids *Python molurus* and *Boa constrictor* (38-55 hours; Toledo et al. 2003; Wang et al. 2003). In the present study, the $\dot{V}O_2$ increment for non-reproductive females at 6-days post ingestion remains elevated by as much as two fold above baseline, while males return to baseline values by five days following ingestion of a 20% body mass meal. The five day duration exhibited by adult male *Lamprophis fuliginosus* in the present study is consistent with previous work in Boa constrictor (i.e., five days) (Toledo et al., 2003), and the longer than six day duration exhibited by females is more consistent with work in *Python molurus* (i.e., 8 days) (Wang et al. 2003).
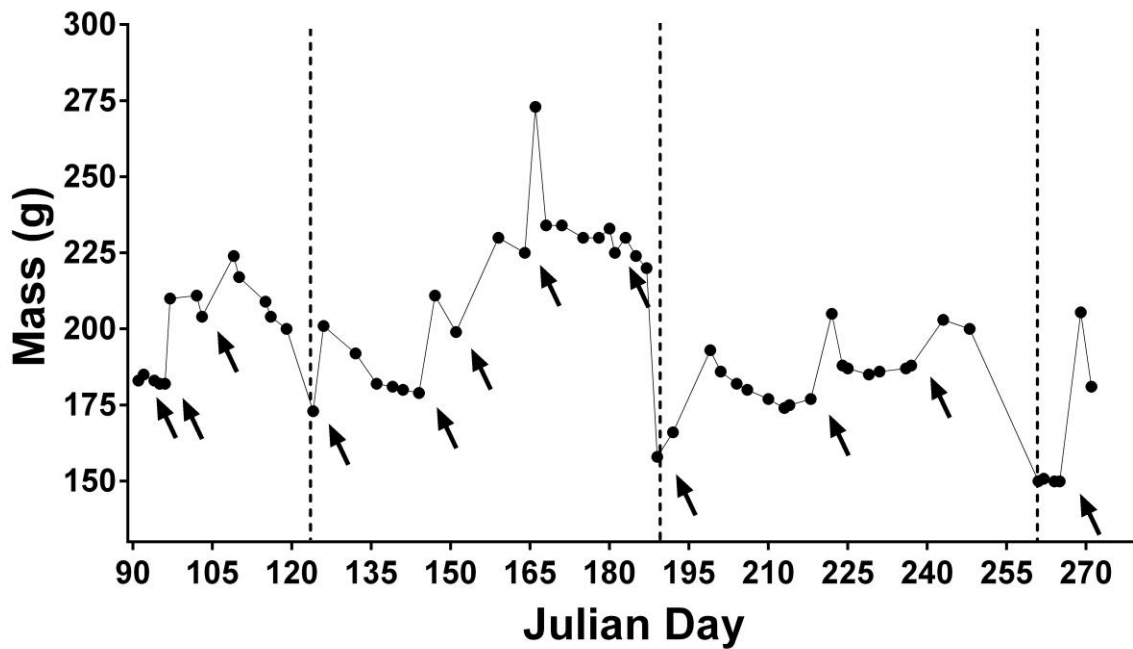
Figure 6.1. One representative adult female *Lamprophis fuliginosus* body mass series during the period of 1 April 2011 to 3 October 2011 (185 days). Dots indicate dates where body mass was determined to the nearest 1 g; feeding is indicated by an arrow, and oviposition by a dashed line. Julian days are the number of days since 1 January 2011.
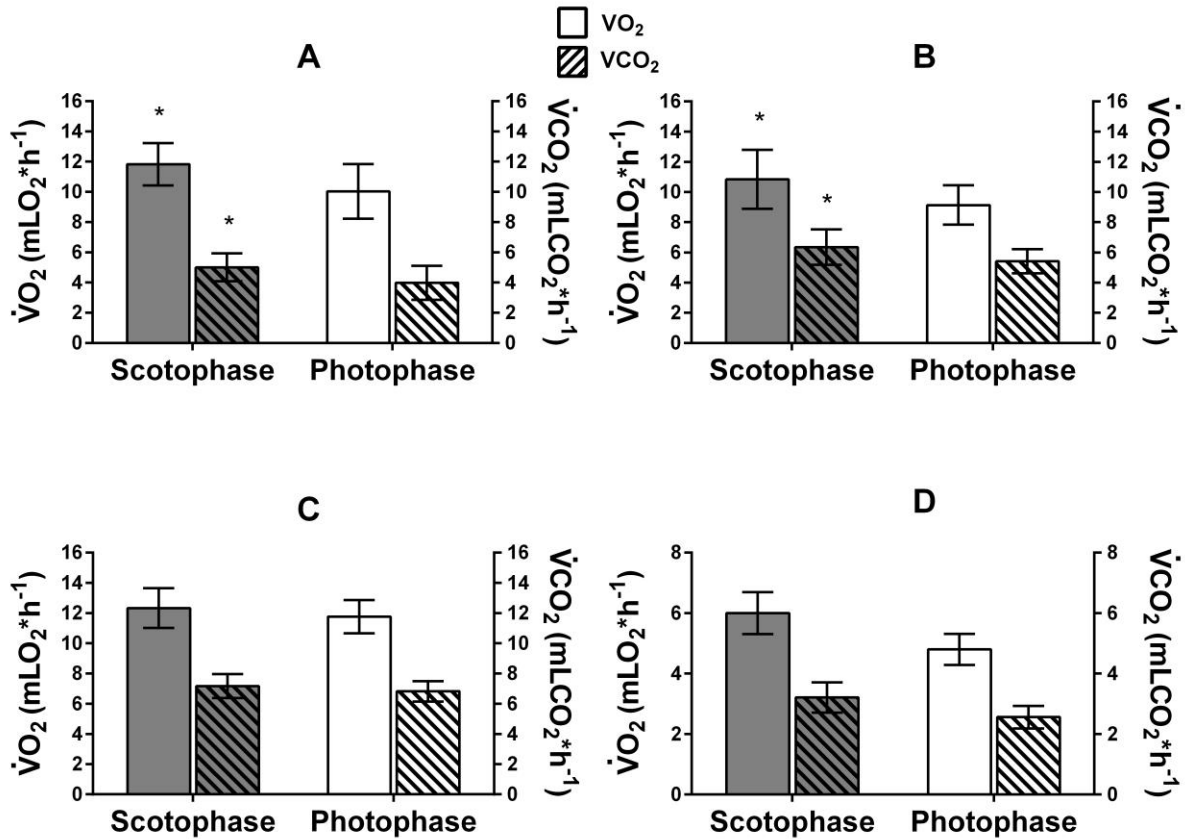
Figure 6.2. Average $\dot{V}O_2$ and $\dot{V}CO_2$ values for the following sets of data: (A) non-reproductive females (Circadian: All dark) (n = 7); (B) Non-reproductive females (Circadian: 12L:12D) (n = 7); (C) Reproductive females (Circadian: 12L:12D) (n = 7); (D) Males (Circadian: 12L:12D) (n = 6). Values represent the mean ± 1 S.E.M. of all values obtained during both photophase 07:00-18:59 (non-shaded bars) and photophase 19:00-06:59 (shaded bars) for 96-168 hours. An asterisk (*) indicates a statistically significant ($p < 0.05$) difference.
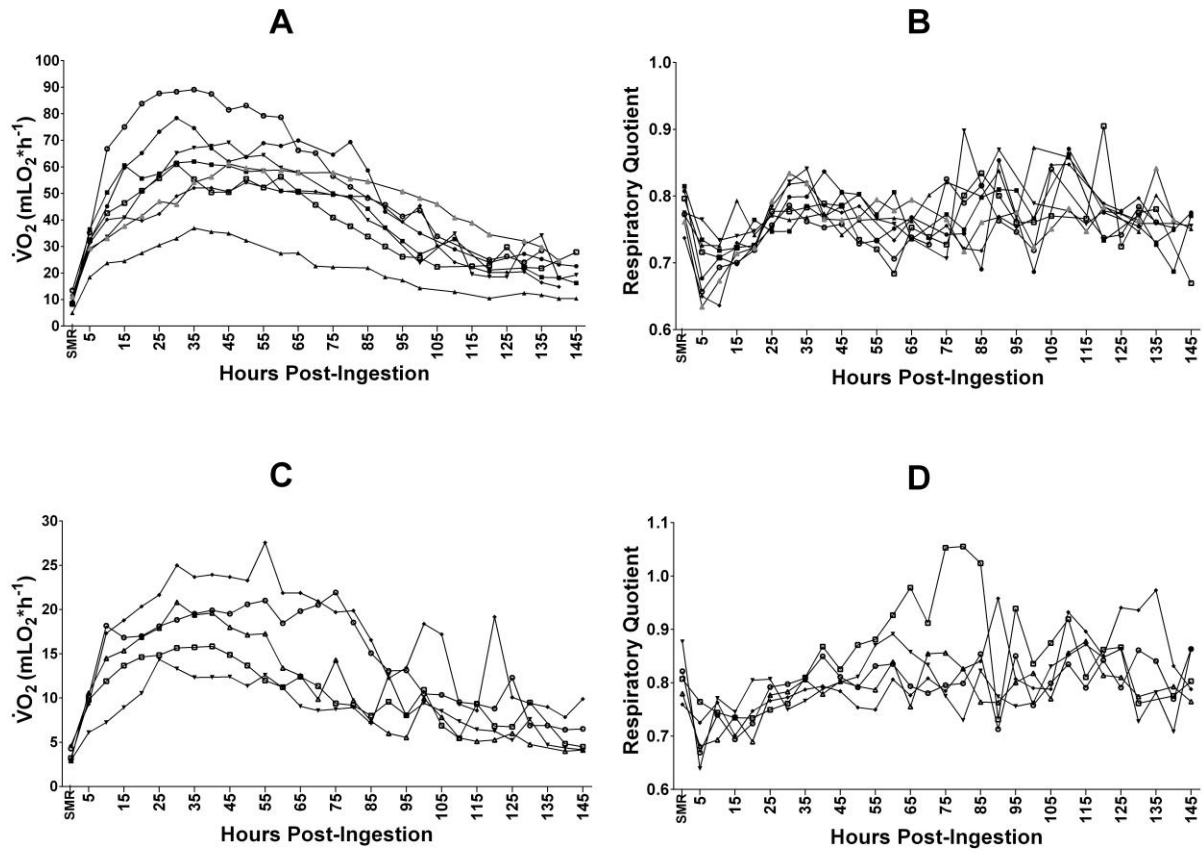
Figure 6.3. Post-prandial $\dot{V}O_2$ and corresponding RQ values for non-reproductive females (n=8) (A, B), and males (n=5) (C, D). For A and C, $\dot{V}O_2$ values represent whole-animal values, plotted at 5±1 hour intervals, until 145 hours post-ingestion. In B and D, the corresponding RQ values are plotted on the Y axis at 5±1 hour intervals. The first value on the X axis represents the baseline (SMR) value obtained for each of the animals for both $\dot{V}O_2$ and RQ.

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Table 6.1. Summary of adult male and female *Lamprophis fuliginosus* morphometric information.

Variable	Male SVL (cm)	Female SVL (cm)	Male mass (g)	Non-reproductive Mass (g)	Reproductive mass (g)
Mean	62	91	88	252	301
Range	57-66	79-108	68-106	130-411	151-518
± 1 S.D.	3	8	18	85	101
Sample size	6	13	6	13	13

Table 6.2. Subset of reproductive cycle data over a 185 day period (1 April to 3 October 2011).

Variable	Clutches (*n*)	Cycle Length (days)	Clutch size (*n*)	Clutch mass (g)	Pre-oviposition Mass (g)[a]	RCM (%)[b]
Mean	2.5	78	10	68	291	24
Range	2-3	59-109	8-15	36-114	146-502	21-27.5
± 1 S.D.	0.5	22	2	25	111	1.8
Sample size	10	10	9	10	9	9

[a]Female pre-partum mass is the body mass of the fasting female 3 - 8 days prior to oviposition

[b]Relative clutch mass (RCM) was calculated by dividing the clutch mass (within 48h of oviposition) by the female's pre-partum body mass

APPENDIX C

SDA CURVES AND MEAL SIZE EFFECTS IN *THAMNOPHIS MARCIANUS*

INTRODUCTION

For males, in addition to the 10% body mass meal treatment (Chapter 3), we also carried out separate tests to determine whether there was a relationship between meal size and the peak post-prandial $\dot{V}O_2$ increment in *Thamnophis marcianus*. Moreover, the SDA curve for *Thamnophis marcianus* has not been previously described. Therefore, for meal size treatments of 10% (males and non-reproductive females), 20% (males), and 30% (males), we described the components of the SDA curve and the peak factorial increments in $\dot{V}O_2$. Virtually no studies have examined the interaction effects between activity and digestion for different meal size treatments in any vertebrate lineage (Table 1.5). Therefore, we also sought to explore the patterns of interaction between physical activity and digestion (10%, 20%, and 30% meal size treatments) in male *Thamnophis marcianus*.

METHODS

Meal Size Effects

In brief, males were first measured under standard conditions to establish baseline gas exchange values under identical conditions as described in Chapter 3. The mean of the three lowest consecutive values (four hour duration) was chosen to represent RMR. Within one day of these trials, males were offered a meal equivalent to 10% (i.e., DIG10), 20% (i.e., DIG20), or 30% (i.e., DIG30) of body mass in the form of thawed rodent. Following voluntary ingestion, snakes were re-inserted into the metabolic chambers (see Materials and Methods in Chapter 3).

Gas exchange values were obtained once every 80 minutes, for 6 to 11 minutes at a time, for up to 24 hours following ingestion. The mean of the three highest consecutive $\dot{V}O_2$ (and corresponding RQ) values were considered to represent peak post-prandial values.

To evaluate the interaction effect between ACT and DIG, during various meal sizes, we repeated the previously described (see Materials and Methods for Chapter 3) exhaustive activity protocol within 28 hours following ingestion. We also recorded TTE for these trials. If the male vomited the meal at any point during the trial, the data was not considered for analysis.

SDA Curve

We carried out a final set of experiments to describe the components of the SDA curve for this species. Non-reproductive females (fasting 14 to 24 days) and males (fasting 16-19 days) were measured at rest for 20 to 37 hours (females) or 15 to 25 hours (males). The mean of the three lowest consecutive values (four hour duration) was chosen to represent RMR. Immediately following the RMR trial, snakes were removed from the metabolic chamber, and placed into their normal sized enclosure. Within 24 hours, snakes were offered rodent meals equivalent to 10% (males and females), 20% (males), or 30% (males) of their body mass.

Within four hours following voluntary ingestion, snakes were placed into the previously mentioned respirometry setup. To prevent contact between the snake and any excrement produced during the digestive process a wire grating was inserted into each chamber that elevated the animal approximately 1 cm above the floor of the chamber. Gas exchange values were obtained once every 80 minutes (6 to 11 minutes at a time), for 140 to 144 hours (females) or 121-144 hours (males) following ingestion, a sufficient quantity of time for closely related species to return to postabsorptive levels (Britt et al., 2006; Bessler et al., 2010). Following the

conclusion of the trial, the snakes were removed, and any excrement that had been produced was left inside of the chamber for gas exchange measurements. We found that male excrement (for any of the meal size treatments) did not produce any noticeable $\dot{V}O_2$ or $\dot{V}CO_2$ signals, but female excrement was found to produce a noticeable effect on baseline values (i.e., empty metabolic chambers had a $\dot{V}O_2$ and/or $\dot{V}CO_2$ signal).

Therefore, we carried out a subsequent experiment to quantify the $\dot{V}O_2$ or $\dot{V}CO_2$ signals associated with excrement for female *Thamnophis marcianus*. In brief, we repeated the above experiment in the same females, and following voluntary ingestion, placed the snakes into enclosures within the walk-in environmental chamber (held at $29\pm1°C$ in constant darkness). Each enclosure contained a wire mesh bottom that facilitated removal of excrement with minimal disruption to the snake. Approximately once every 3 to 12 hours, for up to 144 hours following ingestion, investigators checked for the presence of excrement using a red infrared bulb. If excrement was detected, the investigator carefully removed the entire portion and transferred it to a metabolic chamber in the previously mentioned respirometry setup. Flow rates remained similar to those experienced by the female during the post-prandial trial. The $\dot{V}O_2$ (and corresponding $\dot{V}CO_2$) values for the excrement was subsampled once every 80 minutes (for 6-11 minutes) until the conclusion of the trial. The $\dot{V}O_2$ and $\dot{V}CO_2$ values obtained for the excrement were calculated using the same equations previously described, and subtracted from the post-ingestion $\dot{V}O_2$ and $\dot{V}CO_2$ values obtained from the females (to the nearest ±1 hour).

Statistical Analysis

For males, we carried out a non-parametric repeated measures ANOVA (Steel-Dwass Method) to evaluate whether $\dot{V}O_2$ and RQ values associated with REST, DIG 10%, DIG 20%, and DIG 30% were significantly different. Prior to analysis, $\dot{V}O_2$ values were transformed into mass-specific values (i.e., $mLO_2/g/h$), because male $\dot{V}O_2$ varies significantly with body mass. We carried out a separate analysis of variance (ANOVA) to evaluate the duration of the post-prandial $\dot{V}O_2$ curve for males (10% and 20% body mass meal) and females (10% body mass meal). Comparisons were made between $\dot{V}O_2$ values elicited during RMR, and then at approximately 24, 48, 72, 96, 120, and 140 hours following ingestion. Statistical significance was set to $p = 0.05$. Results are presented as mean $\pm$ 1 S.E.M. The statistical tests were performed using JMP Software (SAS Institute, Inc., Cary, North Carolina, USA).

RESULTS

Meal Size Effects

The $\dot{V}O_2$ and corresponding RQ values associated with REST and the peak values attained for each of the three meal size treatments (DIG10, DIG20, and DIG30) are presented in Figure 7.1. Our results indicate that males exhibit statistically significant ($p<0.0049$) factorial scopes associated with the 10% (4.5 fold), 20% (5.5 fold), and 30% (6.7 fold) meal size treatments. Furthermore, our results indicate that the peak $\dot{V}O_2$ values associated with DIG20 ($p=0.0316$) and DIG30 ($p=0.0092$) were significantly larger than DIG10 values. There was no significant difference ($p=0.9840$) between DIG20 and DIG30 treatments. Our results also indicate that RQ values were significantly larger during DIG30 than REST, but did not differ between any of the other treatments ($p>0.8010$).

For males, the $\dot{V}O_2$ and RQ values associated with REST, ACT, and ACT+DIG (10%, 20%, and 30% body mass meals) are presented in Figure 7.2. During the ACT+DIG trials for the 20% and 30% meal size treatments, the majority (i.e., 13/18) of males vomited the meal, limiting the sample size (i.e., ACT+DIG20, n=3; and ACT+DIG30, n=2). Therefore, we present the data to describe possible trends, but did not carry out formal statistical tests. The factorial metabolic scopes associated with these treatments were: 9.1 fold (ACT), 8.7 fold (ACT+DIG10), 9.7 fold (ACT+DIG20), and 8.7 fold (ACT+DIG30). We also found that males exhibited time to exhaustion (TTE) values that were similar among the three treatments: ACT+DIG10 (24±3 minutes) (n=10), ACT+DIG20 (27±6 minutes) (n=3), and ACT+DIG30 (21±9 minutes) (n=2).

SDA Curve

The SDA curves for females (10% body mass meal) and males (10%, 20%, and 30% body mass meals) are presented in Figure 7.3 (females) and 7.4 (males). Females (n=6) reached peak $\dot{V}O_2$ values by 19±2 hours following ingestion, and $\dot{V}O_2$ remained elevated for five days (p=0.0230) following ingestion. Female RQ values decreased from the average resting values of 0.74±.03 to an average low value of 0.59±.05 within the first 10-15 hours following ingestion, and thereafter average RQ values varied between 0.73±.04 and 0.85±0.09 for the remainder of the trial (Figure 7.3B).

Males reached peak $\dot{V}O_2$ by 16±5 hours following ingestion of a 10% body mass meal (n=6), by 18±2 hours following a 20% body mass meal (n=6), and by 20±4 hours following the ingestion of a 30% body mass meal (n=2) (Figure 7.4A, 7.4C, and 7.4E). Male $\dot{V}O_2$ values remained elevated for two days (p=0.0004) following the 10% body mass meal, and for six days (p=0.0070) following the 20% body mass meal. For all meal size treatments, RQ values did not

shift as dramatically for the males. RQ values at RMR began at 0.78±.01, and remained, on average, between 0.64 and 0.85 throughout the duration of the trial (Figure 7.3B, 7.4B, 7.4D, 7.4F).

DISCUSSION

Our results indicate that for males, a trend exists for increased peak $\dot{V}O_2$ following the ingestion of progressively larger meal sizes (Figure 7.1). This finding is consistent with previous work on *Thamnophis sirtalis* (Bessler et al., 2010), *Lamprophis fuliginosus* (Roe et al., 2004), and *Python molurus* (Secor and Diamond, 1997). The 4.5 fold factorial scope associated with the 10% body mass meal is most consistent with the ~4-5 fold increments exhibited by *Natrix natrix, Acanthophis praelongus* and *Python molurus* (Christian et al., 2007; Hailey and Davies, 1987; McCue et al., 2005). Following the ingestion of a 20% body mass meal, the 5.5 fold increments exhibited by male *Thamnophis marcianus* in the present study are most consistent with those exhibited by *Python molurus* and *Lamprophis fuliginosus,* (Wang et al., 2003; Roe et al., 2004). For the 30% body mass meal treatment, the 6.7 fold increment reported for male *Thamnophis marcianus* in the present study was considerably larger than values reported for *Thamnophis sirtalis* (~4 fold) (Peterson et al., 1998).

The durations required to attain peak values following the ingestion of a 10% body mass meal in the present study (16-19 hours; Figure 7.3A and 7.4A) is consistent with the ~24 hour period previously reported for *Thamnophis elegans, Lamprophis fuliginosus,* and *Nerodia sipedon* (Britt et al., 2006; Roe et al., 2004; Sievert and Andreadis, 1999). For the 20% body mass meal, the 18 hour duration required to attain peak $\dot{V}O_2$ (Figure 7.4C) is similar to the previously reported durations for *Lamprophis fuliginosus* (Roe et al., 2004). Finally, for the 30%

body mass meal treatment, the 20 hour duration (Figure 7.4E) is consistent with previously published values for *Thamnophis sirtalis* (Peterson et al., 1998). The SDA response lasted for two days for males, and five days for females following a 10% body mass meal (Figure 7.3A and Figure 7.4A). For a 10% body mass meal, the duration for males in this study is most consistent with previous work in *Crotalus atrox* (i.e., ~three day duration), and the female duration is most consistent with work in *Agkistrodon piscivorus* (i.e., six days) (McCue and Lillywhite, 2002). For a 20% body mass meal, the six day duration is consistent with previous work in juvenile *Lamprophis fuliginosus* (i.e., six days) (Roe et al., 2004).

There is limited information on how meal size may affect the interaction effect between physical activity and digestion, but it is important to examine such patterns because snakes forage opportunistically under natural conditions, and may be forced to perform physical activity following the ingestion of various meal sizes. Our study represents a preliminary examination of the possible patterns of interaction between activity and digestion for various meal size treatments. Overall, our results indicate that the resulting factorial scope for ACT (~9 fold) was quantitatively similar to those elicited by ACT+DIG (~9-10 fold) for all meal size treatments (Figure 7.2). Our results may indicate a trend of prioritization for O_2 delivery towards active skeletal muscles over the digestive processes, regardless of the size of the ingested meal (Bennett and Hicks, 2001). From our data, two trends suggest a prioritization towards activity performance over digestion: 1) Most males vomit their meals during ACT+DIG20 and ACT+DIG30 trials, instead of refusing to perform further activity, and 2) the five males that successfully completed the ACT+DIG trials at the 20 and 30% meal size treatments did not exhibit a decrement in time to exhaustion.
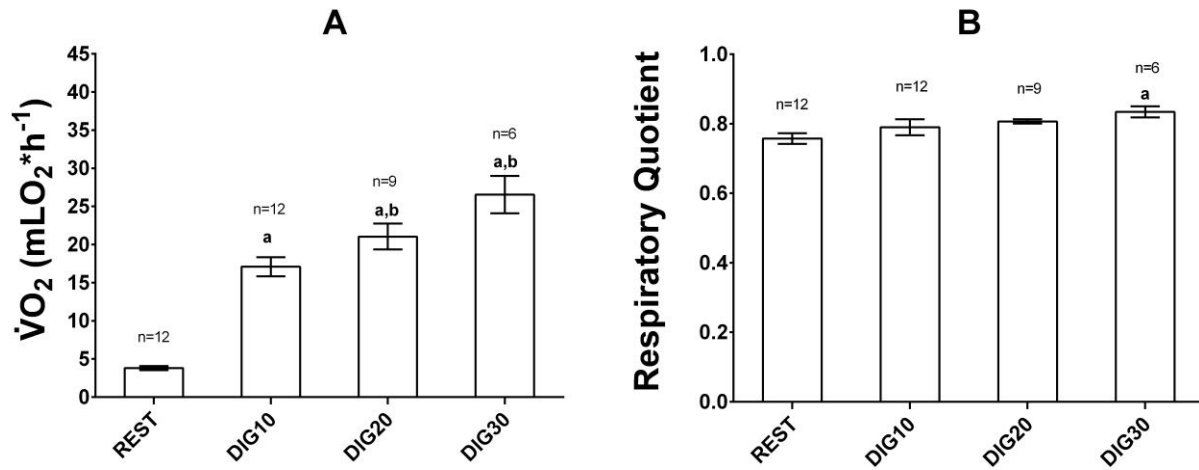
Figure 7.1. Male whole animal $\dot{V}O_2$ (A) and respiratory quotient (RQ) (B) values associated with rest (REST), and peak values attained during the digestion of a meal equivalent to 10% (DIG10), 20% (DIG20), or 30% (DIG30) of body mass. Lowercase letters above the values represent a statistically significant difference: (a) = significantly larger than REST values; (b) = significantly larger than DIG10 values. Sample sizes are located above each value. All values represent mean ± S.E.M.
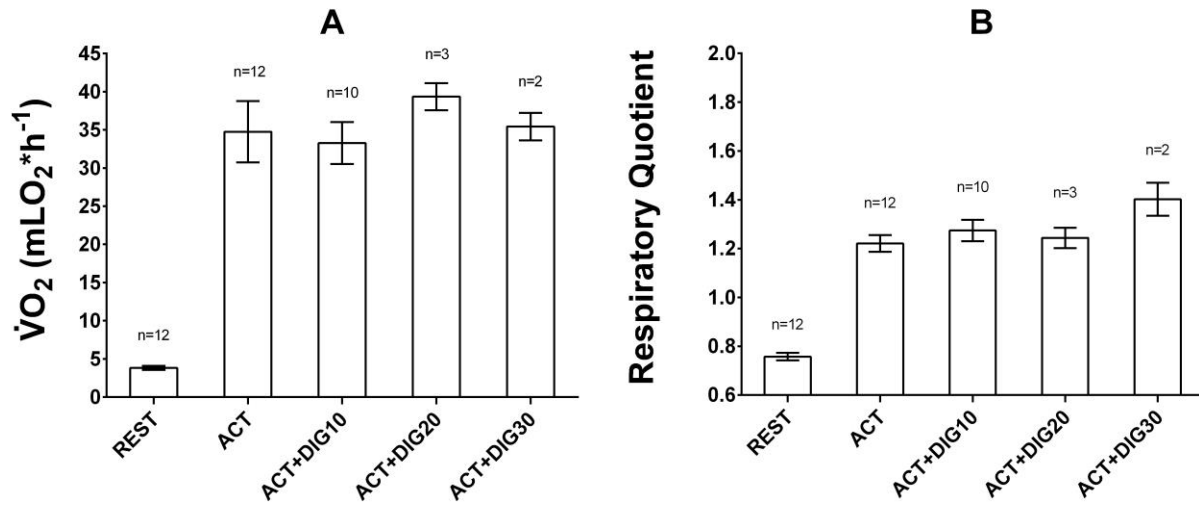
Figure 7.2. Male whole animal $\dot{V}O_2$ (A) and respiratory quotient (RQ) (B) values associated with rest (REST), fasting exhaustive activity (ACT), and post-prandial exhaustive activity following the ingestion of a 10% (ACT+DIG10), 20% (ACT+DIG20), or 30% (ACT+DIG30) body mass meal. Formal statistical tests were not carried out for the present data set. Sample sizes are located above each value. All values represent mean ± S.E.M.
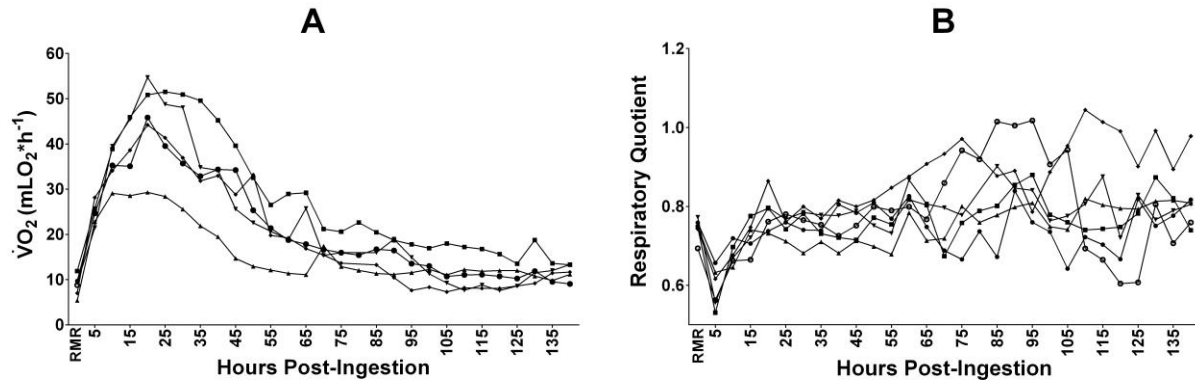
Figure 7.3. Post-prandial $\dot{V}O_2$ and corresponding respiratory quotient (RQ) values for non-reproductive females (A, B) following the ingestion of a 10% body mass meal. $\dot{V}O_2$ and RQ values represent whole-animal values, plotted at 5±1 hour intervals until 140 hours post-ingestion. The first value on the X axis represents the baseline (RMR) value for each animal.
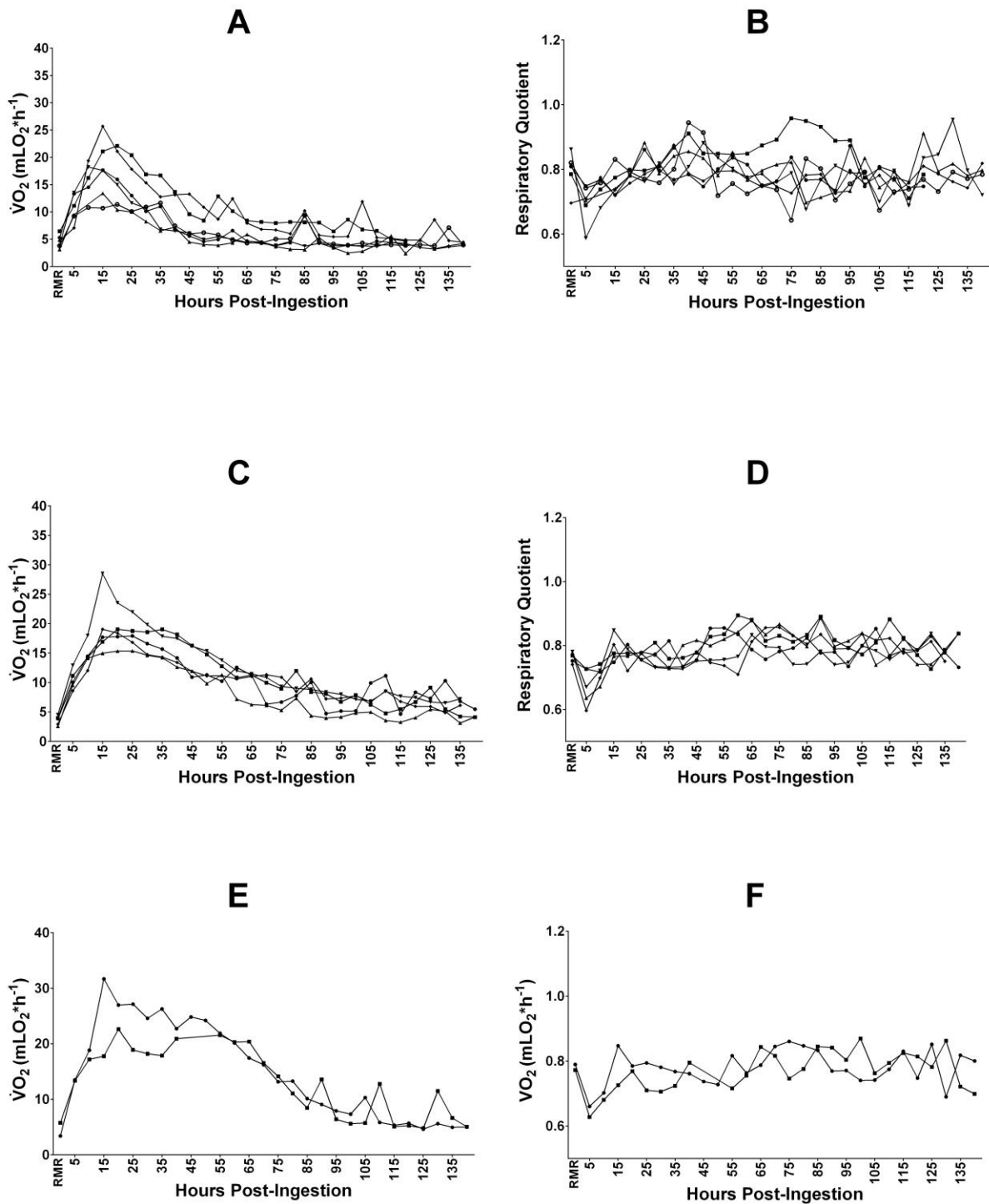
Figure 7.4. Post-prandial $\dot{V}O_2$ and corresponding respiratory quotient (RQ) values for males following the ingestion of 10% (A, B), 20% (C, D), or 30% (E, F) body mass meal. $\dot{V}O_2$ and RQ values represent whole-animal values, plotted at 5±1 hour intervals until 140 hours post-ingestion. The first value on the X axis represents the baseline (RMR) value for each animal.