Shedding in the Timber Rattlesnake: Natural Patterns, Endocrinological Underpinnings, Temporal and Energetic Effort, and Integration as a Reptilian Life History Trait

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Biology

by

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August 2023
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Abstract

The semi-frequent replacement of the epidermis (ecdysis) is a characteristic trait of reptiles. Whereas all reptiles regularly engage in some degree of skin shedding, skin morphology in snakes necessitates the synchronous replacement of the entire epidermis and facilitates the subsequent removal of the old layer as a single sheet. To date, the ubiquitous process has garnered little attention from researchers because snakes shed with unpredictable timing and frequency and are exceedingly cryptic during ecdytic cycles; previously impeding detailed physiological or ecological investigations of the process in the clade. Because of the lack of study, ecdysis is often viewed as a maintenance function; occurring whenever change in body size necessitates the generation of a new epidermal layer. However, recent observations that skin shedding plays a role in conspecific sexual signaling in some snakes suggest that the predominate view of ecdysis as a growth function may be overly simplistic. By studying population-scale patterns of shed, I was able to describe the timing and frequency of ecdysis in a population of Timber Rattlesnakes, solving a long-standing problem in continued study of ecdysis; predicting the occurrence of shed events. Coupling my knowledge of patterns of shed timing with novel methodologies for inducing shed, I was able to induce ecdytic cycles in a laboratory setting and herein provide the first measurements of the metabolic effort and duration of shedding in any reptile. I integrated data on the frequency, duration, and metabolic effort of shed into an individual-based computer model of the Timber Rattlesnake to address larger questions about the selective pressures that may shape patterns of shed in snakes.

I found that Timber Rattlesnakes shed infrequently (1-2 times per year) and often in close proximity to the mating season regardless of sex. However, the physiological conditions that best correlated to shed frequency differed between males (body condition) and females (reproductive condition). Each shed event required a significant metabolic (3% of the total annual energy budget) and temporal (~28 days at 25ºC with ~50% of that including some degree of visual limitation from occluded spectacles) investment. In my computer simulations, I

found that time spent in shed limited lifetime energy budgets (decreasing lifetime resource acquisition via foraging) and that the energetic effort of ecdysis may serve to limit shed frequency in low resource environments. In my observations of patterns of shed in the wild and through simulations of expected female fecundity under alternate shed frequencies, I found evidence that ecdysis may play a vital role in the reproductive biology of rattlesnakes.

Ecdysis is a vital and omnipresent feature of reptilian biology. My data are the first to demonstrate that the frequency of the process is constrained in a population. I provide evidence for the role of growth and body condition, time-energy budgets, environmental conditions, and reproductive events in dictating patterns of shed. As such, patterns of shed may be population specific and serve as an indicator of the important environmental and biophysical forces which shape life histories across populations and species.

Acknowledgements

First and foremost, thank you to my advisor Dr. Steve Beaupre. You have given me direction in my life and helped me to find a passion worth pursuing.

While I felt compelled to dedicate this work to the snakes, I could not have accomplished this without the support of my family. Thank you to my Mom, Ann Mason, for inspiring a love of science and inquiry in me. Although I know I was a tall order, you were right to assume that what made me difficult then has served me well later. To my Dad, John Carnes, for showing me the joy of being alone in the woods and pursuing my own great passions, not what was expected or financially "good" for me. To my partner, Dr. Mary Hunt for her unwavering support, and accepting me for the snake loving wierdo that I am. To my brothers, real and adopted (Zeke, Tolliver, Rew, Julian, Zander, Alex) for listening to me, supporting my interests, and keeping me grounded. To my great friends and lab mates (Larry, Ethan, Allie, Jason, Casey, Brenna) for their insight, camaraderie, sympathy, and beers. To my Fayetteville family (David, Megan, Spencer, Emily) for tolerating snake talk and complimenting my cooking. To my Aunt Mary, who would've loved to see this. To my San Diego family (especially the Rasmussens) for encouraging me to pursue my interests. And to my dogs, Stella and Didgeridoo, for being there, dino time, and getting me out of the house, particularly when I was writing this dissertation. Thank you all for your support and love. You can't pursue your true passions without the people around you, you all make life worth living.

Dedication

For my study animals - who did not volunteer but helped me all the same. I continue to be awestruck by their beauty and promise never to lose sight of what a marvelous group of snakes they are. It is my sincere hope that the data that I collected over these many years results in a continued refinement of our understanding of their biology and ultimately contributes to the successful conservation of the clade.

Table of Contents

Preface **1**

Introduction **2**

Literature Cited *8*

Chapter 1: The Frequency and Timing of Shed Events in the Timber Rattlesnake **10**

Abstract **10**

Introduction **11**

Field Site and Study Organism *16*

Tracking Data *16*

Processing Data *18*

Projection of the Mating Window *19*

Categorization by Reproductive Condition *20*

Statistical Analyses *21*

Results **23**

Discussion **27**

Conclusion **33**

Tables and Figures **35**

Literature Cited **45**

Chapter 2: The use of exogenous thyroid inhibitors and temperature manipulation to induce shedding cycles in Rattlesnakes. **51**

Abstract **51**

Introduction **52**

Methods **56**

Study Organisms and Captive Care *56*

Trial Preparation *57*

Experiment 1: Thyroid Suppression with 1-850 (reversible thyroid receptor antagonist) *57*

Experiment 2: Thyroid Suppression with MLS-000389544 (non-reversible thyroid receptor antagonist) *59*

Experiment 3: Thyroid Suppression x Gonadotropin Upregulation *59*

Experiment 4: Temperature Manipulation *60*

Statistical Analyses *61*

Results **61**

Experiment 1: *61*

Experiment 2: *61*

Experiment 3: *62*

Experiment 4: *62*

Discussion **63**

Conclusion **66**

Tables and Figures **67**

Literature Cited **72**

Chapter 3: The Metabolic Effort and Duration of Ecdysis in Timber Rattlesnakes **78**

Abstract **78**

Introduction **79**

Methods **82**

Study Organism *82*

Open Flow Respirometry *83*

Collection and Preparation of Study Animals *85*

Monitoring and Care *87*

Data Analysis *87*

Volume of CO_2 for Skin Synthesis (E_b) *88*

Volume CO2 of Physical Sloughing (E_r) *89*

Construction of the Mass Adjusted Scaling Equations *90*

Statistical Analyses *90*

Results **90**

Discussion **92**

Time Budget *92*

Mass-Metabolism Relationships *94*

Comparison to Published Estimates *96*

Comparison of Natural and Artificial Hibernation Outcomes *98*

Conclusion **98**

Acknowledgements **99**

Figures and Tables **100**

Chapter 4: Ecdysis as a Constrained Life-History Trait in Squamates: A Dynamic Energy Budget Individual Based Modelling Exercise in a Simulated Rattlesnake **114**

Abstract: **114**

Introduction **115**

General Modeling Approach **119**

Methods **119**

Model Construction *119*

Initializing Parameters *121*

Storage dynamics *121*

Characteristics of the virtualEnvironment *122*

Characteristics of eSnakes *123*

Population Scaling *136*

Modeling Exercises **137**

Sensitivity Analysis for Selection of Mean Foraging Success *137*

Impacts of Shed Frequency on Survival, Energetic Reserves, and Growth Trajectory at Static MFS *139*

Effect of Shed Frequency, Active Season Length, and Mean Foraging Success on Lifetime Energy Budgets. *141*

Effects on Female Fecundity *143*

Affects at the Population Scale with Varying Shed Frequency and Resource Environments *146*

Conclusion **148**

Tables and Figures **150**

Literature Cited **164**

Appendix: *168*

Conclusion **170**

Literature Cited *.....174*

List of Published Papers

Chapter 1

Carnes-Mason, M.D. & Beaupre, S.J. (in Review). The Frequency and Timing of Shed Events in the Timber Rattlesnake.

Preface

Broadly, I am interested in reptilian physiology and conservation. I have always loved snakes and sought to make a career out of it. I pursued graduate studies to work with a species of great interest to me, the Timber Rattlesnake. Through my work, my appreciation of snake physiology has continued to grow, and I am privileged to have spent so much time in the Ozarks pursuing my interests. Timber Rattlesnakes are truly an amazing organism and through my interest I have stumbled upon a basic question of tremendous interest that I cannot believe remained unexplored. The fact that snakes shed is something almost any child can tell you about these amazing animals, but we still know surprisingly little about the how, why, and when of ecdysis. I hope that my obsession will be of interest to other herpetologists. If you study reptiles, shedding is likely of some importance to your favorite organism, and I encourage you to explore its value to your animal (either empirically or conceptually) as you continue down your own rabbit hole.

Introduction

All reptiles engage in some degree of skin shedding (Landmann, 1986). Within reptiles, the epidermal morphology of snakes is unique (specifically the arrangement of rigid keratin filaments) necessitating the semi-frequent, synchronous renewal of the entire epidermis and facilitating the subsequent removal of the old epidermal layer as a single sheet (a sloughed skin; reviewed in Landmann, 1986; Maderson, 1965). While the frequent replacement of the largest organ of the body likely carries a significant metabolic cost and affects the behavior and physiology of the animal, shedding as a life history trait in reptiles has been largely understudied. Detailed work on the cytological events of ecdysis has been conducted (Maderson, 1965, 1984), but limited data are available on the underlying endocrinological mechanisms (Chiu et al., 1967; Chiu & Lynn, 1970a, 1970b, 1972; Chiu & Phillips, 1971; Maderson et al., 1970a, 1970b) and the importance of the process to whole animal energy budgets (Blem & Zimmerman, 1986; Smith, 1976). The majority of previous work has been carried out in captive animals or in small-scale, short-term field investigations, limiting our ability to draw conclusions or appreciate the intricacies of the process at larger scales (population or even taxonomic group). Few data are available on even simple patterns of shed in wild populations, with almost no information on the metabolic effort or even the duration of the process. Without such general information and established processes and methods for obtaining such information, addressing ultimate questions about **Why** snakes shed, what controls the process, or how the biophysical environment may shape patterns of shed across populations (or even taxonomic groups) are out of reach.

Observations of when and how often snakes shed have informed our hypotheses about *why* snakes shed since naturalists and scientists began to keep snake collections and records of behavior. Stabler (1939) recognized that there were seasonal patterns to shedding (suggesting light, temperature, and weather as triggers) and that the time between sheds varied

by species and individual. Continued observation of captive snakes led to the commonly held belief that shed frequency is strictly related to growth rate (Burkett, 1966; Collins, 1992; Fraser, 1936; Heyrend & Call, 1951; Klauber, 1972; Macartney et al., 1990; Smith, 1976), tying timing of sheds to the resource environment and the active season of reptiles (particularly in temperate climates). However, studies from the field of chemical ecology are producing a mounting body of evidence that shedding is an important component of reproductive signaling mechanisms in many snakes (Lemaster & Mason, 2003; Mason & Parker, 2010; Parker & Mason, 2012), suggesting that timing of shed events may be more tightly coupled to phenologically relevant times of year rather than strictly growth, resources, or weather. Such studies are highlighting snakes' dependence upon chemical signaling, revealing them to be highly chemosensory organisms with respect to reproduction. Since snakes lack alternate routes of chemical signaling seen in lizards (e.g., femoral pores; García-Roa et al., 2017) it seems reasonable that signaling (especially reproductive signaling) may be facilitated by ecdysis as lymph fluid and presumptive signaling molecules are exuded between epidermal layers at the culmination of an ecdytic cycle (Duvall, 1986; Maderson, 1986) and released to the environment at sloughing (Kubie et al., 1978; Mason & Parker, 2010). However, testing such hypotheses and addressing WHY snakes shed is hampered by the lack of detailed information on when snakes shed at the population scale and the relative energetic expense of the process that must be paid by individuals.

This dissertation endeavors to develop our understanding of basic patterns of shed, identify potential associated phenological events that may correlate to shed timing, and frame total effort (in energy and time) of ecdysis in a whole organism context to start to address a central, but basic, question; Why do snakes shed? While some portion of this question has been addressed by the cytological studies discussed above (they must shed to renew the integument, prevent water loss, and accommodate changing body size), the ultimate questions

of what role shedding plays in the ecology of snakes (a defining but often ignored trait of the clade) remain unanswered. To contribute to the study of this important facet of reptilian biology, I have taken a variety of approaches to address four relevant questions that I expect will inform our thinking and empower further exploration into the why of shedding.

First (Chapter 1), I used 25 years of radio telemetry and mark-recapture data to construct detailed observational records on when and how often a population of snakes sheds. Such large-scale data are almost entirely absent from the literature (with the few exceptions from Brown, 2016; Macartney et al., 1990; Martin, 2002) but such data provide important insights. By discerning the frequency of shedding in a population, we can better extrapolate the energetic and temporal effort of the process (which scales with frequency; e.g., an animal shedding three times per year pays a higher cost than one which sheds once per year). Information on the timing of the process within the year also provides clues as to the ecological utility of shed events beyond simple tissue maintenance (e.g., reproductive signaling, in response to seasonal shifts, environmental cues). I used my data to test whether shedding is strictly a growth function or may serve alternate or additional functions in the reproductive biology of Timber Rattlesnakes. Under the null hypothesis of shedding as a growth function, I assumed that shedding frequency would be tied to body size (with smaller, faster growing animals shedding more frequently than larger counterparts) and that the timing of shed events would be distributed randomly within the year (as individual snakes reached some pivotal change in body size warranting a shed). I considered non-random patterns in timing, or correlates of shed frequency that did not relate to body size to be in support of alternate explanations of shed patterns in my population.

Second (Chapter 2), I explored potential endocrinological and environmental triggers of ecdysis. The goal of these experiments was to reliably induce shed events in the laboratory in captive rattlesnakes. Success to this end would provide evidence for additional utilities of

ecdysis (e.g., shedding induced by increases in reproductive hormones suggests that reproductive events may be causally related to ecdysis) and provide a reliable method for exploration of the behavioral and physiological changes associated with ecdysis (both in the field and in the laboratory). I tested the use of synthetic thyroid receptor antagonists (thyroid hormones being inhibitory for shedding in snakes; Chiu & Lynn, 1970, 1971), augmentation of gonadotropins, and manipulations of temperature (through replicating brumation conditions followed by rapid increases to summer temperatures) for their utility in eliciting shed events. I hypothesized that increasing shed frequency (beyond that expected under random chance for a binary process) would occur for compounds or methods which were directly related to the inherent triggering mechanisms which snakes in the wild respond to. I assumed patterns of shed which occurred randomly across trials to be the null condition, indicating that internal rhythms of ecdysis were unaltered. Changes in frequency and temporal relations between manipulations and ecdysis were considered as potentially causal if proportions of animals in shed exceeded those expected under the null hypothesis.

Third (Chapter 3), I used open flow respirometry to measure the metabolic effort associated with the synthesis and sloughing of epidermal layers in snakes and used the duration of that effort to quantify the length of the process. I expected that the simultaneous replacement of the entire epidermis and the subsequent movement required to remove that skin would require a significant metabolic effort and would therefore cause a measurable increase in carbon dioxide production in snakes in shed. Furthermore, I anticipated that the total effort of biosynthesis and skin removal would both scale with body mass (assuming the total amount of epidermal tissue to increase with mass in a manner similar to the way that surface area scales with the volume of an object) and that the duration of increased CO_2 production would be indicative of the true duration of the entire shed process. I measured the effort of biosynthesis

and skin removal in nine Timber Rattlesnakes with a range of body sizes and report a scaling equation that allows for the prediction of the metabolic effort of shed given body mass.

Finally, I used a dynamic energy budget individual-based model to integrate the information garnered from the above approaches (Chapter 4). I parameterized a population of simulated Timber Rattlesnakes and modeled the metabolic and temporal effort of ecdysis under varying imposed shed frequencies. I modeled several scenarios, and used the output of my model to inform my understanding of what constraints may govern natural patterns of shed in snakes. Specifically, I tested whether limits in finite time and energy explained the observed patterns of shed in a population and used reproductive output as a metric of fitness. I expected populations parameterized with natural patterns of ecdysis to have the highest survival and fecundity. I interpreted increased fitness or survival that occurred at alternate shed frequencies (e.g., higher or lower than those observed in nature) as indicative of the existence of additional fitness costs or benefits that exist in real populations (thereby constraining shed frequencies to those observed in the wild) not captured by my energy-explicit model of shedding (i.e., shed events may have benefits beyond maintenance, and costs which exceed strict time and energy investments). I tested the null hypothesis of shed frequency as a growth function (expecting observed frequencies of shed to not limit growth rates and reproduction) and used the output of the model to inform my thinking about the forces which may select for observed patterns of shed as a component of a snake's life history.

My data indicate that ecdysis is more complex than the oft-assumed growth function that is predominate in the literature. My multi-disciplinary approach shows that ecdysis is intricately interrelated to the time- and mass-energy-budgets of snakes and offers strong support for additional or alternate roles for shedding (especially reproductive functions) in snake ecology and behavior. While the specific aim of the studies reported here was to test our preconceived notions of the causation and function of ecdysis in a single snake species, my results are likely

broadly applicable to the general biology of all squamate reptiles. Patterns of shed are a central component of the life-history strategy of reptiles, and continued research may reveal a new way in which selective pressures and adaptation shape the unique biology of various reptilian clades.

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Chapter 1: The Frequency and Timing of Shed Events in the Timber Rattlesnake

Abstract

The frequency and timing of shedding in snakes is often assumed to be a growth function. As snakes eat and grow, they are forced to shed to accommodate the increase in body size, relating the resource environment to rates of growth and patterns of shed. Alternatively, it has been suggested that shedding occurs in response to changing abiotic conditions or as a mechanism of semiochemical signaling. Resolution of these opposing hypotheses requires a working knowledge of when and how often snakes shed. To date, descriptions of patterns of the timing and frequency of shed events have been limited to laboratory studies and field observations of limited scope. I used 25 years of radiotelemetry observations of Timber Rattlesnakes in shed to describe the patterns of frequency and timing in a population of snakes. I found that the frequency of shed events was approximately even between males and females, but that while male frequency was related to the resource environment, female frequency was correlated to reproductive condition. Further evaluation revealed that the timing of shed events in lower body condition males was randomly distributed throughout the year, timing in higher body condition males clustered around the mating season, and timing in females related to reproductive condition with gravid females shedding early in the year and non-gravid females shedding close to the peak in male-female courtship. My study suggests that shedding patterns in males are coupled to the resource environment and offer support for shedding as a growth function, but in females shedding may be more tightly coupled to reproduction, serving alternate or additional roles in adult female snakes.

Introduction

Ecdysis is a fundamental aspect of reptilian life history. All reptiles engage in some degree of skin shedding and replacement (Landmann, 1986) but there is variation among groups. The whole-body, synchronous skin shedding observed in snakes has drawn the attention of researchers since the 1930's (Benedict, 1932; Lange, 1931; Schaefer, 1933; Stabler, 1939). Subsequent research has elucidated the underlying histological (Bechtel, 1957; Maderson, 1965) and endocrinological (Chiu et al., 1983; Chiu & Lynn, 1970; Schaefer, 1933; Yau, 1992) mechanisms which govern shed events. However, debate still exists about which biotic or abiotic factors may induce individual animals to undergo a shed event. It has been suggested that shed events occur in response to: growth (Heyrend & Call, 1951; Smith, 1976); a change in permeability, water loss, and humidity (Carlson et al., 2014; Maderson, 1985, 1984; Maderson et al., 1982); an injury or general wear and tear to the existing skin (Smith & Barker, 1988); temperature (Alexander & Brooks, 1999; Gibson et al., 1989; Maderson, 1984); signals from conspecifics (Lillywhite & Sheehy, 2016); (Heyrend & Call, 1951; Smith, 1976) or reproductive events (Aldridge & Brown, 1995a; Crews & Garstka, 1982; Kubie et al., 1978; Nilson, 1980; Schuett, 1992). The conflicting lines of evidence found in the literature suggest that ecdysis may be a more complex phenomenon in reptiles than is generally assumed. Discerning the mechanisms that drive shedding cycles may require a more complete understanding of the importance of ecdysis at the individual and population levels. The frequency at which a species sheds and the timing within a year of those shed events are two basic characteristics that determine the proportion of available energy devoted towards maintenance and upkeep of the dermis, and the utility of shed events in the animals' ecology (e.g., chemical signaling, seasonal responses to water loss, activity budget "decision" making). Framing shed events in a broader ecological context, and ultimately determining their function and causation, requires a robust understanding of the basic patterns of shed frequency and timing and their variation within populations.

Shed events are an obligate expenditure for snakes, requiring resources in the form of time and energy (Beaupre & Duvall, 1998). Here I define "effort" as a proportion of an organism's total energy budget (in kJ) and "cost" as an impact on an individual's lifetime fitness (in number of offspring) (following Hirshfield & Tinkle, 1975). The simultaneous replacement of the largest organ in the body requires significant metabolic effort (Smith, 1976; Taylor & Davies, 1981) occurring at least once per year, but as often as every 18 days (~20 times per year) (Maderson et al., 1970). Blem and Zimmerman (1986) used bomb calorimetry of shed skins to estimate the energetic content of the sloughed tissue. They found that the dry mass of a shed skin as a function of body mass was:

$$\text{skin mass} = 0.6117 + 0.0029 * \text{Body Mass (g)} \qquad \text{EQ. 1}$$

with the energetic density of skin ranging between 19.9-25.1 kJ/g. The derived values suggest that a 500g animal will slough approximately 50kJ of dead skin and replace this shed skin with approximately the same amount (plus additional tissue reflective of any growth that occurred between sheds) of new tissue per shed event. As noted above, this calculation approximates the energetic content of the shed skin but does not include the total effort associated with the skin's production. The true effort associated with a single shed event is certainly higher than Blem and Zimmerman's estimates. The total effort (in kJ) of a single shed event includes the energetic content of skin, the metabolic expenditure required for tissue synthesis, activity related effort associated with the physical removal of the shed skin (i.e., increased movement and rubbing against surfaces to peel the old skin away), and possible increases in resting metabolic rate resulting from animals seeking out warmer microhabitats (Gibson et al., 1989). In addition to effort, there are implicit fitness costs associated with a shed event as animals modify their behavior and time expenditure to accommodate the temporary change in physiology. Snakes in shed often seek out shelter (Hirth et al., 1969), exploit alternative thermal microclimates (Gibson et al., 1989; Kitchell, 1969), and decrease movement and foraging rates (Bellairs, 1969; Parker,

1963; Turmo, 1996) all of which may decrease the time available for, and ultimately decrease their chances of, reproduction, incurring a cost in the form of total offspring production. Changes to behavior during the shed cycle are often attributed to an increase in vulnerability to predation (King & Turmo, 1997) as the eyes become opaque; potentially making refuge seeking an adaptive strategy to limit cost through the preservation of future reproductive value. The concurrent reduction in energy expenditure that results from decreased locomotion may also serve as a mechanism for conserving energy; allowing refuge seeking to serve an additional function as a strategy to limit effort in response to the increased metabolic expenditure of skin synthesis (Blem & Zimmerman, 1986). Clearly, shed events are an important piece of the time-energy budget of snakes having both immediate energetic consequences and potentially impacting an individual's fitness. However, without a clear understanding of how often snakes shed, it is difficult to assess the magnitude of the metabolic effort or the degree to which ecdysis may impact fitness and energetic status by limiting the time available for other behaviors.

In addition to energetic and time constraints, there is growing evidence that shedding plays an important role in the reproductive ecology and social interactions of some species. Studies of *Vipera berus* have reported a correlation between shed timing and reproductive cycles (Andren, 1982) and courtship and gametogenesis (Nilson, 1980). Schuett (1992) noted similar patterns in temperate, North American pit vipers, suggesting that the summer mate searching pattern of males evolved in response to female patterns of vitellogenesis, shedding, and chemical signaling; an argument that has since been supported by Aldridge & Brown (1995) in *Crotalus horridus*. Other work in *Thamnophis sp*. has demonstrated that the act of shedding serves as a mechanism for the release of pheromones to attract mates (Crews, 1991; Crews & Garstka, 1982; Kubie et al., 1978). In these instances, it seems that the timing of the shed event is crucial and serves to fulfill some imperative social function. A better understanding of

the timing of shed events and how they vary among individuals, reproductive classes, years, and populations may improve our understanding of snake reproductive ecology.

Most of our knowledge about shedding patterns is derived from laboratory studies and small-scale field observations because shed events are difficult to observe in the wild. While the entire process of skin synthesis and sloughing can take between 2 and 3 weeks (Maderson, 1984; Maderson et al., 1970), visual signals (i.e., bluing of the eyes, dull skin) of an impending shed are limited to around five days (Cazalot et al., 2015; Tusler et al., 2015). Reliably categorizing an animal in the wild as "in shed" depends on the observation of these external visual cues during this brief window of time, making records from the field rare. Shedding animals also modify their behavior during this period, seeking refuge under cover objects and in burrows (Hirth et al., 1969; Klauber, 1972); further decreasing the probability of observing an animal in a state of ecdysis in the wild. The difficulty of observing a shedding snake in the field has led researchers to rely on aggregations of shedding individuals such as at den sites in the spring (Parker & Anderson, 2007), seasonally attractive thermoregulatory locations in mid-summer (Ashton, 1999), locations frequented by reproductive animals (Nilson, 1980), and social gatherings of unknown importance (Lillywhite & Sheehy, 2016). While these studies have advanced our understanding of shed dynamics in snakes, they focus on specific ecdysis events and are unable to capture shedding events occurring at other times of the year or in other areas within an animal's range. Collection of a long-term data set with frequent observations of individuals is the only approach suited to addressing this gap in our understanding of shed patterns. Because observing an animal in the wild in shed is relatively rare, patterns of frequency and timing of shed events can only emerge with sufficient data. A long-term approach is necessary (Tinkle, 1979) to better understand the intricacies of this characteristic reptilian process.

I used 25 years of radio telemetry and morphometric data collected in a population of Timber Rattlesnakes (*Crotalus horridus*) in Northwest Arkansas to describe the shedding patterns of a wild population of snakes. The unique morphology of rattlesnakes (genus *Crotalus*) makes them ideal for studies of shed frequency and timing for two reasons. First, with each shed, rattlesnakes add a segment to the base of their rattle (Zimmermann & Pope, 1948), providing an attached record of at least some portion of the animal's shed history. The rattle serves as a living record, allowing field researchers to determine if a period of refuge-seeking is associated with a shed event or some other need and provides a history of shed events between long periods without observation (i.e., winter). Second, as large-bodied snakes, rattlesnakes are well suited to radio telemetry studies (Beaupre & Duvall, 1998), allowing for repeated observations of individuals before, during, and after shed events. I analyzed a combination of field radio tracking data and laboratory processing data to investigate the yearly patterns in shed frequency and timing in Timber Rattlesnakes. Here I detail the patterns that emerged and provide evidence for relationships between body condition and shed frequency and between reproductive condition and frequency and timing of shed events.

Methods

I used 25 years of observational data from a population of Timber Rattlesnakes (*Crotalus horridus*) in Northwest Arkansas (Beaupre, 2008; Beaupre & Douglas, 2010; Beaupre & Zaidan, 2001; Gardner-Santana & Beaupre, 2009; Lind et al., 2016; Lind & Beaupre, 2014, 2015; Van Dyke et al., 2012; Van Dyke & Beaupre, 2011; Wills & Beaupre, 2000; Zaidan & Beaupre, 2003) to detail the large-scale patterns of shed frequency and timing in the wild. My data set consisted of two separate types of data; 1) Repeated observations of radio-tagged individuals in the field (Tracking Data; TD), and 2) Morphometric data collected in the laboratory from animals captured at spring emergence, during summer activity, and during the late-summer mating season (Processing Data; PD).

Field Site and Study Organism

The field site consists of ~15,000 acres of nearly contiguous woodland managed and protected by the Arkansas Game and Fish Commission (AGFC) and Arkansas Natural Heritage Commission (ANHC), with the long-term study population residing in a smaller (~2,000 acres) subset of the total area. The habitat is a mix of open and encroached woodland (Hickory, Oak, and Pine), limestone bluffs, pockets of xeric limestone prairies, and intermittent and permanent streams (Marbut, 1914). Human activity within the site is limited to a handful of roads and trails and is primarily used in the spring and fall for hunting (Arkansas Game and Fish Commission, 2021).

Timber rattlesnakes are a large-bodied, reptilian top-predator in the Ozark Highlands ecoregion. As ectotherms, the active season of Timber rattlesnakes in Arkansas is limited to mid-April to late October by cooler winter temperatures. *C. horridus* seek refuge throughout the winter in the many limestone bluffs present in the study area and emerge in early April. After emergence, snakes disperse to forage throughout the area until reproductive activities begin in late-July/early-August. During reproduction, males make long, ranged movements seeking females, and females sit and wait for males ("Prolonged Mate Searching Polygyny": Duvall et al., 1992). The population studied here has been observed throughout every active season with minimal interference or human impact since the project's inception (Autumn, 1995).

Tracking Data

A subset of the population has been continuously monitored via radiotelemetry since 1995. Healthy adult animals (weighing more than 250 g) were outfitted intraperitoneally (Reinert & Cundall, 1982) with a radio transmitter (model SI-2T, Holohil Systems Ltd., Carp, Ontario, Canada) and tracked ~1-2 times per week for the lifetime of the transmitter (24 months). At the end of a transmitter's lifetime, tagged snakes were either retagged or the tag was removed depending on condition of the animal (Beaupre, 2008; Gardner-Santana & Beaupre, 2009; Wills

& Beaupre, 2000). The dataset analyzed here consists of 10,530 observations of 175 individuals. Each observation corresponds to a location event in the field and includes date, time, GPS location, body temperature, habitat descriptors, behavior category (one of eight categories: (Beaupre, 2008)), and notes about the condition or behavior of the animal. I used mass (g) and SVL (snout vent length; cm) data collected in the lab (processing data) at the nearest date prior to each field observation to calculate a body condition estimate (as; $\frac{Log(Mass)}{Log(SVL)}$) for that radio-tagged animal at the time of the observation. For the analyses reported here, only observations where an individual's behavior was categorized as "Ecdysis", or the notes of an animal's behavior include reference to bluing of the eyes or basal rattle segment were included (547 observations across 97 individuals). Since shed events typically last several weeks (Maderson, 1985; Maderson et al., 1970) individuals were often categorized as "in shed" at multiple successive relocations. For this study, I was only interested in unique shed events, so I identified the earliest observation of a particular shed event and discarded all subsequent observations occurring within 4 weeks of that observation for that individual. By discarding subsequent observations of the same snake after their initial categorization as "in shed", I avoided categorizing repeated observations of an individual as separate shed events, preventing an overestimation of shed frequency. Any observation of an animal undergoing ecdysis occurring >4 weeks from the onset of the last shed was considered a separate shed event. It should be noted that a consequence of this approach is that date ranges of shed events reported here are for the first observation of an animal entering shed and not the date range of the sloughing of the skin (which often occurs between 5 and 10 days after the bluing of the eyes; Carnes-Mason personal observation). I recorded 232 unique shed events among 93 individuals (1-9 observations per individual) between 1995 and 2020.

Processing Data

Over the course of the study, animals were collected during spring emergence from hibernation (a period of elevated density at known locations), in association with radio-tagged animals during the late-summer mating season (a period of high social interaction between individuals), opportunistically during radiotelemetry relocation efforts, or through directed habitat searches. Once collected, animals were transported to the laboratory where morphometric measurements could be taken safely (following Beaupre & Greene, 2012), reproductive condition could be assessed (all individuals probed for sex; adult females screened for developing follicles/embryos via manual palpation and/or ultrasound), and newly captured individuals were marked with a unique ID via a subdermal PIT (passive integrated transponder) tag. As part of this semi-annual processing, paint was applied to the three rattle segments closest to the tail (excluding the live basal segment) following a color code corresponding to the last three digits of an individual's unique 9-digit PIT number; allowing for the easy identification of known animals in the field. Rattle painting also allows for an accurate count of shed events that occur between two subsequent capture events as a new segment is added at each shed between the base of the tail and the painted rattle segments. For the analyses presented here, I used the dates, number of days, and number of sheds (as determined by the paint method described above) between subsequent captures to estimate a shed frequency in number of sheds per active season. I included any record where the time between capture was greater than one year and calculated the shed frequency for those observations as;

$$\text{Sheds per Active Season} = \frac{S}{\left(\frac{D}{365}\right)} \qquad \text{EQ. 2}$$

Where S = the number of sheds observed between capture events and D = the number of days between capture events. I assumed that all observations that occurred in time intervals of less than one year were representative of a single active season (rather than $\frac{D}{365}$ active seasons)

and only included observations where that assumption could be met with reasonable certainty. The more simplistic approach for shorter interval observations (<1 year) was used to avoid inflation of shed frequency estimates caused by extrapolating values from fractional active seasons via equation 2 (a single shed observed in a 20-day interval gives an estimate of 18.25 sheds per year, a nonsensical estimate for these animals). Observations that spanned between two active seasons (i.e., October 2004 to May 2005) were discarded unless a single shed event occurred during that time that could be attributed to one year or the other (multiple events could not be attributed to a single active season). Observations where zero sheds were observed were discarded because the tracking data only includes observations of animals in shed (variable relocation intervals prevent the assumption that all shed events were observed; non-shed observations were discarded), making their inclusion in the processing data a source of error in any comparison between the two.

Projection of the Mating Window

To contextualize shed events in relation to reproductive activities, I wanted to compare the timing of sheds to the late summer window when mating behaviors are most frequently observed. From the tracking data, I compiled any observation of a snake whose behavior was classified as "reproductive" and was noted to be either; 1) in close association (<5m apart) with conspecifics in the late summer, 2) actively engaging in male-female courtship (body bumping, chin rubbing, active pursuit, coiled in physical contact, or actively copulating; Hayes, 1986), or 3) competing for access to a female (male-male combat). Since gravid females were often classified as "reproductive" at the majority of relocations during their reproductive bout, these observations were discarded as "passive reproduction" (i.e., a gravid female not actively mating, courting, gestating, or in parturition but instead engaging in other non-reproductive behaviors such as foraging or thermoregulating while pregnant) unless they were observed in courtship or close association with a conspecific. Using the observations of snakes engaged in mating and

courtship (N= 91), I constructed a histogram (observations binned by week) to visualize the distribution of mating activities within the active season. I calculated a mean window of courtship (following a BCa Bootstrapping procedure; Carpenter & Bithell, 2000) and used that range of dates for comparisons of dates of shed to the "peak" of courtship season.

Categorization by Reproductive Condition

The aim of this study is to describe patterns in shed frequency and timing and test whether energetic status of individuals or the timing of reproductive events explain the observed patterns. Female Timber Rattlesnakes have a complex reproductive cycle; individuals must develop follicles (primary vitellogenesis) in one season, successfully overwinter, and then, in the following season, yolk follicles (Secondary vitellogenesis) in the spring before gestation and parturition in the summer and autumn (Aldridge & Duvall, 2002). Female Timber Rattlesnakes in Northwest Arkansas do not appear to reproduce more frequently than once every two years, likely because of the temporal limitations imposed by their patterns of folliculogenesis. In many populations (particularly in the northern portions of their range) active season length (Beaupre & Duvall, 1998; Brown, 1991; Martin, 2002) and resource abundance (Beaupre, 2002) further limit reproductive frequency, as individuals may take multiple seasons to replenish energetic stores in preparation for the production of their next litter. Using my metric of BCI (discussed above) gravid females are often in disproportionately elevated body condition when compared to the rest of the population while post parturient females are often in the worst body condition (Brown, 1991; Duvall et al., 1992; Graves & Duvall, 1993; Lind et al., 2016; Schuett, 1992). Due to the relationship between energetic status and reproductive cycles as well as the many discrete reproductive categories which may impact a female's participation in reproductive activities (Duvall et al., 1992), I classified females by reproductive condition and considered them separately for some analyses. I used evidence from both data sets (tracking and processing) to categorize females by reproductive category based on observations of litter production,

ultrasound imaging of follicular development, and palpation of follicles or embryos. Animals were classified as gravid or non-gravid in every year that they were observed in the tracking data. Within the non-gravid category, females could also be classified as reproductive (possessing either primary or secondary follicles) or non-reproductive (lacking primary or secondary follicles). I also classified any snake observed as gravid in one year as "postpartum" in the following year, although it should be noted that postpartum individuals could also be classified as reproductive or non-reproductive, these categories were not mutually exclusive. In some instances, females would fall into a different reproductive category in a late summer processing event compared to a spring processing event (i.e., non-reproductive in April, reproductive in August), I assumed that the more advanced reproductive condition (gravid > reproductive > non-reproductive) was the most accurate description for that animal in that year. Among adult males, reproductive activities are frequently annual (except for snakes in exceedingly poor body condition; Beaupre, 2008; Lind & Beaupre, 2015) and consist of mate searching, mate guarding, and male-male combat (Beaupre & Duvall, 1998). In Rattlesnakes, body condition is a good measure of reproductive condition in males, as larger, heavier (higher BCI) individuals tend to win combat (Gibbons, 1972; Schuett, 1992) and presumably experience an increased access to females (Duvall et al., 1992). I did not attempt to further categorize males by reproductive condition as I did with females and instead differentiated males by BCI in relevant analyses (assuming BCI to be a correlate of reproductive success).

Statistical Analyses

I assumed a 5% type I error for all statistical tests. All statistical analyses were conducted in R 4.0.2 (R Core Team, 2020). I separated shed observations by sex because *C. horridus* is known to be sexually dimorphic in body size (Gibbons, 1972; Klauber, 1972) and because patterns in shed frequency and timing emerged which varied between the sexes. When appropriate, I further separated observations within sex by reproductive condition

(females; gravid, non-gravid, reproductive, non-reproductive, postpartum) and shed frequency (1 shed per year or 2 sheds per year). I tested the distributions of all groups for normality (following Shapiro & Wilk, 1965; Table 1) and tested distributions of residuals for normality for relevant analyses (those employing regression); residuals from all analyses were approximately normally distributed so I employed parametric statistics throughout. I was interested in 1) differences in shed frequency and timing between groups (males vs. females; females in varying reproductive conditions) and 2) the effects of body condition index (BCI) on shed frequency and timing within groups. I used t-tests to test for differences in mean shed frequency between males and females. Chi-square contingency table analyses (Zar, 2010) were used to test for differences in shed frequency between females in different reproductive conditions. I took a linear mixed effects models (function "lmer", package "lmerTest") approach, coupled with a one-way analysis of variance (ANOVA) with random effects (using individual as a random factor) to test for a difference in shed timing between groups (males vs. females, females in varying reproductive condition, 1x shedders vs. 2x shedders) and effects of BCI on shed timing within groups. My mixed effects models account for the repeated sampling of individuals over time in the data set by including individual ID as a random factor to ensure that significant relationships were the result of the independent variable of interest and not individual variation (i.e., some individuals naturally shed more or earlier than others). Binary logistic regressions (McDonald, 2014, pgs 238-246) were used to test for an effect of body condition on shed frequency within groups. Randomization tests (Manly, 2007; distributions of 5000 simulated means) were used to compare the average date of shed events within groups (sex, reproductive condition, single and double shedders) to the average date of observed courtship events. Means are presented as mean ± 95% Confidence Interval for normally distributed data. Confidence intervals were estimated using a BCa bootstrapping approach for subsets of the population that did not follow a normal distribution (Carpenter & Bithell, 2000).

Results

From the tracking data set, I isolated 244 unique shed events from 94 individuals (207 individual-years) to estimate shedding frequency as average shed events per year. Males shed an average of 1.18 ± 0.075 (N=100) times per year, females shed an average of 1.13 ± 0.064 (N=107) times per year (**Error! Reference source not found.**; TD: All Animals). Average shed frequency did not differ significantly between males and females (Welch Two Sample t-test; t=0.97, df=197, p-value >0.3). All observed animals shed either once or twice per year. A single male was observed to shed three times in 2014, but the time between the first two events in that year were exactly four weeks apart (the cut-off for distinguishing unique shed events) and the second event lacks clear notes about the condition of the animal. In two other instances processing records (Processing Data) suggest males that shed three times in a single year, but these observations could not be attributed to a single season with certainty. Because of the uncertain nature of the observations of males who shed three times per year, they were removed from the remaining analyses as outliers, although it is possible males may shed three times per year infrequently (three individual-years out of 207) in this population.

To create a second estimate of shed frequency (sheds per active season) to validate the estimate generated from the tracking data, I used 1,065 observations of 507 individuals captured in the field and processed in the laboratory (Processing Data). I estimated average sheds per active season for all processed animals, only processed animals implanted with radio transmitters, and only processed animals that were not implanted with radio transmitters (Figure 1). Among all processed animals I observed an average of 1.165 ± 0.070 (N=128) or 1.10 ± 0.068 (N=171) sheds per year in males and females respectively. Shed frequency per active season estimates produced from processing data did not differ between sexes within groups (all animals: t=1.31, df=288, p>0.1), radio-tagged animals (t=1.34, df=232, p>0.1), non-tagged animals (t=0.38, df=47, p>0.70)) or within sexes between groups (two sample t-tests, p-values

all > 0.85). Estimates of shed frequency produced from the tracking data did not differ significantly from processing data estimates when compared within sexes between groups (two sample t-tests, p-values all > 0.45) (Figure 1; Table 2).

The radio telemetry data and my estimates of shed frequency indicate that most animals shed once per year, with only a fraction of animals shedding twice. To estimate the timing (by Julian Day) of shed events I categorized all individuals (Tracking data) in each year they were observed as having either one or two sheds in that year. I categorized distinct shed events based on number of sheds observed for that individual in that year and which shed event it was (1^{st} or 2^{nd}). I cross-referenced observations in the field (tracking data) against rattle counts between successive capture events (processing data) to improve the accuracy of my categorizations. In some instances (n=19 for males, n=11 for females) only a single shed event was observed in the field that year, but the processing data revealed an additional shed event earlier or later in the season that was not observed during radio-telemetry efforts (Processing-Corrected Tracking Data yielded 232 unique shed events from 93 individuals (199 individual-years); Figure 2). I used the processing data-corrected categorizations of tracking data shed events (1 or 2 sheds per year) for all remaining analyses (below). All events fell into one of three categories; 1) The only shed of an animal who shed once (1 of 1), 2) The first shed of an animal who shed twice (1 of 2), and 3) The second shed of an animal who shed twice (2 of 2) (Table 1). I found that 1 of 1 Males shed significantly (>8 days by 95%CI) later than the first shed of 2 shed males (1 of 1 vs. 1 of 2: 1-way anova with random effects ($F=10.5$, $df=75$, $p < 0.001$)) with no effect of individual ($p> 0.3$) but significantly (>15 days by 95%CI) earlier than the second shed of 2 shed males (1 of 1 vs. 2 of 2: 1-way anova with random effects ($F= 20.2$, $df=83$, $p < 0.0001$)) with no effect of individual ($p > 0.7$) (Figure 3). Comparison of the timing of shed events revealed a similar pattern in Females; single shedding animals shed significantly (>26 days by 95%CI) later than the first shed of double shedding animals (1 of 1 vs. 1 of 2: 1-

way anova ($F=26.3$, $df=94$, $p < 0.0001$) with no effect of individual ($p > 0.2$)) and significantly (>12 days by 95%CI) earlier than the second shed of double shedding animals (1 of 1 vs. 2 of 2: 1-way anova ($F=14.2$, $df=100$, $p < 0.0005$) with no effect of individual ($p > 0.05$). The timing of shed events did not differ by shed category between sexes (Males vs. Females: 1-way anova (1of1: $F=0.6$, $df= 130$, $p > 0.4$); (1of2: $F=1.9$, $df= 40$, $p > 0.1$); (2of2: $F=0.3$, $df= 56$, $p > 0.5$) with no effect of individual in any case (p-values > 0.2)).

I used a chi-square contingency table analysis to determine if females in different reproductive conditions shed with different frequencies. In a comparison of Gravid, Reproductive, and Non-Reproductive females who shed either once or twice per year the chi-square test statistic was significant ($x^2 = 12.5$, $df=2$, $p < 0.005$) indicating that there is a difference between shed frequency in females of differing reproductive conditions. Comparison of relative contributions to the chi-square statistic revealed that the lack of gravid female 2x shedders was the primary driver of the significant relationship. Comparisons of only Reproductive, Non-reproductive, and postpartum individuals by a similar chi-square contingency table revealed no significant difference ($x^2=0.87$, $df=2$, $p > 0.6$). The data suggest that gravid females shed less frequently than females in any other condition.

I used binary logistic regression to assess whether body condition was a predictor of likelihood of an animal to shed once or twice per year. Body Condition Index was a highly significant predictor of shed frequency in males (binary logistic regression: $n=92$, $x^2 = 10.14$, $p < 0.001$)(Figure 4) but not females (binary logistic regression: $n=103$, $x^2= 1.45$, $p > 0.2$)(Figure 5; panel D), regardless of reproductive condition (binary logistic regressions: reproductive ($n= 20$, $x^2= 0.001$, $p > 0.9$), non-reproductive ($n=40$, $x^2= 0.002$, $p > 0.9$), postpartum ($n= 14$, $x^2= 0.87$, $p > 0.3$)(Figure 5; panels A - C).

I used linear mixed effects models and 1-way analysis of variance with random effects to investigate whether the timing of shed events is related to sex, reproductive condition, or body

condition. I compared the date of shed events in single shedding animals to determine if there was a difference in the timing of shed events between females in varying reproductive condition. I found that reproductive condition was a significant predictor of the timing of single shed events (anova, $F=6.3$, $p<0.001$) while individual was not significant as a random effect ($p>0.4$)(Table 1; Figure 6). A tukey post-hoc analysis revealed that the primary drivers for this significant relationship were the differences between gravid and reproductive females ($p < 0.05$) and gravid and non-reproductive females ($p<0.001$), but not postpartum females ($p > 0.1$) (Figure 6). I also compared the timing of the 2nd shed in 2 of 2 individuals (males, non-gravid, non-reproductive, reproductive, and postpartum females) and found that the timing of the 2nd shed event did not differ by group ($F=0.32$, $df=4$, $p > 0.8$) (Figure 7). Finally, I tested for an effect of body condition on shed timing in 1 of 1 animals. Body condition did not predict the timing of shed events in any group (1-way Anova with random effects; Males ($F=0.74$, $df=36.5$, $p > 0.3$), Reproductive ($F=0.14$, $df=11$, $p > 0.7$), Non-Reproductive ($F-1.8$, $df=22.5$, $p > 0.1$); 1-way anova (no repeated individuals); Postpartum ($F=3.3$, $df=5$, $p > 0.1$), Gravid ($F=0.06$, $df=14$, $p > 0.8$)) and individual ID did not influence the relationships.

I generated a histogram of observations of *C. horridus* engaging in reproductive behaviors (N=91) (Figure 2; bottom panel). The average Julian date of reproductive activity was 231.09 (non-normal distribution (Shapiro wilks', $p < 0.01$); 95% CI by BCa bootstrap: 228-234, approximately August 16-22). Shed events in 1 of 1 and 1 of 2 groups, regardless of sex or reproductive condition, occurred significantly earlier than the average date of courtship (Randomization tests; p-values all < 0.001). The second shed of all two shedding animals of either sex or reproductive category occurred at the same time as the average date of courtship (Randomization tests; p-values: males > 0.4; Non-Reproductive Females > 0.8; Post-partum Females > 0.1; see Table 1 for average calendar dates of shed) except reproductive females

who shed prior to the mean date of courtship (mean julian date = 214 (2 August), ~ 2 weeks prior to the average courtship date; Randomization Test: p-value < 0.01)(Figure 3; Figure 7).

Discussion

Among snake biologists, the common perception is that shed frequency is related to growth rate, and growth rate is tied to resource availability (Burkett, 1966; Collins, 1992; Fraser, 1936; Heyrend & Call, 1951; Klauber, 1972; Macartney et al., 1990; Smith, 1976). A logical extension to the proposed relationship is that as animals increase in body size, growth rates decrease (Beaupre et al., 1998); thereby decreasing the frequency of shed events. Furthermore, if growth rate, body size, and resource availability are the only factors influencing shedding we would expect the timing of shed events to be distributed randomly within the year, whenever individuals reach a threshold value of change in body size that warrants a shed event. Even though no such threshold of change in body size has been reported (Reiserer, 2016), adult snakes are often observed to have shed frequencies more tightly coupled to seasonal events (Stabler, 1939), and shed events have utility in the chemical signaling and reproductive ecology of snakes (Lemaster & Mason, 2002; Mason & Parker, 2010; Parker & Mason, 2012), the association between growth and shedding remains a pervasive, but perhaps incomplete, explanation for observed patterns of shed. The data presented here are the first to report long-term patterns of both the timing and frequency of shed events at a population scale. The patterns that emerged do not support the dogmatic view of shedding as strictly a growth function in all adult snakes. Differences in the timing and frequency of shed events between males and females in varying reproductive condition, cannot all be explained by the null hypotheses that shedding should be 1.) less frequent in larger (and therefore slower growing) animals, and 2.) randomly distributed throughout the year. Instead, my data offer support for a more complex arrangement of sex-specific drivers which include energetic condition, season, and reproduction.

In my population of Timber Rattlesnakes, all animals shed either once or twice per year (with the few possible 3x exceptions discussed above) and average shed frequency does not differ significantly between the sexes (Figure 1). I used multiple approaches to calculate shed frequency and found that the analysis of tracking data produced a similar estimate of shed frequency in both males and females when compared to processing data of; 1) all; 2) radio-tagged only, and 3) non-tagged animals. The agreement of shed frequency estimates derived from tracking data and processing data (regardless of radio-implant status) suggests that although radio telemetry may influence the behavior of an animal (Withey et al., 2001) and skin injury (such as radio-implant surgery) can result in a shed event (Smith & Barker, 1988), surgical intervention and regular observation did not affect the broad patterns in shed frequency in my tracking data sample.

In adult male Timber rattlesnakes in my population, shedding frequency (mean = 1.18 ± 0.075) is correlated to body condition (a metric of relative resource availability and foraging success (Aubret et al., 2002; Beaupre, 2008; Beaupre & Douglas, 2009; Bonnet & Naulleau, 1995; Lind & Beaupre, 2015; Naulleau & Bonnet, 1996)). Males in lower body condition (with fewer available energy stores) are more likely to shed only once per active season with the timing of these single shed events assuming a random distribution. Higher BCI males may shed two times per season, but their shed events are more tightly clustered, occurring post hibernation in May or June and during reproductive events in late-July or August. My findings offer support for the null hypotheses discussed above; animals with fewer energetic reserves (and presumably lower growth rates) shed less frequently than their well-fed (faster growing) counterparts (growth rate-feeding relationships; Beaupre, 2008). Patterns in timing of single shedding males also support the null, occurring randomly within the active season, while the distribution of the timing of shed events in multiply shedding males is better described as bimodal. The bimodal distribution of shed timing seen in higher BCI males is likely an artifact of

the temporal cost of ecdysis; given the limited active season window coupled with the ~3 weeks it takes to shed, the random timing expected under the growth-based model may manifest as approximately bimodal as random events are constrained to two broad time periods separated by 1 month. A truly non-random distribution of timing of sheds may be of interest, particularly if the association with the courtship window is causal, but without directed experimentation to test this alternative hypothesis I am unable to reject the null hypothesis of random, resource-driven timing in 2x males (i.e., random but bimodally constrained). Regardless of the determinant underpinnings of the distributions, double shedding males shed earlier in the year than single shedding males, suggesting that some external factor(s) present at the beginning of the active season serves to induce earlier shedding in individuals who will shed multiply. An omnipresent abiotic factor (i.e., light, temperature) seems an unlikely explanation because different individuals are effected differently. It seems more plausible that my metric of body condition fails to capture some aspect of the resource environment which varies between individuals such as the date of first prey capture within the year (i.e., animals who eat earlier shed earlier). Under this explanation, we might imagine that energetic condition and the timing of energy acquisition interact to dictate when and how often in a year an animal can shed, with the added caveat that any animal (regardless of energetic status) that sheds too late in the spring may not have enough time before the end of the active season to shed again (a notion supported by elevated shed frequencies in *C. horridus* at more southern latitudes with longer active seasons; Brown, 1991; Martin et al., 2021). At present I lack sufficient data to test this hypothesis, but data on the timing of spring resource acquisition by individuals should be collected in future long-term studies of snake populations to address this possibility. Overall, my data show some support for the null model of growth driven shedding in males, but the relationship may be more nuanced with energetic status and resource acquisition, rather than strictly change in size, underpinning the interactions.

Patterns of shed timing and frequency in adult female Timber rattlesnakes are not dependent upon body condition and the resource environment as they are in males, even though average shed frequency (mean = 1.13 ± 0.064) does not differ significantly. My data demonstrate a strong relationship between female reproductive condition and both the frequency and timing of shed events. Gravid females were only observed to shed once per active season, with the timing of that single shed event occurring significantly earlier than their non-gravid counterparts (Figure 6). Earlier, infrequent shedding in gravid females has been suggested to occur in *C. horridus* (Martin et al., 2021) and been reported in a related species (*Crotalus oreganus*; Macartney et al., 1990); my observation offers support for a potential (so far untested) generality among the Rattlesnakes that would benefit from continued collection of shed data in additional study systems. Gravid females make a significant maternal investment that requires tremendous effort (kJ necessary to produce and yolk follicles; (Beaupre et al., 2017)). During pregnancy, gravid females only ingest meals opportunistically (Lourdais et al., 2002) (prioritizing the occupation of suitable gestational habitat over prey rich environments), relying on existing energy stores to develop offspring and survive overwintering after parturition (Aubret et al., 2002; Lourdais et al., 2002). During gestation, females alter their behavior, reducing time spent foraging or engaging in courtship (typical mid-late summer activities for females). Gravid females instead devote time towards maintaining an elevated internal temperature, favorable to offspring development (Gardner-Santana & Beaupre, 2009; Graves & Duvall, 1993), through extensive thermoregulation in microhabitats that also provide shelter from predators (Charland & Gregory, 1995). Decreasing shed frequency may serve as a mechanism for energy conservation, as females who shed less frequently in gravid years can improve their residual reproductive value and experience better post-partum body conditions and overwinter survival by deferring regular tissue maintenance to the next active season. The timing of lone shed events in gravid females (late May, early June) seems to coincide with reported increases in progesterone concentration (a hormone responsible for maintaining

pregnancy in many vertebrates) in the closely related *Crotalus atrox* (Taylor et al., 2004). The early date of shed seen in gravid females may serve some purpose in signaling their reproductive condition to conspecifics in a similar manner to the pheromone signaling occurring in the late summer (Graves et al., 1986; Schuett, 1992), or it may simply be an adaptation to decrease effort and potential predation risk later in the season when mobility is compromised (Brown & Shine, 2004; Shine, 1988) and gestation is the priority.

In non-Gravid groups (post-partum, reproductive, non-reproductive) individuals shed once or twice per year in similar proportions, but the frequency of shedding is independent of body condition. I was unable to identify any single variable that predicts shed frequency when gravid females were excluded, but the strong effect of reproductive condition seen with gravid females suggests that some metric of an individual's reproductive history and the timing of their next reproductive bout may prove fruitful in future studies. The timing of shed events in non-gravid females relates strongly to the distribution of observations of courtship events. Single shedding non-gravid females shed later than gravid females, but prior to the onset of reproductive events (Figure 6), while the second shed of multiply shedding non-gravid females occurs immediately prior to (reproductive) or during (post-partum, non-reproductive, non-gravid) the mating season (Figure 7; Figure 8). The aggregation of non-gravid female shed events around the mating season may be advantageous because of the presumed reproductive utility of sheds; signaling receptivity (Mason & Parker, 2010) and attractiveness (Garstka & Crews, 1981) in females and eliciting courtship from males (Aleksiuk & Gregory, 1974). Furthermore, as Schuett (1992) has proposed, the timing of the mating season may have evolved in response to patterns of female estrus and the release of pheromones via skin shedding. While I lack endocrinological data regarding fluctuations of female reproductive hormones in *C. horridus*, anecdotally males are frequently observed sitting with females in shed during the mating season and do not disperse or copulate until after the female has shed (Carnes-Mason and Beaupre;

personal observations). Regardless of function, it appears that reproductive condition is responsible for observed patterns of shed frequency and timing in females in my population rather than the often-assumed relationship between growth and shedding. An empirical test of this hypothesis would require high frequency (preferably remote) monitoring to determine whether frequency or timing of shedding correlates to involvement or frequency of involvement in mating behaviors in females; an experiment beyond the scope of this study but worthy of future attention.

My study is the first to demonstrate that although shed frequencies in adult Timber Rattlesnakes are similar between sexes, their driving forces may differ. Males seem to partially conform to my preconceptions in that their rates of shedding are tied to body condition while female shed frequency seems more tightly correlated with reproductive events. However, the interrelatedness of shedding and reproduction in females may not be entirely surprising given the sexually dimorphic patterns of energy allocation seen in *C. Horridus* (Beaupre, 2002; Beaupre et al., 2017). It has been established that juvenile snakes shed more frequently than adults (Beaupre et al., 1998; Jenkins et al., 2009; Klauber, 1972), and that at sexual maturity males, but not females, continue to allocate large proportions of energy towards growth (Beaupre, 2002; Beaupre et al., 1998), thus it makes good sense that male shed frequency remains tied to body condition and resources in adulthood. Growth in adult females, however, plateaus at maturity as individuals allocate the majority of their available energetic surplus towards reproduction with only a small fraction continuing to fuel gradual increases in SVL (Beaupre, 2002; Beaupre et al., 1998). The observation that sheds cluster at phenologically relevant times of year may serve as a mechanism for maximizing the payoff of the energetic expenditure of shedding; co-opting the effort to serve a reproductive function. Interestingly, Macartney et al. (1990) reported similar distributions of shed timing in *C. oreganus* although their study only encompassed two active seasons (1981-1982) it may suggest that the observed

patterns are conserved within *Crotalus* and should be detectible across a range of latitudes and elevations. The proposed relationship between reproduction and shed timing in female rattlesnakes is a hypothesis that remains to be formally tested, but future work should not discount the possibility that male shed timing (particularly in double shedding males) may also be tied to reproduction. Since BCI in males is a good proxy for reproductive condition (i.e., males in good body condition are also more likely to engage in reproductive behaviors, experience elevated testosterone levels (Lind & Beaupre, 2015), and achieve increased reproductive success (Andren, 1982)) it is possible that the effect of body condition reported here is actually an effect of reproductive condition in males. It is unknown whether freshly shed males are more attractive to females in *C. horridus*, but such a finding would suggest that reproduction also plays a role in shed dynamics in males.

Conclusion

A clear understanding of patterns in shed frequency and timing is a prerequisite to asking broader questions about the causation of shed events and the social role they may play in snakes. Here I have reported the most detailed attempt at understanding the patterns of natural shed events at a population level to date. There are clear relationships between body condition and shed frequency in males and between reproductive condition, shed timing, and shed frequency in females. The relatively low frequency of shed events coupled with their relation to the energetic condition (as measured by either BCI or reproductive categorization) of the animal suggests that shedding is an important piece of the energy budget in Timber Rattlesnakes. More complete measurements of the energetic effort required per shed event which include the energetic content of skin, the metabolic expenditure related to its production and physical sloughing, and ecdysis related thermoregulation will be an important next step in incorporating shed events into our understanding of the dynamic energy budgets of snakes. As a process that requires significant effort, patterns in shedding probably carry fitness costs.

Trade-offs between time and energy available and decisions about if and when to spend resources (energy and time) on shedding likely play a role in defining life history strategies among snakes. Further exploration into the patterns of shedding in this and other species may continue to reveal the importance of shedding in whole-animal time-energy budgets and the relative fitness costs associated with this often-underappreciated aspect of reptilian physiology.

Tables and Figures

Table 1: Division of Individuals by reproductive category, shed frequency, and shed event reporting sample sizes, Shapiro wilks' test of normality, average julian date of shed event, and the confidence interval around the mean. Note that the significant values in the Shapiro wilks' column are marked; in these instances 95%CI's were estimated using a BCa bootstrapping approach.

Reproductive Condition	Sheds per year	Shed within the year	N	Shapiro Wilks	Average Julian Date of Shed	Average Calendar Date of Shed	95% CI (or bootstrap Equivalent)
Male	1	1	54	0.207	194.7	13 July	±10.28
	2	1	24	0.279	167.5	16 June	±8.31
		2	34	0.310	228.4	16 Aug	±9.06
All Females	1	1	78		199.4	18 July	192.3-206.8
	2	1	18	0.810	159.4	8 June	±7.2
		2	24	0.092	224.9	12 Aug	±5.5
Gravid Females	1	1	16	0.065	177.7	26 June	±14.3
Non-Gravid Females	1	1	62		205.0	24 July	197-211.9
	2	1	18	0.810	159.4	8 June	±7.2
		2	24	0.092	224.9	12 Aug	±5.5
Reproductive, Non-Gravid Females	1	1	13	0.114	206.4	25 July	±14.4
	2	1	3	0.747	149.0	29 May	±14.8
		2	7	0.357	214.1	2 Aug	±10.4
Non-Reproductive, Non-Gravid Females	1	1	25	0.207	215.9	3 Aug	±10.4
	2	1	12	0.988	160.7	9 June	±8.1
		2	12	0.093	231.7	19 Aug	±6.7
Post-Partum Females	1	1	8	0.166	202.5	21 July	±20.9
	2	1	4	0.841	172.2	21 June	±8.5
		2	7	0.318	221.7	9 Aug	±12.3

Table 2: Groups, Sample Sizes, Averages, and Variance of Shed per Active Season or Year Estimates. "TD" = Tracking Data, "PD" = Processing Data

Data Subset	Sex	N	Average Sheds per Year	95% C.I.
TD: All Animals	F	107	1.13	±0.064
	M	100	1.18	±0.075
PD: All Animals	F	171	1.10	±0.068
	M	128	1.165	±0.070
PD: Non-Tagged	F	31	1.12	±0.239
	M	20	1.18	±0.228
PD: Tagged	F	140	1.10	±0.065
	M	108	1.16	±0.072

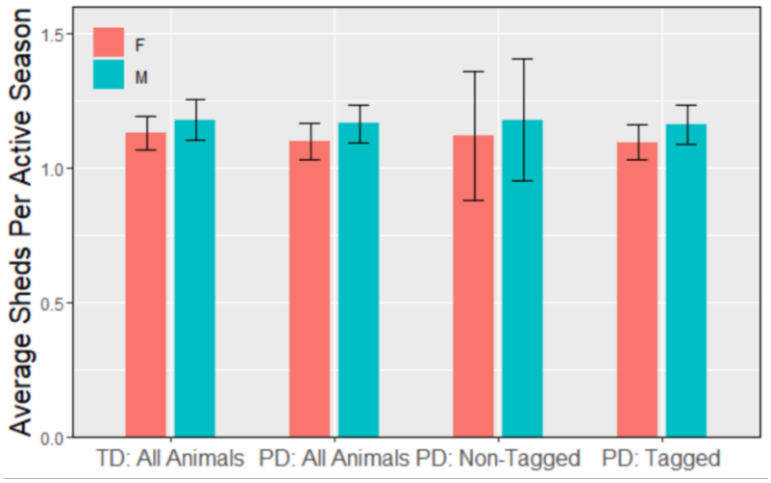

Figure 1: Average number of sheds per active season (Processing data, "PD") or per year (Tracking data, "TD"). Tracking data estimate includes all animals, processing data estimates have been divided into groups of; all animals, only animals without radio tags, and only animals with radio tags. Error bars show a 95% CI around the mean.

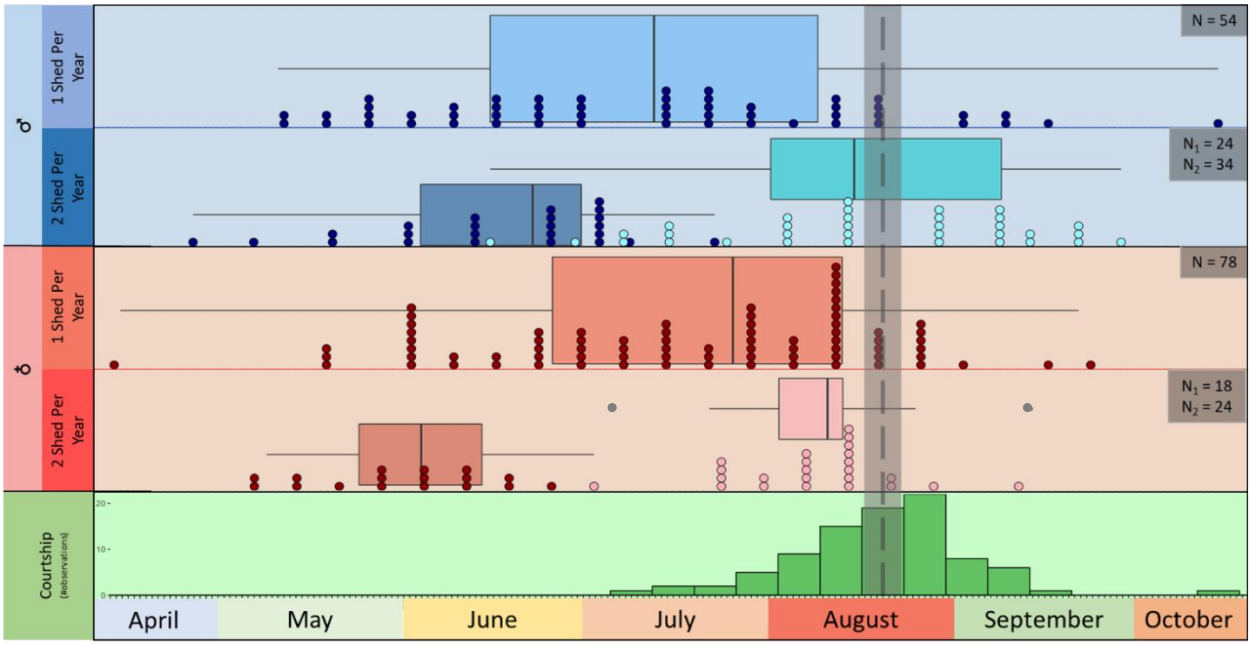

*Figure 2: Infographic showing average timing of shed events by sex depending upon number of sheds observed in a year. Box plots show the mean (black vertical line), interquartile range (IQR) (box), min and max of the data that falls within 1.5*IQR (black horizontal lines), and outliers (grey dots beyond the range of the black horizontal lines). Superimposed dot plots show histograms of observations that make up the data, stacks are 1-week bins of observations. Reproduction (bottom panel) is a histogram of observations of animals engaging in reproductive behaviors (courtship, conspecific associations) binned in 1-week increments. The dashed, grey, vertical line shows the mean courtship date plus or minus a 95% CI (grey shaded area surrounding). Months are displayed along the x-axis, with tick marks representing days. The Y-axis shows the category; blue = males (light blue = single shed per year, dark blue=2 sheds per year), red = females (light red = single shed per year, dark red = 2 sheds per year), green shows courtship frequency.*

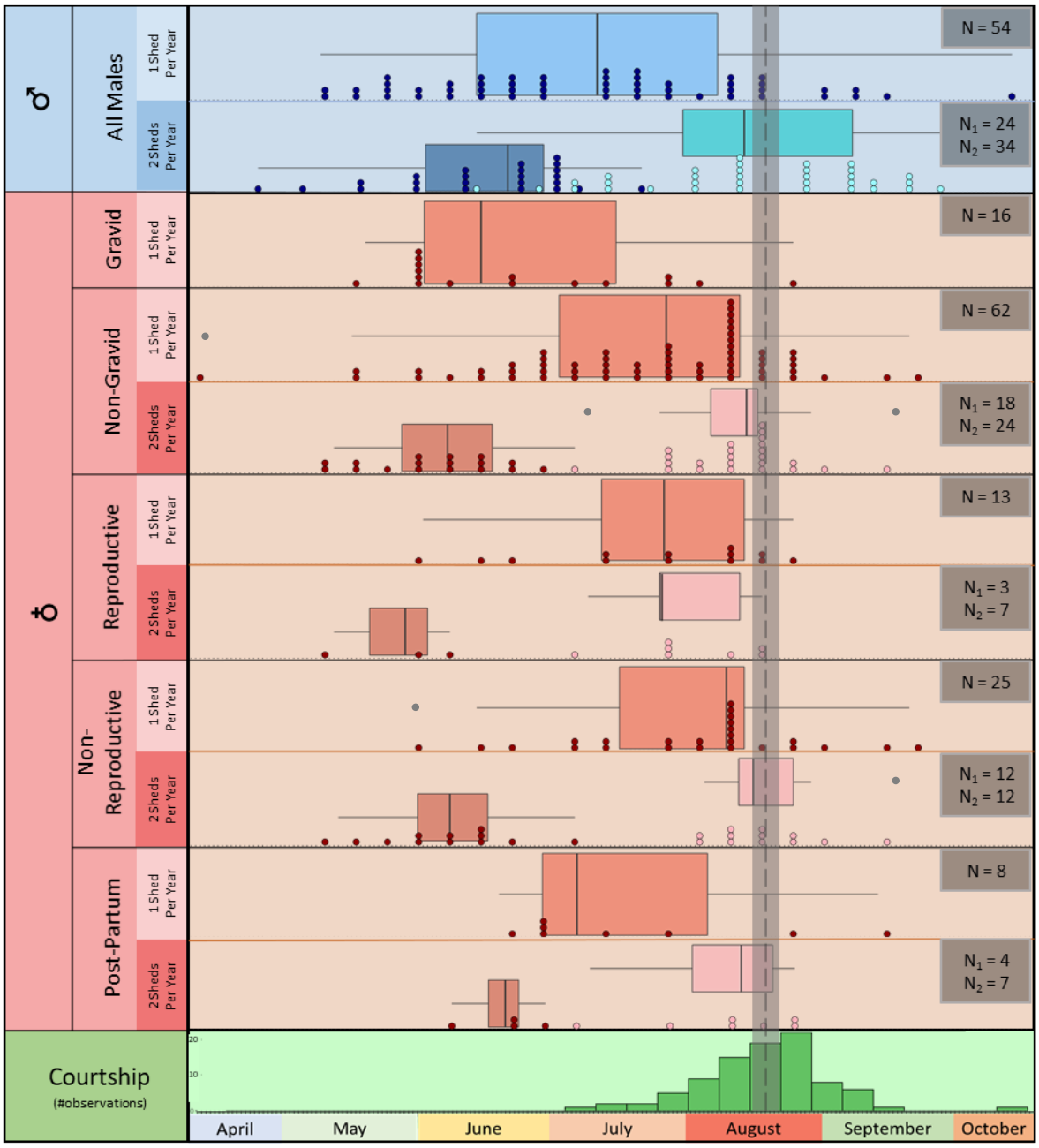

*Figure 3: Infographic showing average timing of shed events by sex depending upon number of sheds observed in a year. Box plots show the mean (black vertical line), interquartile range (IQR) (box), min and max of the data that falls within 1.5*IQR (black horizontal lines), and outliers (grey dots beyond the range of the black horizontal lines). Superimposed dot plots show histograms of observations that make up the data, stacks are 1-week bins of observations. Reproduction (bottom panel) is a histogram of observations of animals engaging in reproductive behaviors (courtship, conspecific associations) binned in 1-week bins. The dashed, grey, vertical line shows the mean courtship date plus or minus a 95% CI (grey shaded area surrounding). Months are displayed along the x-axis, with tick marks representing days. The Y-axis shows the category; blue = males (light blue = single shed per year, dark blue=2 sheds per year), red = females of varying reproductive class (light red = single shed per year, dark red = 2 sheds per year), green shows courtship frequency.*

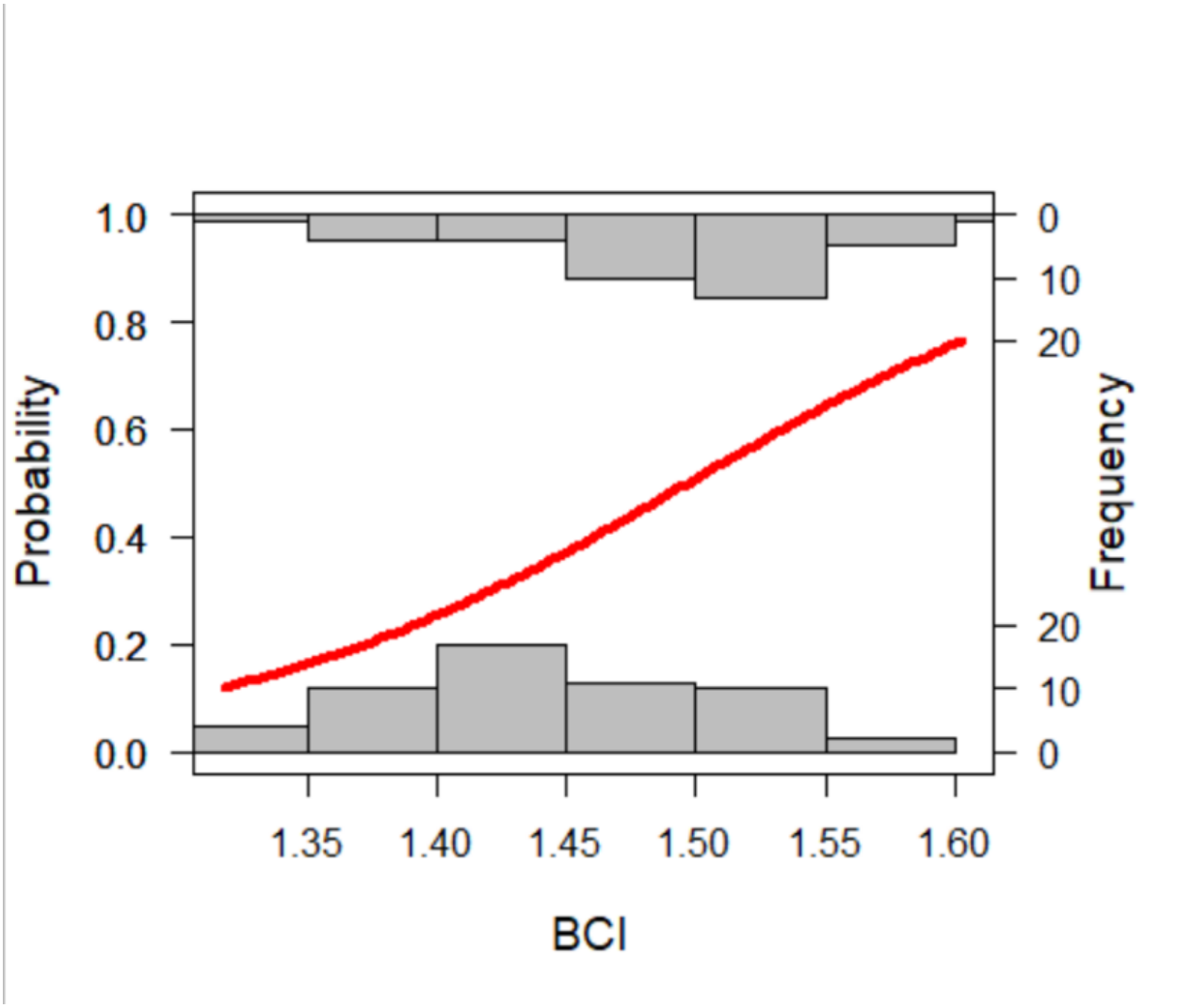

Figure 4: Binary logistic regressions of body condition index (BCI) compared to shed frequency (1 or 2 per year) in Males. The x-axis is the body condition of animals in a year where they were classified as a 1 or 2 shedder. Grey bars depict the frequency of observations in each bin (right y-axis). Distributions on the top of the figure are for 2 shedding individuals, bottom distributions are 1 shedding individuals. The red line shows the probability (left y-axis) that an animal will fall into the top category (2 shedders) or bottom category (1 shedders) depending on body condition.

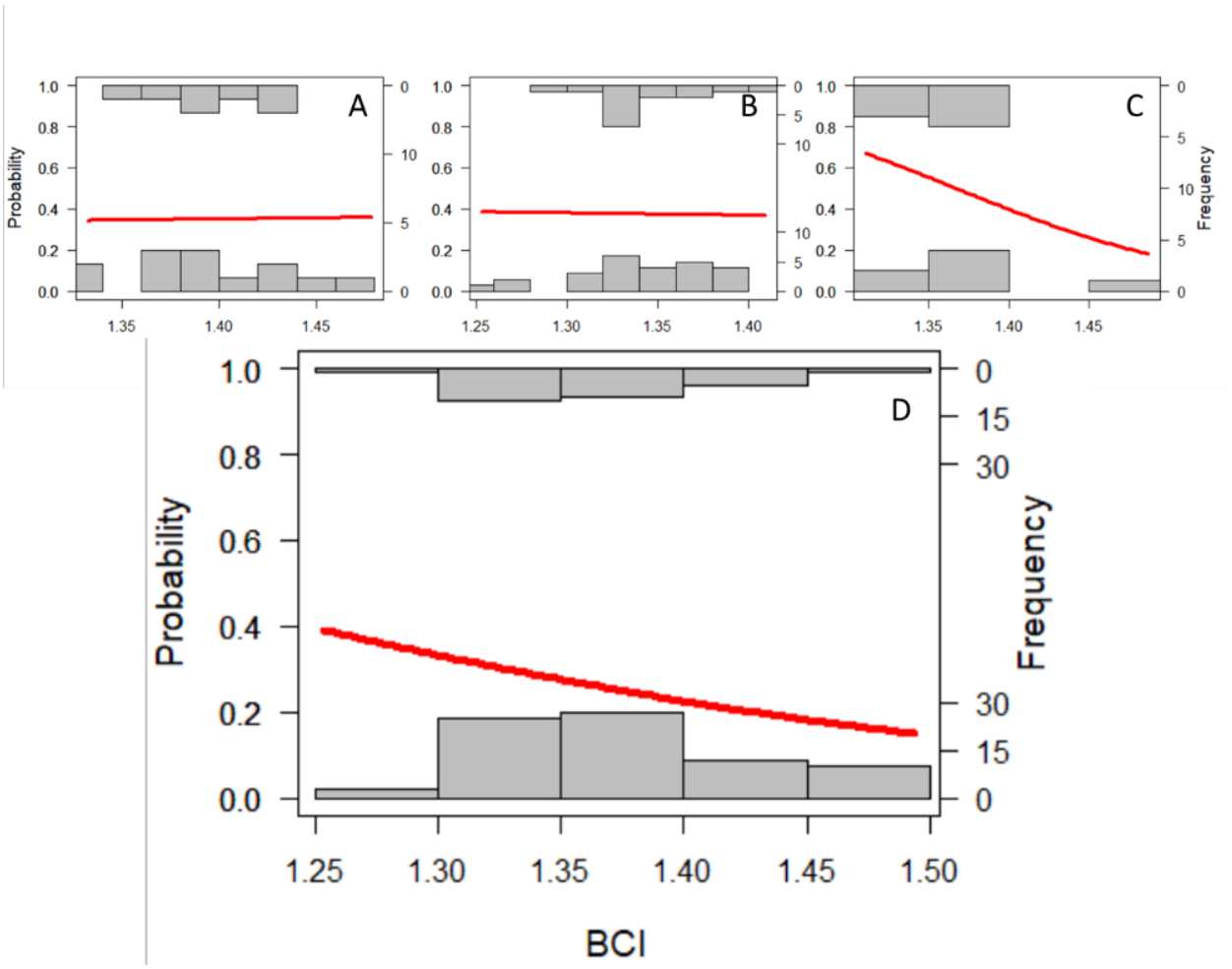

Figure 5: Binary logistic regressions of body condition index (BCI) compared to shed frequency (1 or 2 per year) in Non-Gravid Females. The x-axis is the body condition of animals in a year where they were classified as a 1 or 2 shedder. Grey bars depict the frequency of observations in each bin (right y-axis). Distributions on the top of the figure are for 2 shedding individuals, bottom distributions are 1 shedding individuals. The red line shows the probability (left y-axis) that an animal will fall into the top category (2 shedders) or bottom category (1 shedders) depending on body condition. The main panel (D) shows the binary logistic regression model for all non-gravid females considered together (n=103). Panels A (Reproductive; n=20), B (Non-Reproductive; n=40), and C (Post-Partum; n=14) show binary logistic regression models for each reproductive category subset. Note the lack of significant slope regardless of category.

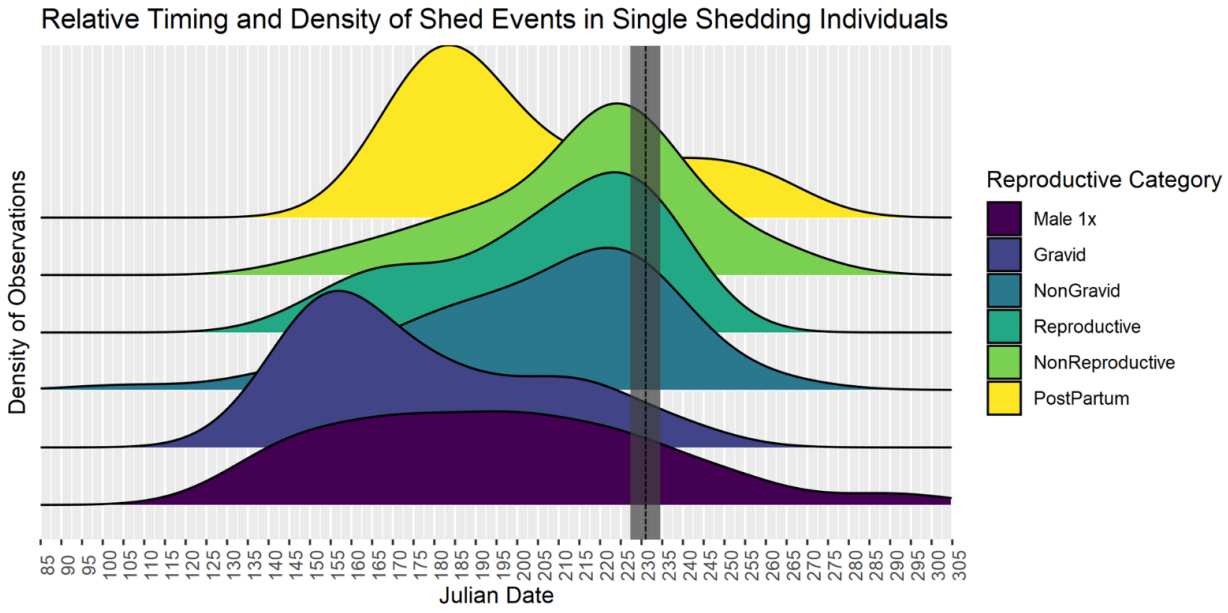

Figure 6: A density ridgeline plot of shed timing in single shedding animals by reproductive condition. Distributions reflect the relative proportion of observations over the date range. Area under each curve is equal to 1 and the height does not reflect the magnitude of observations. Note that not all categories are mutually exclusive. Non-gravid animals include both reproductive and non-reproductive individuals, while postpartum are also either reproductive or non-reproductive. Vertical grey bar and dashed vertical black line show the average dates of courtship events.

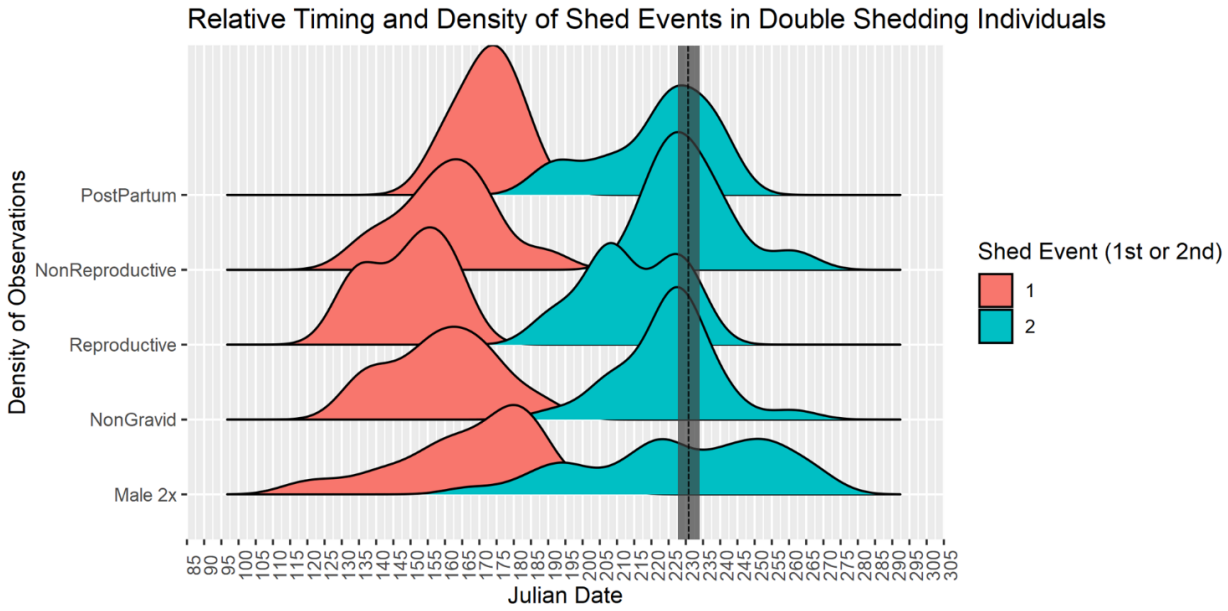

Figure 7: A density ridgeline plot of shed timing double shedding animals by reproductive condition. Distributions reflect the relative proportion of observations over the date range. Area under each curve is equal to 1 and the height does not reflect the magnitude of observations, only the proportion of the total. Note that not all categories are mutually exclusive. Non-gravid animals include both reproductive and non-reproductive individuals, while postpartum are also either reproductive or non-reproductive. Vertical grey bar and dashed vertical black line show the average dates of courtship events.

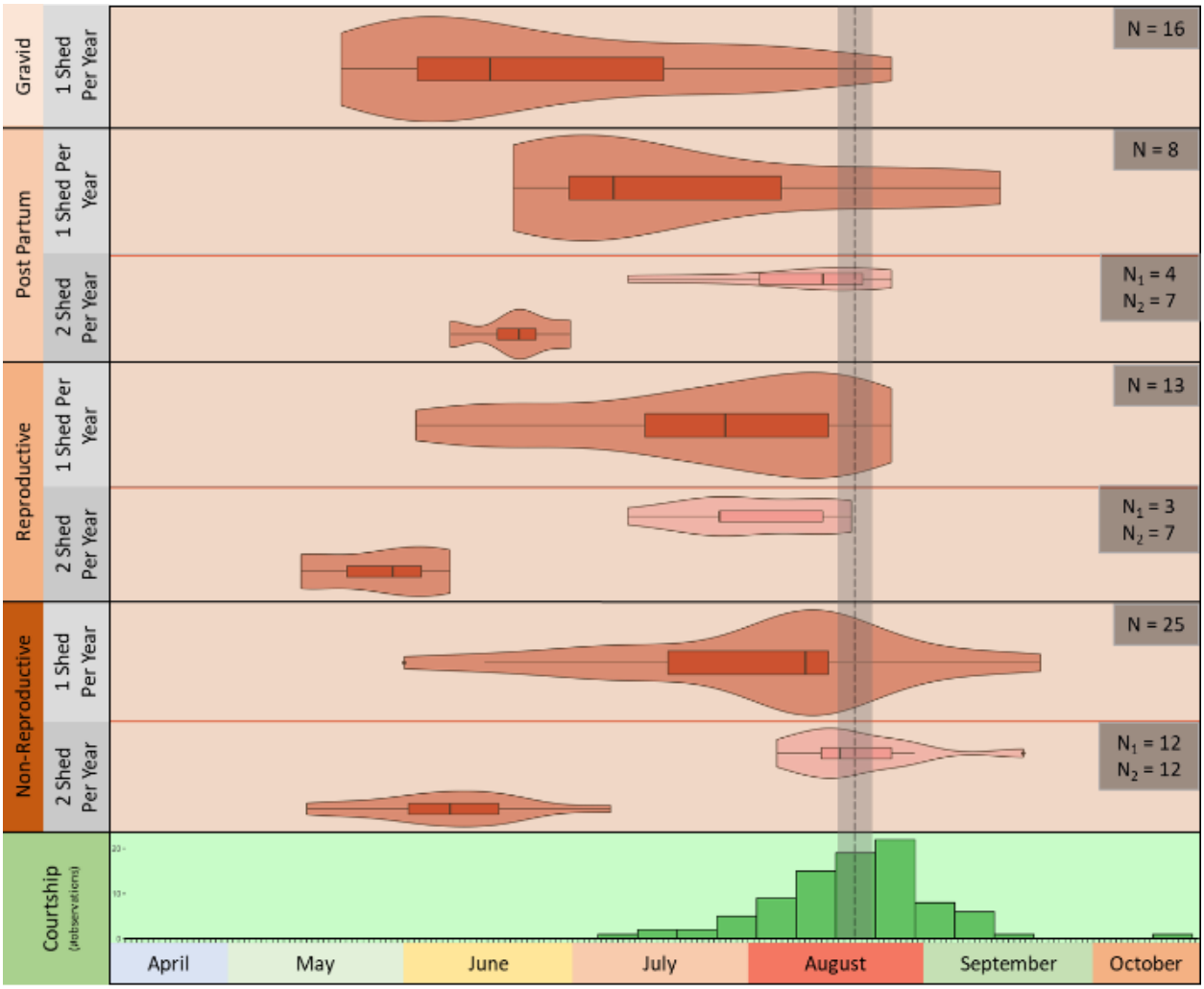

Figure 8: Female shed frequency and timing by reproductive condition. Each panel contains a violin plot depicting the distribution of each sample. Boxlplots nested within each violin plot show the mean (vertical black bar), interquartile range (box) and min/max (black horizontal bars), outliers are shown as single black dots. Courtship observations are depicted as a histogram in the bottom panel. Months along the bottom show time of year and individual tick marks above the months represent individual days. The vertical gray box with a dotted line shows the mean date of courtship observations ± a 95% CI calculated by BCa bootstrap.

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Chapter 2: The use of exogenous thyroid inhibitors and temperature manipulation to induce shedding cycles in Rattlesnakes.

Abstract

Thyroid hormones have been implicated in the control and regulation of shed patterns in squamate reptiles. In snakes, thyroid function suppresses shed frequency, but additional cofactors may be involved. Thyroidectomy is the traditional route towards thyroid manipulation and investigation of shed patterns and possible cofactors. To develop a non-lethal alternative to thyroidectomy, I tested the ability for synthetically derived thyroid receptor (TR) antagonists to initiate shed cycles in snakes. I found that two potential TR antagonists had limited (~20% efficacy), non-significant impacts on the timing and frequency of shedding in two species of rattlesnakes. Further experimentation manipulating the HPG axis with the addition of hCG with simultaneous thyroid receptor antagonization showed no potential for a thyroid-gonadotropin mechanism for the control of ecdysis. Finally, temperature manipulations following simulated brumation periods were used to induce ecdysis. Temperature trials were successful in inducing ecdysis (69% of treatment animals) with likelihood of ecdysis related to time since last shed event, but not body condition. Temperature manipulation shows potential for use in the lab-based induction and study of ecdysis, while further research is needed to develop a non-lethal shed trigger with utility in field-based studies.

Introduction

Changes in concentrations of endocrine compounds and phenological shifts in hormonal assemblages control the timing, intensity, and frequency of physiological processes at virtually all levels (molecular, cellular, organismal, populational; see Norris & Carr, 2021 for a full review). The thyroid gland (present in all vertebrates) releases triiodothyronine (T_3) and thyroxine (T_4) and is thought to primarily regulate metabolism, growth, and development (Nilsson & Fagman, 2017). In lizards, the thyroid gland and the hormones it secretes play a role in governing the occurrence of ecdysis, with complete thyroidectomy resulting in decreased shedding frequency in *Hemidactylus brookii* (Noble & Bradley, 1933). In snakes, however, thyroidectomy significantly increases the frequency of ecdysis (Chiu & Lynn, 1970a, 1970b; Maderson et al., 1970b) while injections with exogenous T4 in similarly treated animals restores natural shedding frequency (Chiu & Lynn, 1971; Maderson et al., 1970a). The diversity in the role of thyroid hormone in the two closely related groups (suborders *Serpentes* and *Lacertilia*) demonstrates that T4 and T3 play a vital but complex role in shedding in squamates. While the underlying mechanisms that facilitate the dichotomy in thyroid-shed relationships across squamates remain unresolved, it is generally posited that thyroid hormones play a stimulatory role in ecdysis in lizards but are inhibitory in snakes (Lynn, 1960).

The nature of the thyroid-ecdysis relationship in snakes remains poorly studied when compared to the body of lizard literature available. Basic patterns of thyroid secretion in snakes are largely undescribed with only a handful of studies quantifying natural patterns of variation in circulating T_4 in captive (Chiu & Lam, 1994; Yau, 1992) or free-ranging (Bona-Gallo, 1980; Wong & Chiu, 1974) animals. In studies reporting thyroid hormone concentrations, a sudden decrease in plasma T4 has been noted to accompany the onset of ecdysis (Bona-Gallo, 1980; Chiu & Lam, 1994), offering support for its inhibitory role. Still, the exact mechanism of thyroid action on shedding in snakes is unknown, further complicated by the observation that

thyroidectomized animals (who presumably have no circulating thyroid) still enter the perfect resting phase (the histological period between epidermal sloughing cycles; Maderson, 1965). As such, thyroid hormones might be best described as “permissive-inhibitory” or “suppressive” in snakes, requiring low thyroid in the presence of some additional factor(s) to permit the initiation of ecdysis. A similar but inverse pattern has been reported in lizards (Chiu et al., 1970), with other suggested cofactors including temperature (Chiu et al., 1986; Chiu & Maderson, 1980), other hormones (gonadotropins: Bauwens et al., 1989; prolactin: Maderson & Licht, 1967; androgens: Maderson & Chiu, 1981), or general metabolic state (Chiu et al., 1986). Given that shed events in snakes have been reported in conjunction with seasonal shifts (i.e., temperature changes; Klauber, 1972; Maderson et al., 1982; Semlitsch, 1979; Stabler, 1939), reproductive events (Chapter 1; Mason & Parker, 2010; Parker & Mason, 2012; Smith & Mason, 1997), and growth and resource availability (Heyrend & Call, 1951; Klauber, 1972; Macartney et al., 1990); I suggest temperature, body condition, or the HPG axis as possible cofactors interacting with thyroid levels to elicit sheds in *Crotaline* snakes.

The in-vivo manipulation of thyroid function via thyroidectomy (Chiu et al., 1983) has limited application in investigations of possible cofactors as radically altering the natural hormonal milieu through the complete removal of thyroid hormones may have complex influences on whole organism function, particularly with reference to specific cofactors (e.g., body temperature regulating effects of thyroid hormones, (Little, 2021); disruption of vital cross talk with female reproductive systems, Ren & Zhu, 2022; regulation of sex steroid synthesis in vertebrates, Duarte-Guterman et al., 2014; interactions with growth factors, Candelotti et al., 2015). Additionally, the use of thyroidectomies (or hypophysectomies) to manipulate hormones in-vivo is destructive, precluding their use as a technique in field based ecological studies of hormone driven physiological processes (e.g., Shedding) or in rare or protected species. A promising alternative approach to thyroidectomy is the use of a T_4 or T_4-receptor antagonist

allowing for the short-term (hours-days) manipulation of thyroid function (Lim et al., 2002) which mimics the naturally observed hormone fluctuations seen in snakes (Bona-Gallo, 1980; Chiu & Lam, 1994). Developments in organic chemistry and compound synthesis (Mackenzie, 2018; Nguyen et al., 2002; Schapira et al., 2003) have resulted in the production of targeted Thyroid hormone receptor antagonists capable of decreasing the amount of receptor-bound hormone and receptor mediated gene-expression, functionally recreating the decreased levels of T_4 reported in shedding snakes. These compounds have been shown to decrease activation of receptors by T_4 (Schapira et al., 2003) effectively (~90%) and briefly (half-life ~ 12 hours) in herpetofauna (Lim et al., 2002). Exploration of the use of synthetic thyroid receptor antagonists to mimic outcomes of thyroidectomy without long term damage to individuals is necessary to validate the technique for use in field studies and protected species.

Reptilian endocrinology is still in its infancy, with the majority of previous focus being on sexual signals (e.g., pheromones; Mason, 1992; Mason & Parker, 2010; Parker & Mason, 2012; Smith & Mason, 1997) and the stress axis (DuRant et al., 2008; Taylor & DeNardo, 2005, 2011). For this study, I narrowed my focus towards a single possible endocrine cofactor of shedding (gonadotropins) because of the apparent relationship between ecdysis, sexual signaling, and reproductive state in snakes (Chapter 1; Aldridge & Duvall, 2002; Martin et al., 2021; Smith & Mason, 1997). Our understanding of the Hypothalamic-Pituitary-Gonadal (HPG) axis and the functional molecules and receptors involved in reptiles is limited but basic research has been conducted (reviewed in Norris & Lopez, 2011). With specific reference to interactions between gonadotropins and shedding, Chiu and Lynn (1970) were able to increase shed frequency of hypophysectomized snakes through the injection of human Chorionic Gonadotropin (hCG). Chiu and Lynn's finding suggests that manipulation of the HPG axis through injections of the exogenous hCG in thyroid hormone limited snakes may affect sufficient increase in concentration of gonadotropins to elicit a shed, supporting a connection between shedding and

reproduction in snakes (Chapter 1). Further support of a complimentary interaction between low thyroid and elevated gonadotropins is necessary to demonstrate a mechanistic connection between thyroid, the HPG, and ecdysis in snakes.

Endocrine compounds are the mechanism by which environmental conditions and physiological state of individuals are translated (directly or indirectly with the aid of the nervous system) into physiological change. Environmental temperatures often cause seasonal shifts in behavior and ecology (Adolph & Porter, 1993), particularly in ectotherms where the rates of all physiological processes are tightly linked to body temperature (Angilletta et al., 2002; Huey & Kingsolver, 1989; Stevenson, 1985). Furthermore, in low-energy ectotherms such as rattlesnakes, we might expect animals in low body condition (i.e., limited resource pools) to forgo optional metabolic expenditures (such as growth and reproduction; Beaupre, 2008; Lind et al., 2016; Lind & Beaupre, 2015) in order to save energy for requisite functions (metabolism and other maintenance costs; Beaupre, 1995; Dunham et al., 1989). Because shedding is an energetically expensive process (Chapter 3) that occurs at a temperature sensitive rate (i.e., warm snakes progress through shed cycles more quickly; Carnes-Mason, personal observation) I expect that temperature and body condition (a reflection of energetic resources) may also act as cofactors with T_4, eliciting shed events when the suppressive effects of thyroid hormones are limited.

To investigate the concurrent role of thyroid hormones with temperature, body condition, or the HPG axis, I used synthetically derived thyroid hormone receptor antagonists to reversibly suppress thyroid function over a short time scale in-vivo in snakes. I selected two species of rattlesnakes for my investigations because infrequent shedding in the clade (Timber Rattlesnakes: Chapter 1; prairie rattlesnakes: Macartney et al., 1990) allows for more reliable attribution of manipulations towards the occurrence of shed events, and because Timber Rattlesnakes are the subject of additional ecdysis related investigations in my current research

agenda. Timber rattlesnakes and prairie rattlesnakes are among the most well studied species within the genus, particularly in the observations of natural shedding frequency (Chapter 1; Macartney et al., 1990). I used captive populations of prairie and timber rattlesnakes to 1) assess the efficacy of an exogenous thyroid receptor antagonist in-vivo; 2) test for an interaction between gonadotropins and thyroid function suppression and ecdysis using novel non-lethal methods; and 3) investigate temperature and body condition as possible cofactors to the onset of ecdysis in snakes.

Methods

Study Organisms and Captive Care

Snakes in the genus *Crotalus* are low energy specialists (Beaupre & Duvall, 1998) with infrequent shedding patterns (Klauber, 1972). Germain to the experiments here, rattlesnakes also have a useful morphological feature, the rattle. At each shed event, a new segment is appended between the tail and the existing rattle strand (Zimmermann & Pope, 1948). The emergence of the new basal rattle segment precedes any other external sign of impending shed (e.g., occlusion of the spectacle, bluing of the ventral scales, dulling of coloration) by 2 weeks (Chapter 3). The emergent basal segment makes rattlesnakes a useful model organism for studies of shed timing and occurrence, providing for a clear indicator of shed onset and reliable attribution of ecdysis towards specific manipulations.

I selected two species of *Crotaline* snakes to expand the conclusions of my experiments beyond the species level. Timber Rattlesnakes (*C. horridus*) and Prairie Rattlesnakes (*C. viridis)* are relatively abundant in the populations of origin from which my study animals were collected (*C. horridus* – Northwest Arkansas; *C. viridis* – South Dakota Central Plateau). Animals consisted of long-term captives (*C. viridis*) and short-term captives (1-2 years; *C. horridus*) collected as part of a series of shed-related investigations in my lab group. All animals were housed in plastic, lid-locking Tupperware containers and housed on absorbent newsprint

with a hide and water dish for the entirety of the experiment. Housing facilities were maintained on a 12L:12D photoperiod between 22-25ºC year-round. Each species was stored in a separate room with its own dedicated air flow, but it should be noted that within species, animals were in a shared space and therefore were able to sense the chemical cues of conspecifics (a possible factor influencing shed occurrence in some populations; Lillywhite & Sheehy, 2016; Loughran et al., 2015). Animals were fed monthly except during trials. All animals were checked daily for visual evidence of impending shed. Shed events (including those occurring outside of experiments) were logged for all animals throughout the duration of the study.

Trial Preparation

All animals were fed a meal one month before the beginning of injections or other experimental manipulations. Feeding was necessary to maintain the condition of animals in captivity, but timing was crucial to avoid any confounding effect of growth or resource acquisition which is thought to be correlated to the onset of shed in free ranging and captive snakes (see Chapter 1 for discussion). Following feeding, all animals were checked weekly for signs of impending ecdysis (emergent basal segments, occlusion of the spectacles; Chapter 3). Animals free of sign of impending shed one month after feeding were included in trials, those in shed were excluded from participation. Immediately prior to manipulations, all animals were weighed (g) and measured (snout-vent length, SVL; cm) to allow calculations of mass-specific dosages and body condition indices (as; $\frac{\log(Mass)}{\log(SVL)}$) reflective of the animal's energetic condition at the start of the trial. For all trials, animals were assigned to treatment and control groups in approximately equal sex ratios with a representative range in body conditions in each group.

<u>Experiment 1</u>: Thyroid Suppression with 1-850 (reversible thyroid receptor antagonist)

I selected a thyroid receptor antagonist (Schapira et al., 2003; 1-850: supplied by KeyOrganics, CAS: 251310-57-3, >97% purity) shown to be effective in amphibians (Lim et al., 2002) and fish (Navarrete-Ramírez et al., 2014) to suppress thyroid function on a limited time

scale in a reptile. Nuclear thyroid receptors are reversibly antagonized by 1-850, allowing for preservation of receptors and degree of inhibition to fade as the compound is washed out (Schapira et al., 2003). I used multiple injection schedules and dosages (below) to attempt to replicate drops in thyroid availability seen in naturally shedding snakes (Bona-Gallo, 1980; Chiu & Lam, 1994). While the antagonist did not directly impact thyroid hormone availability, I hypothesized that 1-850's high affinity for nuclear thyroid receptors would cause a temporary disruption to the negative regulation of shed cycle initiation seemingly maintained by elevated circulating thyroid hormone concentrations (as evidenced by increased shed frequency in Thyroidectomized snakes; Chiu et al., 1983; Chiu & Lynn, 1970b, 1971).

Solubility of 1-850 in physiological saline solution (0.9% NaCl) was low (<5mg/mL), so to achieve the concentrations necessary for my manipulations, 1-850 was dissolved in DMSO (high solubility: >10mg/mL; safe in reptiles (Gad et al., 2006)), aliquoted for mass-specific dosages (1-15 mmol/kg), and mixed with small enough quantities of warmed (37ºC) saline to prevent crash out but keep injection volumes low (<1mL). Injections were given intra-peritoneally (IP) with a 1mL tuberculin syringe with a 0.5" 27-gauge needle on alternate-days. The half-life of the compound has been reported as ~12 hours (Schapira et al., 2003), so I believed an alternate day injection schedule would be sufficient to maintain suppression of the thyroid system for several days (as reported for thyroid suppression accompanying the onset of ecdysis).

I conducted a series of pilot injections with compound 1-850 in both *C. viridis* and *C. horridus*. No toxicity was expected or observed in any treatment animal; dosages included 1, 2, 5, 10, and 15 mmol/kg body mass. I treated all individuals with a series of three injections administered on alternate days (for total injection dosages of 3-45 mmol/kg body mass over 1 week). After validation that dosages would be non-toxic, all trials were conducted at a dosage

of 10mmol/kg body mass. Animals were injected in series then monitored daily for one month for signs of impending shed.

Experiment 2: Thyroid Suppression with MLS-000389544 (non-reversible thyroid receptor antagonist)

Following limited success in my 1-850 trials (see results), I attempted manipulations with an alternative thyroid receptor antagonist, MLS-000389544 ("MLS"; supplied by Enamine ltd., CAS: 573965-48-7, >95% purity). The compound, MLS, is a non-reversible, high permeability (Perez Diaz et al., 2016; Zloh et al., 2016) thyroid receptor (TR) antagonist with high specificity for TR-*beta* (see Aranda et al., 2013 for review of thyroid receptor physiology), functioning by preventing association between the TR and steroid receptor coactivator 2 (SRC2; Hwang et al., 2009, 2011, 2012). Steroid receptor coactivator 2 regulates thyroid hormone mediated gene expression (Norris & Carr, 2021, pg 224), leading us to hypothesize that preventing this interaction may interfere with gene expression which dictates the cellular proliferation associated with ecdysis. The compound (MLS) has been shown to effectively augment gene expression in a vertebrate (zebra fish; Bhumika et al., 2015), but has not been used in a squamate. An advantage to the non-reversible mechanism is the minimization of injections, lowering the likelihood that stress effects may confound my results.

The thyroid receptor antagonist MLS was dissolved in warmed (37ºC) DMSO at 1mg/mL, aliquoted to mass specific dosages, and mixed with physiological saline prior to injection. Solutions were injected IP in a single dose (1, 5, 10, 30, or 60 mmol/kg) with a 1 mL tuberculin syringe with a 27-guage needle and animals were monitored for one month.

Experiment 3: Thyroid Suppression x Gonadotropin Upregulation

The desired effect of this set of experiments was to suppress thyroid function with the preferred TR antagonist 1-850 while simultaneously increasing plasma gonadotropin concentrations through the injection of hCG (hCG supplied by BioVendor R&D, CAS: 9002-61-3,

>98% purity; Figure). This manipulation was intended to mirror the thyroidectomy and hCG injections reported to increase shed frequency and prevent animals from entering an inter-slough perfect resting phase (Chiu & Lynn, 1970a) using a non-lethal alternative to thyroid removal. I used the 1-850 dosage that had some possible, but non-significant effect on shed frequency in *C. viridis* (Experiment 1; 3 x 10mmol/kg on alternate days) for my investigation of gonadotropins as a possible cofactor. All animals were treated with 1-850 on alternate days following a preloading period with hCG injection (1.0-1.5 IU/g body mass hCG;

Table *4*). I prepared and injected 1-850 as above (IP, DMSO and H_20 vehicle, alternating days, 3 total doses). I dissolved hCG in physiological saline and injected for 2 days prior to 1-850 manipulation, administered alongside each 1-850 dose, and continued after thyroid suppression 2 days after the completion of 1-850 doses (6 total doses;

Table *4*; Figure). I injected hCG subcutaneously (Chan et al., 2003), with the preload period intended to allow time for upregulation of downstream products of the HPG axis prior to thyroid suppression.

Experiment 4: Temperature Manipulation

I used a walk-in environmental chamber (Model 13030, BioCold Environmental Inc., Fenton, MO) to acclimatize snakes to a 0L:24D photoperiod under constant temperature (10ºC) experienced by hibernating animals (Agugliaro, 2011; Beaupre, 2008) over a 6-week period. Animals were moved from long-term housing (12L:12D, 22-25ºC) to the environmental chamber, lowered to hibernation conditions over five weeks (derived from Agugliaro, 2011; Table 3), and held under constant temperature in the dark for three weeks. At the end of the holding period, photoperiod was held constant (0L:24D) and temperature was increased to 33ºC over 12 hours (mid-summer body temperatures experienced by Crotalus; Beaupre, 2008). Temperature was held at 33ºC for one week and animals were returned to long-term housing at 12:12 photoperiod and an ambient of temperature 22-25ºC. Snakes were monitored daily for signs of impending

shed. Animals were categorized by whether they initiated a shed during the trial period and whether that shed occurred immediately after or during the temperature increase (e.g., within two weeks of increase from 10 to 33ºC) or shed events were delayed (occurring between 1 week and 2 months following temperature manipulation).

Statistical Analyses

All analyses were conducted in R studio (version 4.2.3). Data sets consisted of binary response variables (shed vs. no shed) and were non-normally distributed. I used statistical tests that do not require normally distributed data or normally distributed residuals of regressions (z-tests, binary logistic regressions). For chemical/endocrine manipulations, I used two-proportion z-tests between control and manipulated groups to test for significant effects of treatment group on shed outcome. In temperature trials, I used binary logistic regression to test for differences between groups and for an inflection point in relationships between body condition and timing since last shed on shed outcome (either shed or no shed OR immediate or delayed shed) following manipulation. I assumed a 5% type I error rate for all statistical analyses.

Results

Experiment 1:

I used thyroid receptor antagonist 1-850 to suppress thyroid hormone action and elicit shed events in two rattlesnake species. I observed limited success in *C. viridis* (sheds in treatment animals = 8/24; sheds in control animals = 3/18). No sheds were recorded in *C. horridus* with 1-850 injections alone (sheds in treatment animals = 0/5; sheds in control animals = 0/5). Shed occurrence in treatment groups of *C. viridis* was non-significant relative to the frequency of shed observed in control groups (2-proportion z-test; $z=1.194$; $p > 0.1$).

Experiment 2:

To minimize injection frequency and investigate the efficacy of an alternate thyroid receptor antagonist, I used the non-reversible antagonist MLS to inhibit thyroid axis function to elicit shed events in a rattlesnake species. Following pilot studies to test for toxicity (no toxicity related mortality was observed at any dose; 1, 5, 10, 30, or 60 mmol/kg body mass in single or dual injections), I carried out a series of small-scale trials in *C. viridis*. No shed events were recorded in relation to MLS injections at any dosage (0/3 at 30mmol/kg; 0/3 at 60mmol/kg; 0/3 at 2x60mmol/kg). Trials were not continued in *C. horridus* due to the lack of effect in *C. viridis*.

Experiment 3:

To test whether the HPG axis in rattlesnakes is a cofactor controlling shed frequency (in conjunction with the suppressive action of thyroid hormone), I treated rattlesnakes with a reversible thyroid receptor antagonist (1-850) and a simultaneous upregulation of the HPG-axis via injections of hCG. Gonadotropin trials were only conducted in *C. horridus* due to the primary focus of the larger body of work (Chapters 1,3) and the cost of materials. An initial success was recorded (3/4 manipulated animals in an uncontrolled (no DMSO-only group) initial trial). Subsequent trials found no evidence of an interaction between 1-850, hCG, and shed frequency (0/8 treatment animals vs. 0/2 control animals).

Experiment 4:

I used a series of thermal manipulations to investigate temperature as a potential trigger for shed events in rattlesnakes. Male and Female *C. horridus* in a range of masses (n=16; 5 male : 10 female : 1 unsexed juvenile) were acclimatized to hibernation temperatures and photoperiod (constant 10C; 24D:0L) and then rapidly warmed to summer temperatures (33ºC) under constant dark for a one-week period (ramp from 10ºC to 33ºC was approximately 6 hours). After returning to ambient temperature and photoperiod (~ 22ºC; 12L:12D), several animals were in visible shed (8 of 16) upon removal from 33ºC; 3 of 16 shed over the

subsequent two months. 11/16 animals (69%) entered shed in response to temperature manipulations. Within animals that shed, group of shed (Immediate; entering shed during high temp vs. delayed; entering shed >1 week after return to ambient) correlated strongly to the timing of the most recent shed event (groups were completely exclusive, linear models failed to converge; Figure). Among all temperature trial snakes, all delayed shed individuals and all animals that did not shed in response to manipulations had shed more recently than 150 days prior to the temperature increase to 33ºC (Figure). Body condition was not a significant predictor of shed occurrence (Binary Logistic Regression: R^2 = 25%; Figure) or timing (immediate vs. delayed; Binary Logistic Regression: R^2 = 1%; Figure).

Discussion

The set of experiments that I carried out were intended to explore a new methodology for the reliable induction of shed events in snakes while simultaneously broadening the existing knowledge of reptilian endocrinology. While existing methods (thyroidectomy/hypophysectomy) can elicit shed events in snakes (Chiu & Lynn, 1970a, 1970b, 1972), their terminal nature prevents exploration of the ecological role of shedding in free ranging snakes or investigations with rare or protected species. Additionally, the removal of key portions of the endocrine system has cascading effects which alter the behavior and metabolic activity in-vivo, preventing controlled investigations of behavior and energetics in surgically altered snakes. Because my larger research agenda centers on the role shedding plays in snake ecology and the fitness consequences of ecdysis as a life history trait, this set of experiments was designed to facilitate future investigations of the energetic, behavioral, and ecological role that shedding plays in snake life histories in the field and laboratory.

I had limited success in the elicitation of ecdytic cycles in my study. Exogenous hormone manipulations were not found to be a causal agent in shed events. Limited successes (~20%) did seem to correlate to manipulations by time (all sheds occurred 14-21 days post

injections) but proportions of shed in treatment versus (admittedly limited) control groups were non-significant. Further investigation is needed, but it is possible that the season of manipulations (and associated circulating hormone levels and degree of tissue-specific receptor expression), individual physiological state, and background hormonal concentrations of individuals may all play a role in whether manipulations are successful (thereby obscuring my results). Future studies should focus on explicitly quantifying circulating hormone levels throughout trials. In-vitro studies are also needed to validate the efficacy of thyroid antagonists 1-850 and MLS in reptilian tissues.

Across trials, temperature manipulation was the most successful, eliciting shed events in up to 69% of treated individuals. Shed events were more likely to occur in close temporal proximity to the temperature increase in animals that had not shed recently (>150 days prior). While shed events can occur at a maximum frequency of once every 28 days in snakes (Maderson, 1984; Maderson et al., 1970b) my data suggest that there may be additional limitations which prevent shedding more frequently than once every three months in rattlesnakes. However, data presented here are preliminary, so I caution against consideration of a temporal threshold value for shed occurrence until more trials corroborate my findings. Additional support for a threshold value from multiple trials with larger sample sizes would warrant further investigation of the underlying mechanisms.

My study found no evidence for an effect of body condition on likelihood of shed cycles in snakes. If shedding were truly a growth function, occurring in conjunction with a change in body size or energetic condition, we would expect to find a pivotal body condition value or change in body size (Meik & Schuett, 2016) that correlates to shed cycle timing. As of yet, no such pivotal conditions have been identified. However, elevated body condition has been found to increase shed frequency in free ranging male timber rattlesnakes (Chapter 1), suggesting that resource pools may influence shedding, but as a necessary fuel for the process (Chapter 3)

rather than occurring as a function of change in body size (e.g., increased length or “fatness”). Regardless of body condition, my temperature data suggest that time since last shed (with a potential pivotal value ~ 150 days) and temperature may be reliable correlates of ecdysis in *Crotalus*.

While exogenous manipulations were unsuccessful, the strong evidence for a role of thyroid hormone in shed control (Chiu & Lynn, 1970a, 1970b, 1972; Maderson et al., 1970a, 1970b) indicates that a set of endocrine conditions are necessary to allow shed events to occur. Since temperature plays a role in augmenting a plethora of endocrine functions in ectotherms (e.g., temperature dependent sex determination, Elf, 2003; phenological changes in reproductive status, Licht, 1972; Licht et al., 1985)) I hypothesize that the temperature manipulations employed here succeeded because they resulted in a set of endocrine conditions which promote shedding. Cold temperatures result in the downregulation of thyroid secretion in reptiles (Licht et al., 1989; Rivera & Lock, 2008), with upregulation occurring in response to warming spring temperatures (Norris & Carr, 2021). A possible cofactor involved in the process is likely a hormone which responds more quickly to increasing temperatures than thyroid tissues (spiking prior to thyroid upregulation and related shed suppression in temperature manipulated animals) and corresponds to fat storage and lipid availability (given my findings that rapid temperature increases elicit shedding and my assumption that animals must have sufficient reserves to pay the caloric cost of shed). I suggest prolactin as a possible cofactor (involved in temperature/ecdysis relationship in lizards; Licht & Jones, 1967); PRL has been implicated in the lipid dynamics of birds and lizards (Meier, 1977) and may respond to increases in temperature under zero light conditions in squamates as dopamine secretion is decreased (a potent PRL-inhibitor; Ramachandran & Kurup, 2011). Future directions of this research involve the purification of reptilian PRL, development of methods for quantifying PRL concentrations in snakes, and exogenous manipulation of PRL in thyroid depressed snakes.

Conclusion

Attempts to induce sheds via the manipulation of endocrine systems (both thyroid and gonadotropin axes) in vivo in two rattlesnake species proved largely unsuccessful. More detailed hormone analyses are required to validate the methods employed here. Use of a binary response variable (occurrence of shed) and small sample size limit my statistical power, but the few successes reported here warrant further research (though larger sample sizes are necessary). Temperature manipulation, however, appeared to be an effective method of shed induction in captive snakes. An alternative method conducive to field manipulations would still prove valuable (via the injection of endocrine compounds in the wild), but my evidence suggests that simple temperature manipulation may be an efficient and cost-effective method for the elicitation and investigation of ecdysis in the laboratory.

Tables and Figures

Table 3: Schedule of Photoperiod and Temperature Adjustments for Shed Induction Temperature Trials

Week(s)	Hours Light	Hours Dark	Temp Day (ºC)	Temp Night (ºC)
1	11	13	18	16.5
2	10	14	16.5	15.0
3	0	24	13.0	13.0
4	0	24	11	11
5-7	0	24	10	10
8	0	24	33	33
9-12	12	12	22	22

Table 4: Injection Schedules for alternating day injections (for 1-850 trials) including the preloading and post injections employed in gonadotropin manipulations when administered in conjunction with 1-850

Day	1	2	3	4	5	6	7	8	9
1-850			x		x		x		
hCG	x	x	x		x		x		x

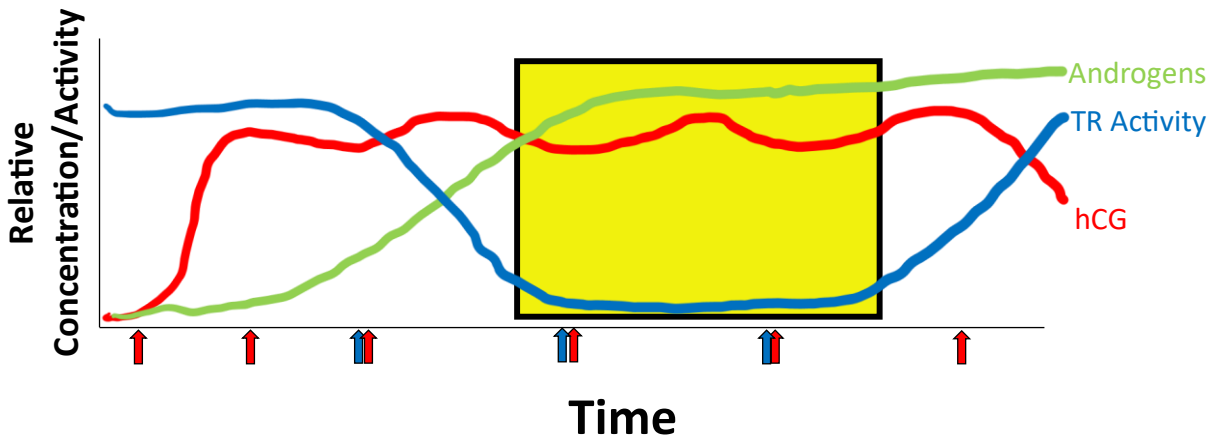

Figure 1: Expected change in endocrine concentrations in response to 1-850 and hCG injections. Injection time points are denoted on the x-axis as color coded arrows (Red=hCG injection; Blue=1-850 injection). The yellow box identifies the desired physiological condition resulting from the manipulations; elevated androgens in response to hCG injections coupled with thyroid receptor activity (and related gene expression) down regulation. I hypothesized that inducing a set of conditions as indicated by the yellow box for several days, animals would initiate a shed event.

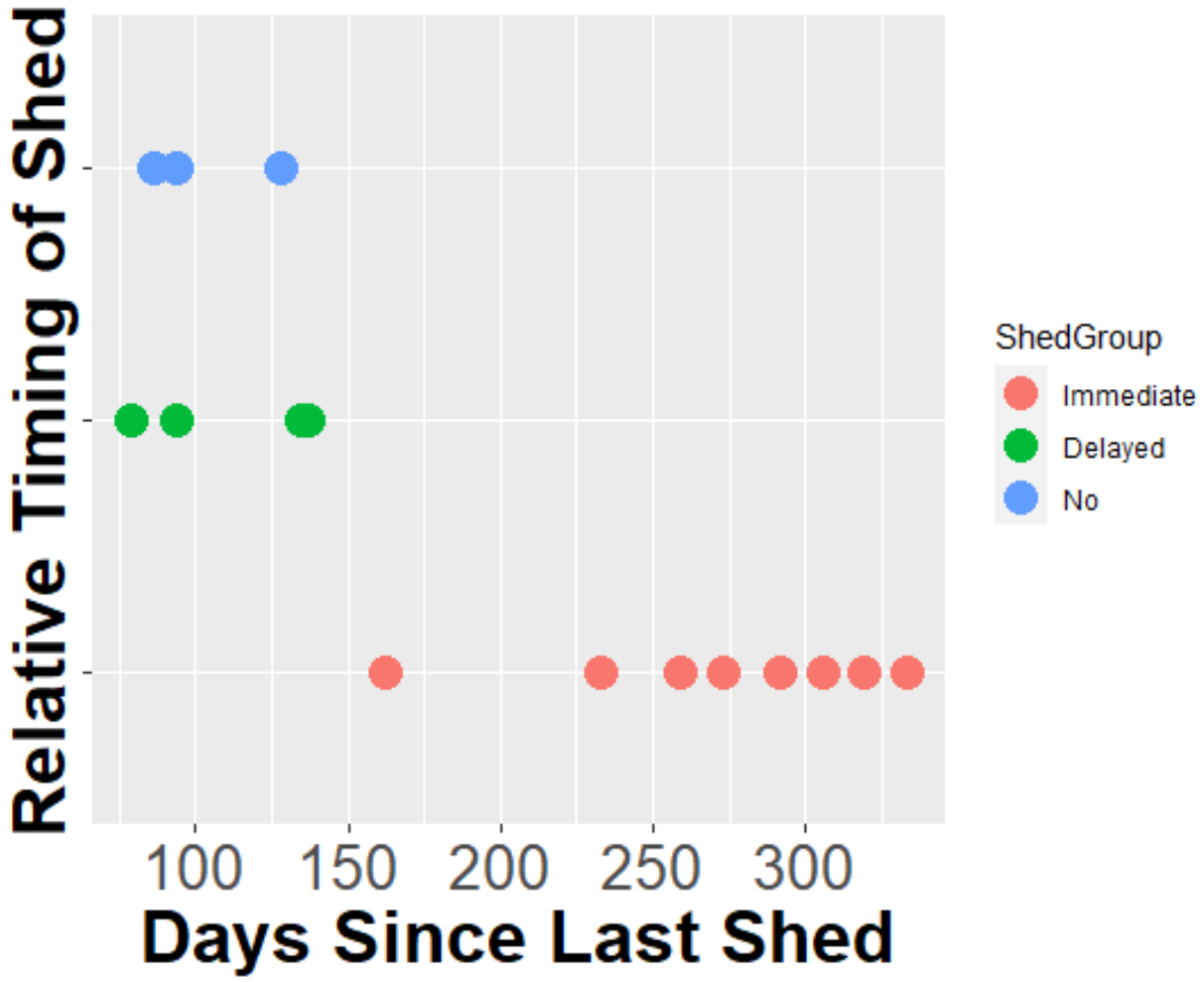

Figure 2: Shed Group (Immediate, Delayed, or No Shed) by time (days elapsed between last shed event and end of temperature increase). There was a clear difference between groups with sheds being delayed or prevented in animals that had shed within 150 days of the trial end.

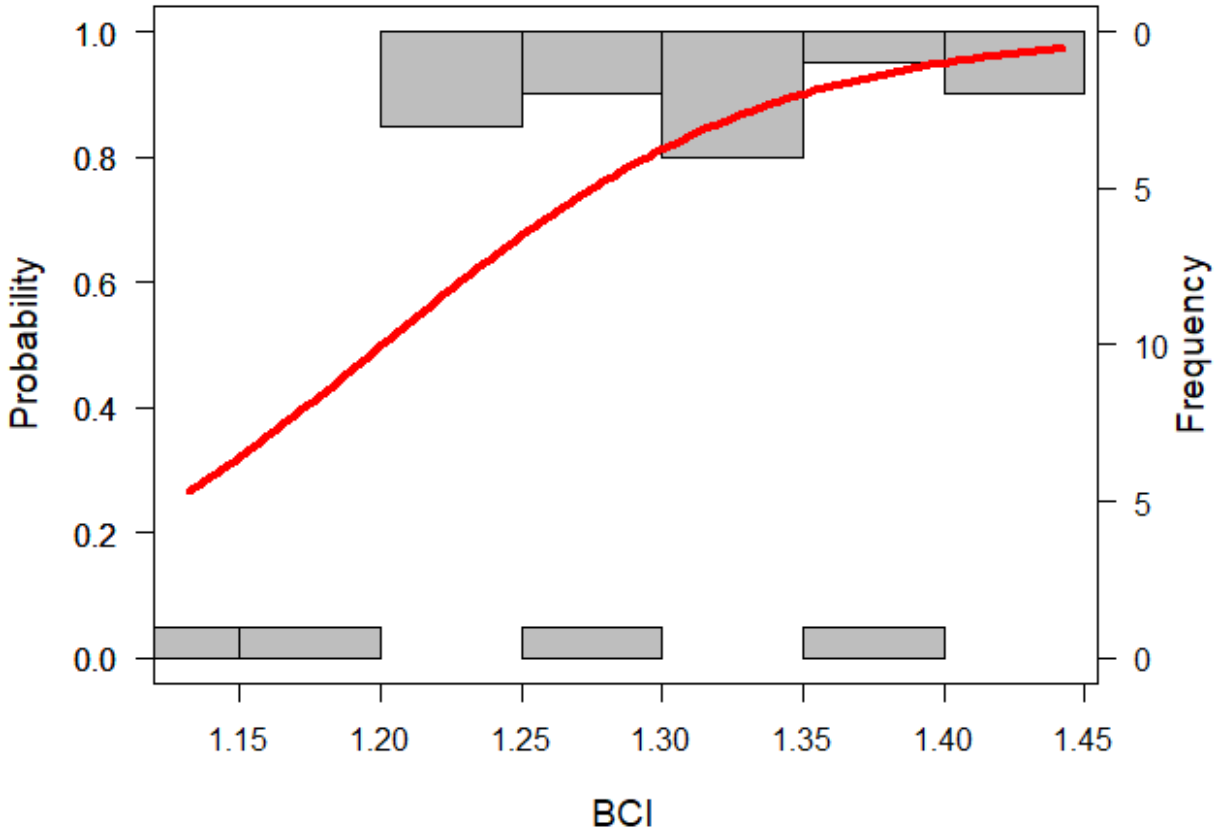

Figure 3: Binary logistic regression of shed occurrence (yes = 1 or no = 0) vs. body condition index in all animals subjected to temperature trials. The top distribution shows body conditions of animals that shed, the bottom distribution is the animals that did not. Logistic regression found no correlation between animals in increasing BCI and likelihood of shed.

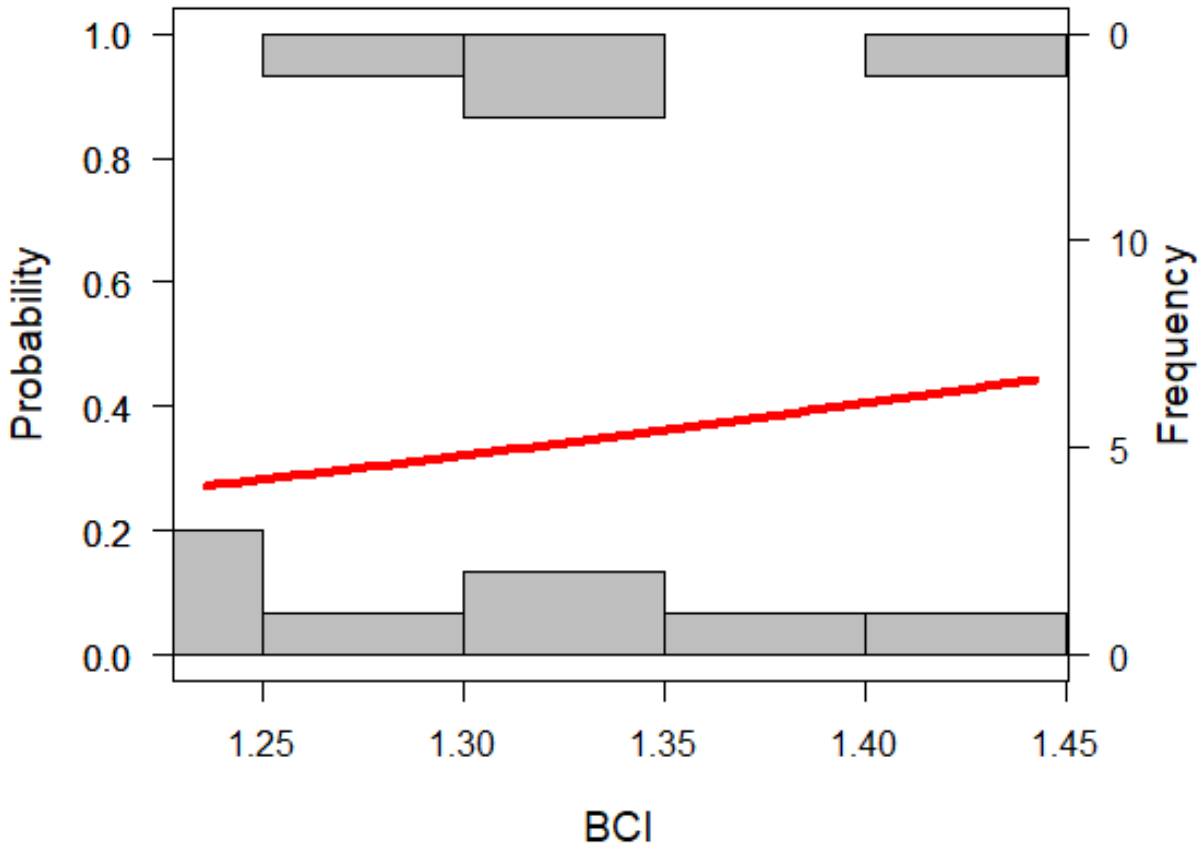

Figure 4: Binary logistic regression of animals that shed following temperature trials showing a lack of relationship between body condition and timing of shed relative to temperature increase in the animals that shed during temperature trials ("Immediate" vs. "Delayed"), animals that did not shed were not considered in this analysis but were included in an alternate binary regression (figure 3). The bottom distribution shows body conditions of animals that shed immediately following temperature spike, top distribution shows body conditions of animals who shed >1 week after. Animals in better body condition were not more likely to shed immediately following manipulations.

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Chapter 3: The Metabolic Effort and Duration of Ecdysis in Timber Rattlesnakes

Abstract

Many temperate reptiles are considered to be low energy systems, experiencing limited energetic resources during active seasons constrained by seasonal temperature fluctuations. Individuals allocate their finite time and energy towards competing functions, where maximizing the efficiency of those allocation decisions influences lifetime fitness. Among reptiles, skin shedding, or ecdysis, is a ubiquitous physiological function, requiring energy for skin synthesis and removal and time during which behavior and morphology are affected. For the first time, I measured metabolic effort exerted by individual Timber Rattlesnakes, during complete shed cycles. Metabolic data allowed us to define the time allocated towards shedding more accurately than any previous study. I produced an allometric scaling equation describing the relationship between body mass and the energetic effort of shed and found that each shed cycle requires approximately 3% of the annual energy budget, occurring over more than 4 weeks at 25ºC. Ecdysis is a significant part of the time-energy budgets of snakes, necessitating further attention in studies of reptilian energetics.

Introduction

Animals have a finite amount of time and energy to allocate towards various competing functions throughout their lives (Congdon et al., 1982; Dunham et al., 1989). The laws of thermodynamics (e.g., the conservation of energy, ever increasing entropy) act to constrain the form and function of organisms; lending tangible consequences to the allocation decision making of individuals (e.g., sub-optimal allocation decisions may lead to population decline or extirpation). The energetic constraints that govern organisms are reflected in patterns of phylogeny and life history diversity, with selection favoring life history phenotypes where the efficiency of resource allocations (in both time and energy) maximizes reproductive output and increases individual fitness (Dunham et al., 1989). The quantification of temporal and energetic effort devoted by individuals towards specific ecological and physiological functions can serve as a metric by which to investigate the ways that bioenergetics constrains organisms on ecological and evolutionary scales.

The strategy of low energy lifestyle employed by some reptiles make them useful model organisms in investigations of time-energy tradeoffs (Beaupre et al., 1993; Beaupre & Duvall, 1998; Brewster et al., 2021; Lind & Beaupre, 2015). Within reptiles, squamates have been the subject of numerous studies of temporal and energetic allocation (Beaupre, 2002, 2008; Beaupre et al., 2017; Beaupre & Duvall, 1998; Congdon, 1989; Nagy, 2000; Nagy et al., 1999; Sears & Angilletta, 2015). Substantial efforts have been made to measure the energetic effort associated with most behaviors commonly seen in lizards and snakes. There are empirical data on the metabolic effort of; hibernation (Agugliaro, 2011; Aleksiuk, 1971, 1976; Costanzo, 1989), rest (Beaupre & Zaidan, 2001; Chappell & Ellis, 1987), growth (Beaupre & Zaidan, 2001; Nagy, 2000; Peterson et al., 1999), digestion (Funk, 2019; Secor, 2009; Zaidan & Beaupre, 2003), movement (Secor et al., 1992; Walton et al., 1990), mate searching (Lind et al., 2016; Lind & Beaupre, 2015), and offspring development (Van Dyke et al., 2012; Van Dyke & Beaupre,

2011). Among squamates, rattlesnakes make exceptional model organisms for the isolation and quantification of activity specific metabolic rates as well as computations of energy budgets (Beaupre & Duvall, 1998) because they perform a small number of mutually exclusive behaviors (e.g., hibernation, foraging, digesting, shedding, mate searching, gestation; King & Duvall, 1990) with relatively small (~100,000 kJ across a 20 year life-span; Beaupre et al., 2017) total energy budgets (facilitating accounting of a majority of energetic expenditures; Beaupre et al., 2017). By measuring the metabolic and temporal effort that rattlesnakes devote towards each requisite behavior, I can address questions about the ways that thermodynamics and energetics constrain life histories and influence patterns in survival and reproduction in reptiles.

To date, little effort has been made to estimate the requisite effort for the most recognizable reptilian behavior; ecdysis. Among reptiles, shedding is unique in squamates in that the integumental layering of multiple keratin types necessitates the periodic sloughing of an entire epidermal layer in a single sheet (as in snakes) or in smaller chunks (as in lizards; Landmann, 1986). Patterns of shed in snakes facilitate the measurement of the entire associated effort, rather than attempting to disentangle the nearly continuous shedding of single scutes and scales as in turtles and crocodilians or the patchy and prolonged sloughing process seen in lizards. The energetic and temporal effort of ecdysis affects all reptiles, but reliable measurements of the associated efforts are needed to improve our understanding of energy budgets in this species rich clade (Pincheira-Donoso et al., 2013).

The cytological steps of shedding in snakes are well understood (Maderson, 1965; review); briefly, animals undergo a prolonged and variable period known as “perfect resting phase” (stage 1) during which no cell proliferation occurs, once a shed is triggered (by some unknown set of conditions) cell proliferation begins (stage 2; beginning of the epidermal renewal cycle), cell development and differentiation proceed as the underlying tissue layer develops into a replacement epidermis (stages 3-5; includes visible changes that indicate shed), finally

culminating in the removal of the exterior epidermal generation (stage 6). The series of cellular events suggests that the energetic investment in the replacement of the epidermis begins prior to the appearance of visible external cues of an impending shed (e.g., occlusion of the spectacles, dulling of the skin, changes in behavior). Therefore, accurate estimation of the metabolic effort of skin shedding necessitates metabolic measurements of animals; 1) at rest before a shed, 2) during the early stages of the process (which can be difficult to reliably diagnose visually), and 3) during the more conspicuous stages where external cues are visible.

The total energetic effort required at each shed includes the metabolic effort involved in biosynthesis of a new epidermal layer (Energy of biosynthesis; E_b), the energetic effort required to physically slough the old skin (Energy of removal; E_r), and the caloric content of the newly produced tissue (to be discarded at the next shed; Energy deposited in sloughed skin; E_s). The few studies that have attempted to estimate the energetic effort of ecdysis in squamates have used the energetic content of shed snake skin to evaluate the cost of the entire process (measured via bomb calorimetry; Blem & Zimmerman, 1986; Smith, 1976), assuming E_s to be the total or most significant cost while ignoring E_b and E_r. As a result of the lack of reliable, wholistic metabolic estimates, ecdysis is rarely considered in the accounting of the energy budgets of squamates. Incorporation of shedding into our understanding of reptilian energy budgets requires empirical measurements of the missing portions of ecdysis (E_b, E_r).

A vital caveat to the measurement of ecdysis-specific effort is the measurement of the process in the absence of other overlapping behaviors (such as movement or digestion), which may also elevate an individual's resting metabolic rate. While field measurements are precluded by this caveat, controlled laboratory measurements via indirect calorimetry are a suitable option.

To estimate the total metabolic and temporal effort of ecdysis, I used open flow respirometry to measure the metabolic effort of skin synthesis in a sample of Timber

Rattlesnakes. To circumvent difficulties in predicting the timing of shed events in animals enrolled in my laboratory study, I collected field animals at emergence from hibernation prior to seasonal shedding events (Chapter 1) or at induced emergence following an artificial hibernation (Chapter 2; Agugliaro, 2011). I placed pre-shed animals into individual chambers and collected metabolic data before, during, and after a shed event. I theorized that the energetic effort of biosynthesis associated with the replacement of the body's largest organ would be significant and of sufficient magnitude to be detected via open flow respirometry. I predicted that the magnitude of the associated effort would be similar to the effort of protein synthesis for growth (Beaupre & Zaidan, 2001; Nagy, 2000) and be allometrically related to body size; scaling as surface area (of the skin covering) scales to body volume (~2 to 3). I also hypothesized that the true duration of the process exceeds previous estimates, with metabolic effort of synthesis preceding the appearance of the external visual cues (which are the result of progressing tissue synthesis) typically used to delineate the duration of shed events. Here, I present data on the size corrected effort of skin synthesis (energy budget), the total duration of the shed process under constant temperature (time budget), the effort of physical sloughing of the old skin, and compare my estimates of metabolic effort of shed to preexisting estimates reached by alternate approaches.

Methods

Study Organism

The Timber Rattlesnake (*Crotalus horridus*) is a large-bodied pit viper who's current or historic range encompasses much of the eastern United States. *Crotalus horridus* occupies the forests of eastern North America; possessing a seasonal activity pattern limited by cooler months, particularly at more northern latitudes. Their patterns of shed vary with latitude but adults typically shed between one and three times per active season (Chapter 1; Martin et al., 2021). I elected to use *C. horridus* for the metabolic measurements herein for several reasons. First, the dynamic energy budgets of *C. horridus* are well studied (Beaupre, 2008; Beaupre et

al., 2017; Beaupre & Zaidan, 2001, 2012; Van Dyke & Beaupre, 2011; Zaidan & Beaupre, 2003) allowing for the future integration of cost of shed findings into already developed models of time and energy allocation (Beaupre, 2002; Beaupre et al., 2017). Second, in rattlesnakes the first external signal of an impending shed is the emergence of a new basal rattle segment (see Zimmermann & Pope (1948) for full review); preceding other morphological changes (dulling of color, opaque spectacles) by days or weeks (Carnes-Mason; personal observation). From the literature, it can be inferred that the emergence of the new basal segment tissue coincides with the 2nd stage (of 6; see Maderson, 1965; Maderson et al., 1970b) of shed-related cellular proliferation; earlier than the onset of color change or spectacle opacity in all other snakes which coincide with stage 3 (Table 5). The additional external cue in rattlesnakes allows for improved mapping of cellular events onto traces of metabolic expenditure. Third, as drought-adapted, low-energy specialists, rattlesnakes are well suited to prolonged metabolic measurements in low moisture environments such as those encountered in many open-flow respirometry systems. Current estimates suggest that the entire shedding process takes ~3 weeks (based on x-rays of developing rattlesnake rattle segments; Zimmermann & Pope, 1948), necessitating long term measurements to capture the time before, during and after a shed; *C. horridus* is an appropriate study organism for continuous multi-week metabolic measurements. Finally, the shed patterns of *C. horridus* are well described (Chapter 1) allowing for the collection and measurement of animals prior to any onset of shed related metabolic expenditures following spring emergence.

Open Flow Respirometry

I used a Sable TR-3 open-flow respirometry system to measure metabolic rates via indirect calorimetry using CO_2 production (VCO_2) following Beaupre & Zaidan (2001). As configured, the system allows for the simultaneous measurement of 8 lines (7 animals and one reference baseline) per sampling block. Snakes were placed in gas-tight chambers (“metabolic

chambers"; 1600-2300 mL) and external air was pumped from a remote compressor (at 90-psi), scrubbed of CO_2 and H_2O via a purge gas generator (model FT-IR 75-45; Whatman, Haverhill, MA), and split into eight separate lines through a Sable Systems MF-8 airflow manifold. The flow rate through each line was set manually via a needle valve in the manifold and checked and recorded by a Sable Systems mass flow meter downstream. Flow rates varied between 425-600 mL/min depending on chamber dead-space (calculated from snake size and chamber volume). The metabolic chambers were housed in a single environmental chamber (model 1-35LLVL; Percival Scientific, Boone, IA); set to 14L:10D (6am-8pm), 25ºC) where a dedicated line (of fixed airflow rate; recorded continually by a mass-flow meter) was connected to one port of the chamber (incurrent) while a second line carried gas from an excurrent port of the chamber, through a Sable multiplexer to a syringe barrel for subsampling. Prior to the subsampling apparatus, all gas movement was driven by positive pressure.

The eight-channel multiplexer sequentially cycled through each dedicated line every hour. Within each hour, the empty baseline chamber was sampled for 3.25 minutes at the beginning and end of the hour, while metabolic chambers were sampled for 7.5 minutes each in-between. The two baseline samples served to compensate for within-hour baseline drift post-hoc. During each sampling period, gas was subsampled at the syringe barrel apparatus via negative pressure (at ~190 mL/min; recorded continually by a mass-flow meter) and was drawn through; a naphion column for water expulsion (model MRD110-072TKV1111-0; Perma Pure, Amersham, UK; supplied by a dedicated countercurrent purge line at 2.5 psi), a chemical desiccant (Drierite) to ensure excess water was removed, a CO_2 sensor (LI 6251 Li-Cor infrared gas analyzer; Li-Cor, Lincoln, NE), and then expelled to the room air. The gas analyzer collected data every 5s and logged data to disk using the Universal Interface of the Expedata Software. Time (D/M/Y; H:M:S), CO_2 concentration (ppm), Temperature (ambient room, environmental chamber), and mass-flow rates (both positive and negative pressure portions)

were stored to disk hourly. Hourly rates of CO_2 production (mL/h) were calculated (following Lighton, 2008; Van Dyke et al., 2011) using hourly CO_2 concentrations (ppm) corrected for variable flow rates and subsampling rates with associated mass-flow meter measurements. Metabolic Rate data are presented at standard temperature and pressure.

Collection and Preparation of Study Animals

Metabolic data were collected from two separate sets of Timber Rattlesnakes. The first, "Group A", was collected in the Spring of 2022 at emergence from hibernation. These animals were handled as described below and metabolic data were collected. To improve sample size, I collected a second set of animals, "Group B", opportunistically during the 2021 and 2022 active seasons. Group B animals were subjected to an artificial hibernation, and metabolic data were collected following a warming to summer active temperatures in the laboratory in the winter of 2023 (Chapter 2). The shape, duration, and magnitude of shed-event related CO_2 production was similar between groups A and B, although sample sizes were insufficient to test for an effect of hibernation group (A or B). The morphological changes associated with ecdytic cycles, integrity of sloughed skin, and general appearance of post-shed animals was consistent between groups A and B. I assumed shed events were comparable between groups and present both data sets together, minor differences in animal treatment are described when relevant.

I collected Group A animals (N=7) at emergence from overwinter brumation in natural hibernacula in Northwest Arkansas (determined by season, temperature, and close association of individuals to bluffs and rocky outcroppings possessing the characteristics of suitable hibernacula) and transported them to the laboratory for holding. In the laboratory, animals were initially held in 10G aquaria in a climate-controlled room (22-24ºC) with a large window; ensuring exposure to natural light cycles and some diel temperature cycling. Animals were collected over a 6-week period until natural light cycles reached 14L:10D (16 May, 2022), at which point they

were processed for morphometric characteristics (mass and snout vent length; SVL), examined for any external signs of impending shed, placed in gas tight chambers, and transferred to a light- and temperature-controlled environmental chamber (14L:10D, 25ºC).

Metabolic measurements were collected from group A animals for six days to serve as a baseline for future data collection. After six days, animals were removed from their gas tight chambers, returned to 10G glass aquaria in the holding room (with window) and offered a food item (thawed pre-killed rats or mice) between 7-26% of the animals' body mass. Feeding was deemed necessary based on the predominant theory that shed timing and frequency may be tied to available resource pools or the timing of food acquisition in the spring (Chapter 1; Collins, 1992; Heyrend & Call, 1951; Klauber, 1972; Macartney et al., 1990). After feeding, animals were allowed to digest for 10-14 days (the average time of the specific dynamic action response; Beaupre & Zaidan, 2012) to avoid stress induced regurgitation and prevent excessive CO_2 production that may have overloaded my CO_2 sensor in its described configuration. Once animals no longer had a visible food bolus, they were weighed, examined for signs of impending shed, returned to their gas-tight metabolic chambers in the environmental chamber (14L:10D, 25ºC), and reconnected to the respirometry system.

I recreated hibernation conditions for Group B animals (N=16) using a walk-in environmental chamber (Model 13030, BioCold Environmental Inc., Fenton, MO) and measured energetic effort of ecdysis in the shed events which followed the hibernation event. In preparation for trials, animals were fed a medium sized meal (10-20% body mass), fasted for 1 month (~22ºC, 12L:12D; ensuring animals were not in shed prior to temperature treatments), acclimated to decreasing temperature and shortening photoperiod (Chapter 2; Agugliaro, 2011), held at 10ºC under constant dark for one month, and then rapidly warmed to summer active temperatures (33ºC) for one week, before being placed in respirometry chambers in a full height incubator and maintained at 25ºC; 14L:10D. Of the Group B snakes, a subset showed no sign

of impending shed and were in visibly healthy condition (N=9 of 16) following artificial hibernation, the remaining seven snakes were in shed prior to removal from the environmental chamber (see Chapter 2 for details). I selected seven animals (of the nine suitable individuals) which best filled gaps in the distribution of body sizes (from Group A animals), placed them into gas-tight metabolic chambers (described above) and collected metabolic data in an identical fashion to Group A (see above). Snakes were not fed after the artificial hibernation event because the animals' dietary history was known and the timing of shed events was not correlated to feeding in Group A animals (see below).

I logged CO_2 production hourly following the sampling protocol for the entirety of each of two experimental runs (Group A, Spring/Summer 2022; Group B, Winter/Spring 2023). Once an animal sloughed its skin it was maintained in its metabolic chamber for an additional 2 weeks (to capture any post shed cost associated with the continued maturation of the new epidermal layer), then it was weighed and removed from the respirometer.

Monitoring and Care

Snakes were checked daily for signs of impending shed (emergent basal segment, dulling of the skin, opaque eyes), dehydration, or expedient decline in body condition (an indicator of poor health warranting removal from the trial for the safety of the animal). Apart from the food administered at the start of the study (Group A only), no animal was fed for the remainder of the experiment. Water was offered through the gas inlet ports of the metabolic chambers (~15-50mL depending on animal size) approximately biweekly.

Data Analysis

Data were collected using Expedata, the proprietary software of Sable Systems Inc. During each sampling interval, 90 measurements of ppm CO_2 produced were recorded per individual (7.5 minutes per individual per hour / 5 seconds between samples). Prior to export from the Expedata environment, the baseline endpoints (3.5 minutes of CO_2 and H_2O free air)

were used to correct each hour-long block for baseline drift (Expedata function; *correction drift*), ppm values for each gas were converted to volume (mL) at STP following Lighton (2008; equations 11.7, 11.8). All 90 measurements per individual during each hour were averaged to a single mean value for each variable for each individual for that hour (i.e., 1.2 mL CO_2 per hour). Data were batch processed, exported as .csv files, and imported to the R software environment (Base-R version 4.0.2).

In R, I plotted average volume of CO_2 produced per hour by time (in number of hours since run start) for each individual across the length of the study. Clear baseline resting metabolic values emerged for each individual as well as an associated curve attributable to the shedding process (Figure 1,Figure Figure). During measurements events such as daily run turnovers, watering events, visual inspections for sign of shed, and automated light cycling resulted in temporary increases in metabolic rates (assumed to be the result of minor stress and increased alertness or physical activity) above the apparent resting metabolic rate. To smooth the curves I removed data collected in the 2 hours following a turnover/visual inspection/watering event and the 1 hour after each change in light (6am and 8pm). To reduce noise across the samples, I constructed blocked averages of CO_2 production for each 5-hour portion of time (i.e., values for 1pm-5pm were averaged to give a single value, centered on 1pm on the x-axis).

Volume of CO_2 for Skin Synthesis (E_b)

Once each curve was smoothed, I fit a linear model to the CO_2 production associated with a period of 1-3 weeks of sustained resting metabolic rate, prior to the onset of ecdysis-related increases in metabolism (Figure 1**Error! Reference source not found.**; red line). The slopes of my linear models were slight (all < 0.001) and non-significant (All $p > 0.05$), and so I assumed the y-intercept (experiment start) for each line (slope=0) was a suitable value for a resting metabolic rate "baseline" to which shed related increases could be compared. To

estimate total CO_2 produced for each shed event, I calculated the area under the curve (integrated under the trapezoidal rule; R package pracma, function “trapz”) between shed start (determined by visual inspection of the data for an inflection point after which CO_2 production deviated from previous baseline values and made up the beginning of the shed-associated curve) and 2 weeks post slough for each 5-hour CO_2 production mean minus the value of the RMR baseline (Figure 1; green area). The metabolic effort above the curve associated with the spike in physical effort of skin removal (Figure 1; purple shaded peak) was removed from each estimate of total area under the curve of CO_2 produced to give a biosynthesis specific estimate (E_b) for each individual.

Volume CO2 of Physical Sloughing (E_r)

I recorded the date of each animal’s shed to pinpoint the period of time during which animals invested energy towards the physical removal of the skin (E_r). For all individuals, there was a clear spike in increased activity lasting 4-50 hours associated with the date of sloughing. To estimate the volume of CO_2 associated with the activity of skin removal, I subsampled the time range of that activity spike, as well as 4 hours before and after its onset (pre- and post-slough periods; Figure). For E_r estimates I used single hour average CO2 production (rather than the 5-hour smoothing approach taken for total effort estimates) to avoid underestimation of this brief and variable (between hours) process. I constructed a linear model between pre-slough and post-slough CO_2 (Figure 1, blue line; Figure) and assumed the area under the curve above that line to be equal to the effort of physical removal (E_r; Figure 1; green shaded area). Because biosynthesis (E_b) is ongoing during the physical removal of old skin, I used the equation for the E_r specific linear model, to subtract the mean CO_2 production associated with RMR and E_b during the sloughing spike and achieve a cleaner estimate of Er (Figure 1; isolating purple shaded peak by subtracting the area below blue line in area C from total CO_2 production in the same time interval; integrated under the trapezoidal rule; R package pracma, function

"trapz"). I subtracted my estimate of E_r from the total CO_2 production estimates (above) to split CO_2 production for each individual into component pieces, E_b and E_r.

Construction of the Mass Adjusted Scaling Equations

Using my estimates of CO_2 produced, I constructed an allometric scaling equation for E_b which predicts metabolic effort of that component piece of ecdysis given body mass. E_r was not allometrically related to body mass (see below). For the analysis of allometric relationships, data were log transformed to linearize the relationships and linear models were fit to the data. The equation for each line was anti-log transformed (following Bennet & Dawson, 1976) to give the resulting allometric scaling relationships.

Statistical Analyses

I assumed a 5% type I error for all statistical analyses. Means are presented as mean ± 95% confidence interval unless otherwise noted. For analyses that employed linear regression, I tested the distribution of residuals for normality using Shapiro-Wilk tests. Because residuals of linear models were all approximately normally distributed, I used parametric statistics. I tested for relationships between shed timing and meal size by ANOVA.

Results

I collected juvenile, subadult and adult *C. horridus* (approximate age range estimated from size and rattle string characteristics; 3-20 years old) with a range in mass representative of the population (117.81g – 1114.8g; mean = 363.7 ± 620.14; based on long-term mark-recapture data). Animals were held in metabolic chambers for 52-125 days (Group A); 51-88 days (Group B). In Group A six of seven animals shed during trials (Figure). The seventh animal (female, 120g, fed 13% body mass) did not undergo ecdysis and was removed from the respirometer at 125 days (Figure). In Group B, three of seven animals shed during trials (Figure Figure).

Of the nine recorded shed events, the average length of the shed process (from increased metabolic rate to physical skin removal) was 28.4 ± 4.39 days (range 24-32; Table 5).

In Group A animals, shed events were clustered in the late summer, occurring between August 2nd and September 3rd (85-117 days post feed, mean = 104.2 ± 25.5 days). The metabolic initiation of ecdysis occurred approximately 8-12 weeks after feeding in Group A (mean 76.2 ± 26.1 days, range 57-89 days), well after a return to RMR following specific dynamic action.

Analysis of Variance revealed that meal size (in grams or as a percentage of body mass; Figure) was not a significant predictor of the duration of shed events (p-values: grams > 0.15, percent > 0.06) or the timing of shed events relative to feeding (p-values: grams > 0.67, percent > 0.72).

The first external indication of an ongoing shed event (the appearance of an early emergent rattle segment) in all animals was not detectible until 8.7 ± 8.6 days after the increase in metabolic activity associated with epidermal renewal (range 3-18 days). Using morphological features to determine the intervals of the six cytological stages of ecdysis revealed that the duration of each phase was variable among individuals (Table 5) even though total duration was relatively consistent across my sample.

The total metabolic effort of skin synthesis was allometrically related to body mass (Table 2), whereas effort of physical removal was best described as random with respect to body mass (variance of allometric slope includes 0; Table 7). Total metabolic effort ($E_b + E_r$) ranged from 536 to 6,026 mL CO_2 produced (Figure ,Figure) with energetic effort of removal (E_r) making up 2-21% (mean = 7.75% ± 11.5%) of the total effort measured. The allometric scaling relationship relating body mass to metabolic cost of biosynthesis (E_b) is given by (Table 2);

$$E_b = 15.2 * Mass^{0.88} \qquad \textit{Eq. 1}$$

where E_b is the cost of skin synthesis in mL CO_2 (1 mL CO_2 = 27.42 J; Gessaman & Nagy, 1988), and Mass is the snake's body mass in grams (Table 6; Figure).

E_r was independently variable and not related to body size or E_b. The mean E_r for all shedding observations was 118.1 ± 64.4 (1 SE; n=9) mL CO_2 which is the equivalent of 3.2 ± 1.7 (1 SE; n=9; assuming 27.42 J/mL CO2; Gessaman & Nagy, 1988) kJ. Mean E_r ranged from 2.5% to 27.4% of observed effort of biosynthesis (E_b).

Discussion

Time Budget

Shedding is an important component of the time budget of snakes. Individuals alter their behavior, decrease activity, and seek refuge during the phase of the shed when the spectacles become opaque (Gibson et al., 1989; Hirth et al., 1969; Turmo, 1996). It has been suggested that the one- to two-week period of refuge seeking occurs as a response to increased susceptibility to predation (R. B. King & Turmo, 1997), increased energy demands (Blem & Zimmerman, 1986), or in pursuit of more favorable microclimates suitable for skin sloughing (Gibson et al., 1989; Kitchell, 1969). Regardless of the motivators, snakes seldom move or forage during at least part of the shedding period, limiting the amount of time that they can allocate towards other functions such as mate searching, courtship, and reproduction and potentially reducing their total yearly energy intake. As such, understanding the total duration of shed events allows us to evaluate the degree to which ecdysis limits the time budget of snakes.

Currently, estimates of shedding duration are derived from observations of spectacle occlusion (Cazalot et al., 2015; Tusler et al., 2015; 5-10 days), cytological evaluation of epidermal tissue samples (2-3 weeks; Maderson, 1984; Maderson et al., 1970b), and, in rattlesnakes, x-ray photography of the emergent basal rattle segment (*C. atrox*, 23 days; Zimmermann & Pope, 1948). However, each of these estimates relies on the appearance of morphological changes that only become evident sometime after ecdysis related cellular proliferation begins. Of the available estimates, those derived from microscopy of skin biopsies (Maderson et al., 1970) are likely the most accurate. The cytological approach can account for

changes not visible to the naked eye, but still relies on some degree of observer assessment for categorization into one of the six somewhat overlapping stages of ecdysis (as defined by Maderson, 1984). Accurate estimation of the timing of the initiation of the process is a prerequisite for measuring total duration and is sorely needed for studies attempting to identify the causal mechanisms that elicit shed events in squamates (requisite environmental, seasonal, or biophysical conditions). Using measurements of metabolic effort, I was able to measure the duration of the entire process, including the early stages of upregulated cellular proliferation that may be overlooked by other estimates. According to my data, at 25ºC the average shed event takes nearly a week longer (28.4 +/-4.4 days) than estimates derived from tissue biopsies (Maderson et al., 1970) with some additional metabolic effort occurring after physical sloughing. I attribute the metabolic effort post-slough to the continued maturation of the newly developed epidermal layer (Landmann, 1986). While body temperature throughout the epidermal renewal cycle likely plays a role in dictating the duration of the process (as it does for most physiological processes in ectotherms), to my knowledge it has never been reported in conjunction with studies of the duration of ecdysis; preventing direct comparisons between my estimate and others. Repeated measurements at alternate temperatures are necessary to better quantify the duration of sheds and their impact on the time budget, but my metabolic method provides higher resolution information on the duration and timing of initiation of ecdysis than any previous study.

A direct relationship between feeding, growth and shedding in snakes has long been assumed (Burkett, 1966; Collins, 1992; Heyrend & Call, 1951; Klauber, 1972; Macartney et al., 1990; Smith, 1976) but recent evidence suggests that the cues that initiate shedding are more complex (Chapter 1; Reiserer, 2016). In my data, there was no clear relationship between the timing of feeding and the timing of shed events in natural shedders as all sheds initiated between two- and three-months post feeding. Likewise, analysis of variation in shed timing in relation to the magnitude of meal size (as percent total body mass or raw mass) by ANOVA

revealed no significant effect of energy intake on the immediacy or duration of shedding in Timber Rattlesnakes. My data do not support a strict feeding-shedding-growth relationship (in agreement with Chapter 1) as singly fed animals did not initiate shed events soon after feeding and digestion, but most did eventually shed in close temporal proximity to one-another under identical conditions (during summer 2022, all Group A animals shed within 30 days of one another in apparent synchronization).

Given my limited sample size, conclusions about the duration of portions of the shed cycle are best viewed as preliminary. However, several interesting patterns are suggested by the present data set (Table 5). First, it is noteworthy that total length of shed was relatively well conserved at 25ºC (28.4 ± 4.4 days). Also of note is the tight clustering of the length of time after the spectacle clears until the skin is removed (3-5 days). Other portions of the process appear more variable at 25ºC, such as duration of spectacle occlusion. It seems reasonable that the portion of the ecdytic cycle that impairs an individuals' vision should be temporally minimized by natural selection, but further investigations of variation in the duration of spectacle occlusion among individuals, populations, or environmental conditions are needed.

Mass-Metabolism Relationships

Allometric scaling relationships describe how a characteristic of an organism scales with body size. Because the metabolic effort required to produce epidermal tissue is likely directly correlated to the total amount of tissue synthesized, I anticipated a relationship between body mass and total energetic effort required to produce a new epidermal layer. I used log-log transformations to linearize the curvilinear relationship between mass and metabolic effort and interpret the resulting linear relationship and its "allometric slope" (i.e., the exponent to which the equation scales) as descriptive of that allometry. I found that the allometric slope of metabolic effort of ecdysis was steep (0.88) for E_b, but the range of estimates (-0.29 – 0.67) for E_r included

0; indicating that physical effort of removal may not be a function of body size in Timber Rattlesnakes.

From my data, I expected that the total surface area of epidermal tissue synthesized would scale with body mass and therefore E_b would scale to approximately 0.67 (Heusner, 1982), the rate at which surface area scales with volume. My allometric scalar of 0.88 appears to exceed that which would be expected under the assumption that metabolic effort of skin renewal is a surface area function but given variance terms (Table 6), I cannot refute a strict surface area to body mass ratio (95% CI range of allometric scalar = 0.55-1.21). Additional data are necessary to improve the precision of my equations, but confirmation of the reported allometric scalar above the expected 0.67 value may be a reflection of the 3-dimensional structure of epidermal tissue and the numerous physiological changes which accompany/facilitate ecdysis (e.g., production of lymph tissue to lubricate skin removal, mobilization of lipids to increase the resistance of skin to water loss).

Conversely, the derived E_r scalar was not significantly different from 0, suggesting that the effort of removal is not related to body mass. E_r relies upon the ease of removal of the outer epidermal layer and is therefore dependent upon relative humidity, hydration state of the animal, and availability of suitable surfaces to rub against to aid in removal. In my experiment, animals were housed in smooth plastic containers and watered biweekly, possibly altering the total effort of removal based on individual success in finding purchase to begin the sloughing process and temporal relation to the time of last water offering. The data indicate that the variation in E_r observed in my experiment may reflect the ease with which an individual was able to slough their skin, rather than a body mass relationship. Low sample size hampers the power of my conclusions, with each individual point contributing significantly to the derived relationship, but it seems logical that total effort of removal should vary with the ease of removal. More data may be needed to detect an underlying body-size relationship, but I suggest the use of the average

effort of removal (118.1 mL CO_2; 3.2 kJ) as a suitable estimate of the effort of physical removal until more data are available.

Comparison to Published Estimates

The total metabolic effort required per shed event includes the metabolic effort of tissue biosynthesis in the production of a new epidermal layer, the energetic content of the newly deposited skin (lost at the next slough), and the energy lost as physical effort required to remove the old skin. Here, using open-flow respirometry, I report the first empirical measurements of E_b and E_r. Following my equation, for a 500g animal the expected metabolic effort of each component is 3,615.03 mL CO_2 (E_b) and 118.1 mL CO_2 (E_r). Given a conversion of 27.42 Joules/mL CO_2 produced (Gessaman & Nagy, 1988), E_b= 99.0 kJ and E_r = 3.2 kJ per shed event.

Prior to this work, consideration of the energetic aspect of ecdysis has relied solely on measurements of the energetic content of shed skins (Blem & Zimmerman, 1986; Smith, 1976). Even by these conservative estimates, ignoring cost of synthesis and physical removal, species that shed frequently allocate a significant proportion (2-11%) of their total energy budget towards shedding (Smith, 1976). While these few bomb-calorimetry studies concluded that shedding occupies a significant portion of the total energy budget, the energetic cost of ecdysis has been largely trivialized, overlooked in the majority of literature as a feature of reptilian biology of minor energetic concern. However, I believe the disregard for cost of shed is problematic for the study of reptilian energy budgets as an estimate of E_b using simple extrapolations of skin dry mass production and cost of tissue synthesis are of significant magnitude. I used Blem & Zimmerman's (1986) equation for the mass of shed skin produced ($Dry\ Mass\ Skin\ (g) = 0.01308 * \ Body\ Mass^{0.86}$) and scaled those values by the metabolic effort of tissue synthesis in Timber Rattlesnakes (10.5 kJ/g; Beaupre and Zaidan, 2012) giving 29.3 kJ for E_b in a 500 g Timber Rattlesnake (though even this estimate is conservative as Blem

and Zimmerman did not account for the energetic content (and dry-tissue mass) of the newly appended rattle segment in *Crotalus*). My empirical measurements of E_b indicate that the true cost of ecdysis is greater still, roughly triple that reached by the mathematical approach to estimate E_s alone outlined above, further supporting my assertion that the cost of shed is worthy of the consideration in squamate energy budgets.

Total annual and lifetime energy budgets for a "typical male Timber Rattlesnake" have been reported in the literature (Beaupre et al., 2017; updated with shed values; Table 8). Total annual energy budget for a ~500g animal was estimated to be 4,790 kJ with 829 kJ of that allotted towards growth (Beaupre et al. 2017). Therefore, by my estimates the cost of skin synthesis and physical removal from a single shed accounts for approximately 2.1% of the yearly energy budget. If shedding is considered a growth function, occurring in conjunction with changes in body size, a single shed event accounts for 12.3% of the growth allotment for the year. While these values are surely non-trivial for a low energy species, Timber Rattlesnakes have been shown to shed multiple times in a single year (although infrequently; Chapter 1; Martin et al., 2021) doubling or even tripling the yearly effort of ecdysis in these animals. Furthermore, the energetic content of the newly deposited skin (approximated following Blem & Zimmerman (1986) and assuming the energetic content of the new skin to be shed during the next molt to be approximately equal to that sloughed in the present cycle; $\text{Total E content (kJ)} = 0.258 * Mass^{0.88}$) for the same 500 g animal is 61.0 kJ. Summation of each portion of the cost of shed ($E_b + E_r + E_s$) brings the total cost per shed event to 163.2 kJ (at 500g), 3.4% of the total energy budget per shed, 19.7% of the yearly growth budget of an adult male timber rattlesnake (6.8% of total and 39.3% of growth budget in an animal who sheds twice per year). In ecological terms, 163.2 kJ of metabolizable energy is equivalent to 30.3 g of mouse tissue (assuming metabolizable energy is 80.6% of ingested energy (Beaupre & Zaidan, 2012); rodents contain 23.311 kJ per gram dry mass and are 71.4% water (Beaupre et al., 2017))

meaning that in my Arkansas population of *C. horridus*, a 500g snake must consume an additional ~2 adult mice (avg mass of an adult *P. maniculatus* in NW Arkansas Ozark Forests ~15.4 g; Sealander, 1964) per shed event per year.

Comparison of Natural and Artificial Hibernation Outcomes

Metabolic effort of ecdysis appeared comparable between Groups A and B. Addition of Group B metabolic data to an earlier model (Group A only) of the logMass vs. $\log E_b$ relationship had minimal effect on the equation of the linear model or the goodness of fit (R^2 = 85% Group A, 80.6% Groups A and B). Discrepancies in feeding in relation to hibernation emergence are unlikely to have impacted outcomes because digestion and processing was completed >2 months prior to the commencement of metabolic measurements in all cases. The largest difference between groups was in binary outcomes of ecdysis. Natural hibernation animals shed with higher frequency (6 of 7) than artificial hibernation animals (3 of 7 in respirometer, although in the larger captive population, 11 of 16 shed but the additional 8 animals were not part of the metabolic measurements reported here; see Chapter 2). I am unsure of the cause of this discrepancy, but internal clocks (artificial hibernation in Group B commenced in November and concluded in January) or timing of most recent preceding shed (see Chapter 2 for discussion) may play a role.

Conclusion

My data suggest that ecdysis is a significant energetic and temporal undertaking for Timber Rattlesnakes, requiring a significant portion of the total energy budget and impacting an individual's behavior for 4-8 weeks of the active season each year (depending on shed frequency). Because of its large impact on time-energy budgets, ecdysis is clearly an important aspect of reptilian life history. However, my use of *C. horridus* as a study organism may bias my wholistic conceptualization of the energetic requirements of ecdysis. Rattlesnakes produce a more water-tight, thicker, and energetically expensive integument relative to other taxonomic

groups (Blem & Zimmerman, 1986; Roberts & Lillywhite, 1983), studies with genera displaying alternate renewal characteristics (more frequent shedding, less water-resistant integuments) are necessary to further detail ecdysis in squamates. Still, the derivation of the scaling equation presented here offers a method by which energetic cost of shed can be more completely estimated and incorporated into studies of reptilian energy budgets. My metabolic approach to the estimation of the time required to produce a new shed also provides more precise measurements that can improve the modeling of reptilian time budgets, but more data are needed on the extent to which different portions of the shed process constrain other activities. More information is needed to discern the effects of temperature on the total effort required to shed, but such studies may reveal that thermoregulation plays a key role in minimizing time-energy budget impacts from this mandatory reptilian process. Modeling exercises incorporating the metabolic scaling equations and temporal estimates of ecdysis into a larger model of Timber Rattlesnake energy budgets are in progress and will be the subject of future communications.

Acknowledgements

I thank Jason Ortega for his invaluable assistance with the initial set up of the open flow respirometry system. All aspects of this work were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC protocols: 20049, 20062, 23011, and 23023)

Figures and Tables

Table 5: Key Cytological and Morphological events of ecdysis with average lengths as determined by observations of external changes. Lengths of phases are counted from the first observation of the key morphological features until the appearance of the key identifiers of the next phase. Note that phase 1 is the time between shed events which can vary in length from days to months. During phase 1 some continued maturation of new tissue occurs as a holdover from the preceding shed event. My estimation of the duration of stages 3 and 4 is limited as I did not use any techniques to determine the intensity of occlusion, instead I lump these two phases together as the time period during which spectacles are occluded. Duration of stage 6 was estimated based on number of hours showing elevated metabolic effort during the time of physical sloughing, it does not include the periods of rest within that time, or the time of continued maturation of the new epidermal layer. Total length from metabolic start to physical shed removal is denoted in blue.

Phase	Key Cytological Events	Morphological Features	Days to start of next phase
1	No Activity; "Perfect Resting Phase"	No Evidence	Variable
2	Cellular Proliferation; Beginning Differentiation of Epidermal Layers	Emergent Basal Segment; Opaque Basal Segment	9.5 ± 9.0
3	Cellular Proliferation; Cellular Differentiations	Beginning of Occular Occlusion	5.8 ± 4.3
4	Development of Division Between Old and New Layers; "Stratum Intermedium"	Peak Occular Occlusion; Dull Color	
5	Lacunar Tissue Dissociates; Keratinization of Inner Layer; Inner and Outer Generations Separate	Occular Occlusion Resolves; Clear Eyes	4.2 ± 1.6
6	Inner Layer Continues to Mature	Skin Physically Sloughed	0.69 ± 1.13
	Total Length:	28.4 ± 4.39 days	

Table 6: Regression results for the effect of body mass on metabolic effort of biosynthesis of ecdysis (mL CO_2).

Variable	Df	Parameter	SE	t-value	P
Intercept	1	1.1823	0.3940	3.001	0.01992
logMass	1	0.8803	0.1648	5.342	0.00107

Table 7: Regression results for the effect of body mass on metabolic effort of skin removal of ecdysis (mL CO_2).

Variable	Df	Parameter	SE	t-value	P
Intercept	1	1.5611	0.5771	2.705	0.0304
logMass	1	0.1947	0.2413	0.807	0.4464

Table 8: Effort of Ecdysis for animals that shed once or twice per year at different size classes. Derived from Beaupre et al., 2017, updated to include ecdysis and its relative importance in the energy budgets of Timber Rattlesnakes.

Mass (g)	Total Annual Energy Budget (kJ)	Total Annual Growth Budget (kJ)	Sheds per Year	Eb (kJ)	Er (kJ)	Es (kJ)	Total Effort of Shed (kJ)	Proportion of Total EB (%)	Proportion of Growth Budget (%)
125 (Juvenile)	1198	449	1	29.2	3.2	8.9	41.2	3.4	9.2
			2	58.4	6.4	17.7	82.5	6.9	18.4
521 (Adult, Subadult)	4790	829	1	102.5	3.2	30.3	136.1	2.8	16.4
			2	205.0	6.4	60.7	272.1	5.7	32.8
1241 (Large Adult Male)	11613	830	1	220.0	3.2	64.2	287.4	2.5	34.6
			2	440.0	6.4	128.4	574.8	4.9	69.2

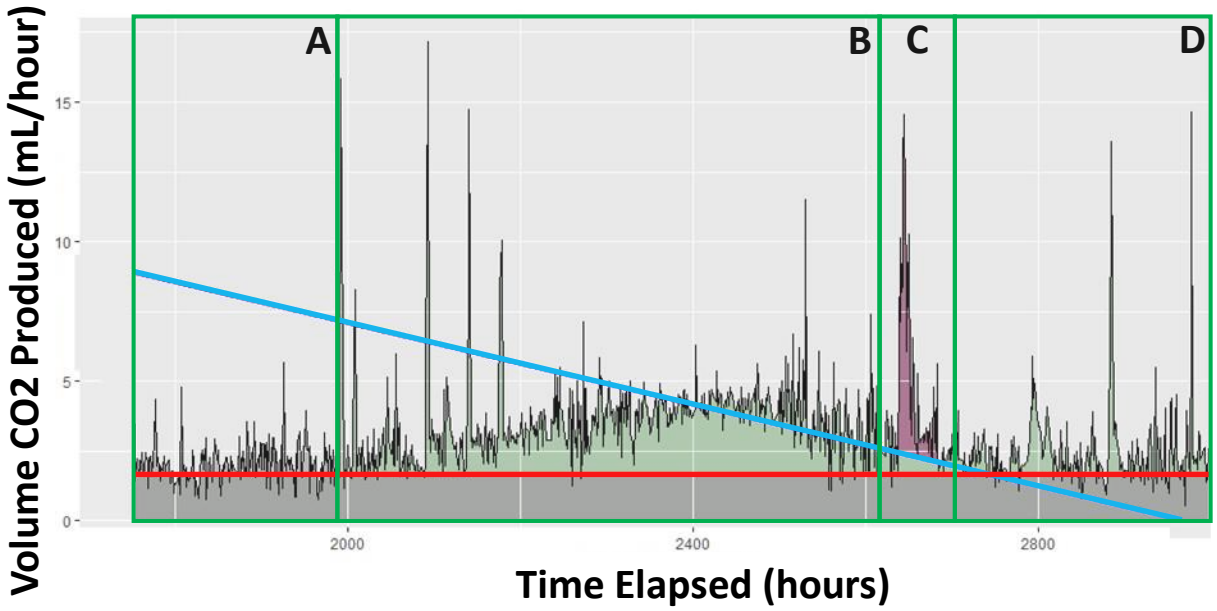

Figure 1: CO_2 production in a single animal during a single shed cycle depicting the data analysis approach for defining, isolating, and measuring the metabolic cost of biosynthesis and removal (E_b and E_r). The black line shows the average CO_2 production (mL/hour) through time, with green boxes A-D showing distinct portions of the metabolic trace – A) Before Shed, B) Biosynthesis of new shed, C) Physical removal of old skin and continued biosynthesis, and D) Post slough return to baseline metabolic rate. I fit a linear model (red line) to the pre- and post-shed response baselines (areas A and D). The area below the linear model for baseline (grey box below red line) depicts the resting metabolic rate (RMR), the average CO_2 production occurring in animals at rest not undergoing significant metabolic processes. A separate linear model (blue line) was fit to the areas of the curve before and after the physical removal of the skin (peak in area C). The area shaded in green denotes the volume of CO_2 produced above RMR in association with tissue biosynthesis (E_b). The area shaded in purple denotes the volume of CO_2 associated with physical removal of the old skin (E_r). Note that the total metabolic activity in area C includes RMR, Eb and Er occurring simultaneously – necessitating the use of multiple linear models to disentangle the composite portions of the shed response.

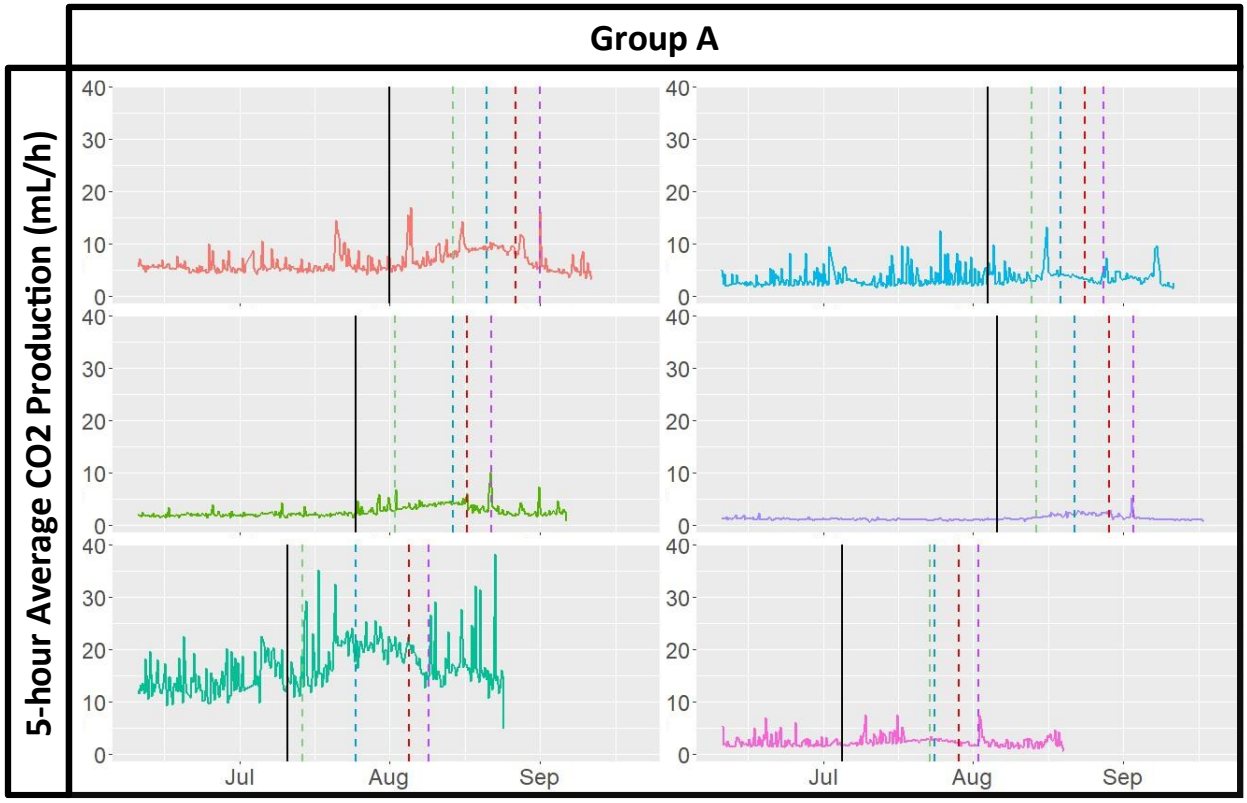

Figure 2: CO_2 Production of Group A animals that shed during the trial at rest, during ecdysis, and post shed. Lines are based on 5 hour lumped averages of CO_2 production. Each panel is a different individual. The vertical blackline denotes the "metabolic start of ecdysis", the time at which the slope of the baseline changed and the time from which metabolic effort was calculated. Vertical dashed lines denote the day that various visual cues or important events of the shed were first noted, green=first visual sign (usually emergent basal segment), blue=eyes begin to cloud, red=eyes clear, purple=physical sloughing. Note the resting metabolism for each animal, the large curve above resting that correlates to the metabolic process of ecdysis, and the spike associated with the purple line that corresponds to physical sloughing.

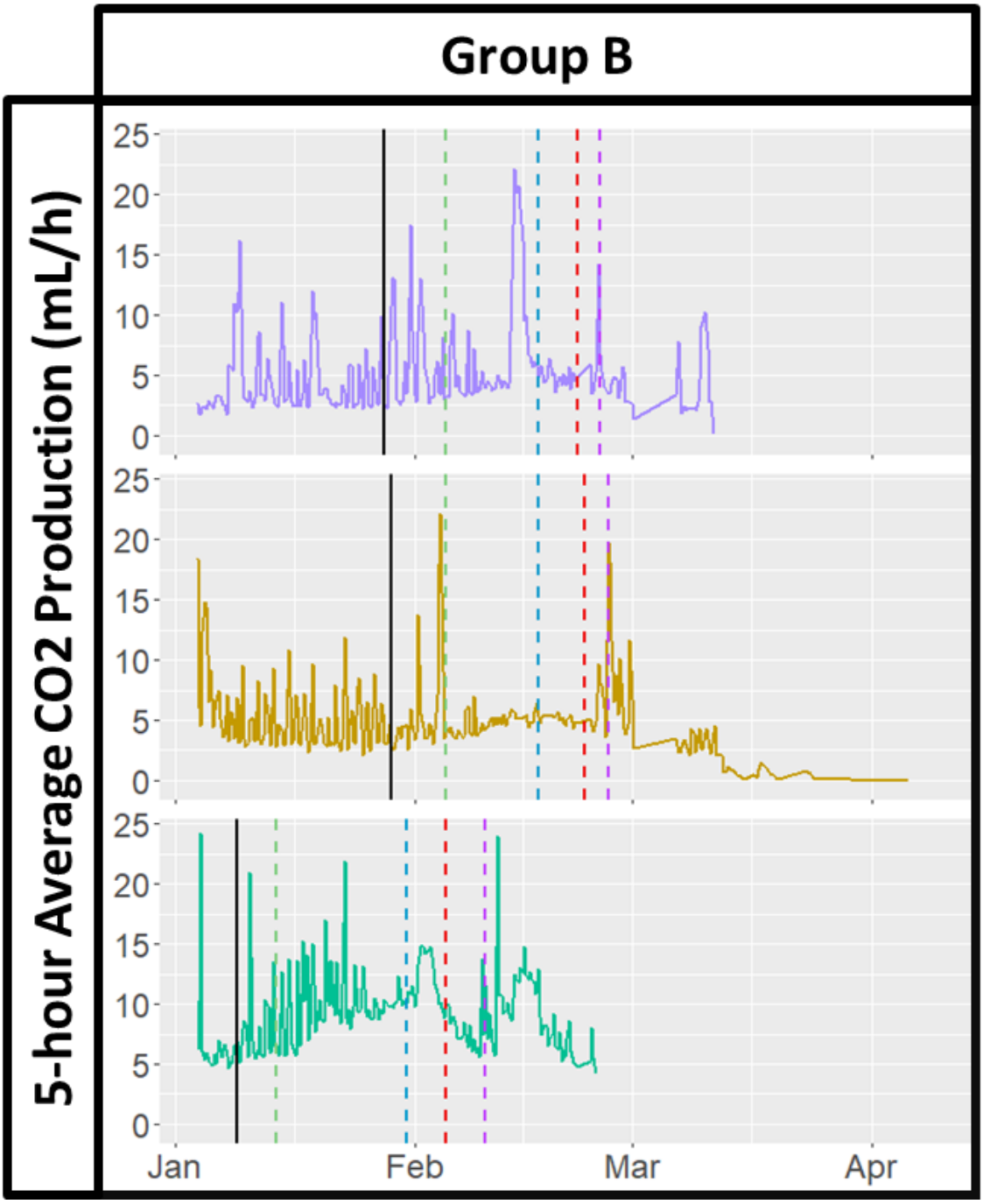

Figure 3: CO_2 Production of Group B animals that shed during the trial at rest, during ecdysis, and post shed. Lines are based on 5 hour lumped averages of CO_2 production. Each panel is a different individual. The vertical blackline denotes the "metabolic start of ecdysis", the time at which the slope of the baseline changed and the time from which metabolic effort was calculated. Vertical dashed lines denote the day that various visual cues or important events of the shed were first noted, green=first visual sign (usually emergent basal segment), blue=eyes begin to cloud, red=eyes clear, purple=physical sloughing. Note the resting metabolism for each animal, the large curve above resting that correlates to the metabolic process of ecdysis, and the spike associated with the purple line that corresponds to physical sloughing.

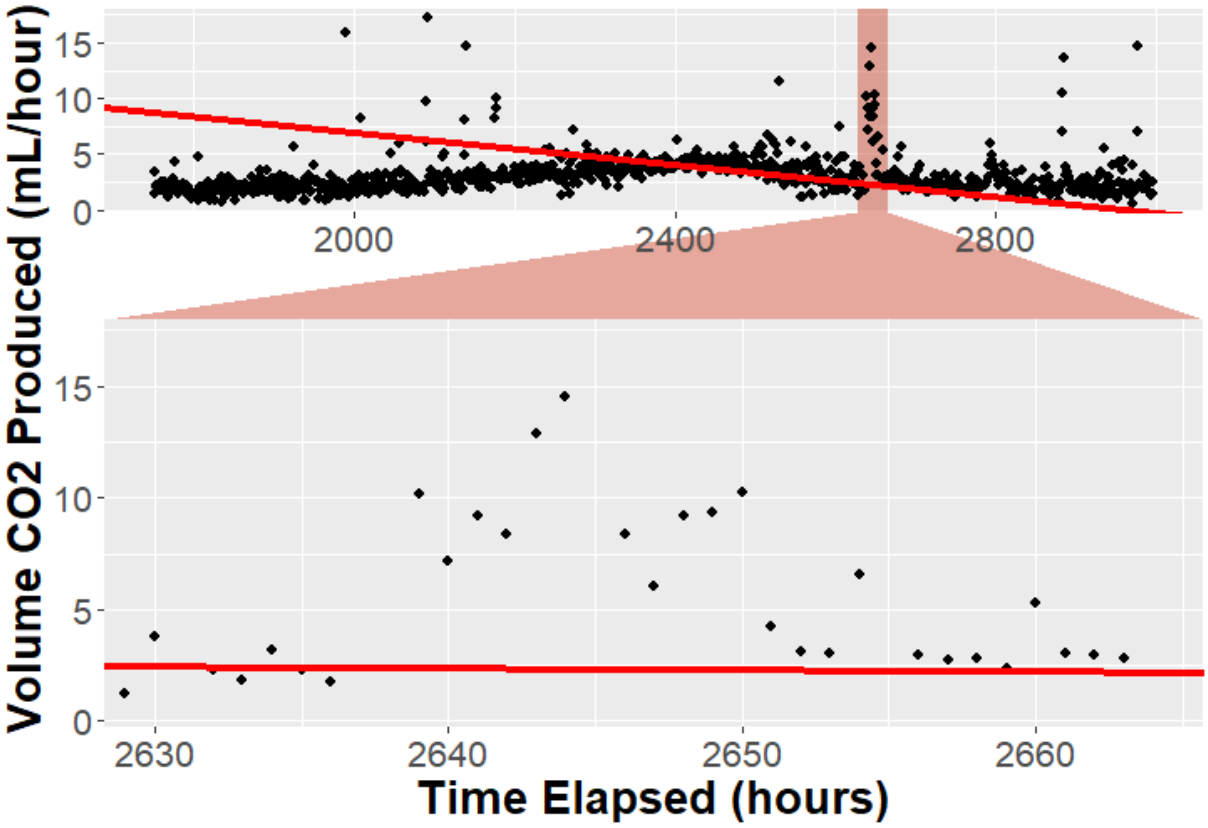

Figure 4: Example of E_r curve fitting procedure. The top figure shows hourly mean CO_2 production values (in mL/hour) with a linear model (red line) fit to the angle of the curve associated with skin synthesis at the time of physical sloughing. The inset (bottom panel) shows the general fit of the line during the several hours surrounding physical sloughing. Area under the curve but above the trend line was used to calculate CO_2 production above E_b attributed to E_r.

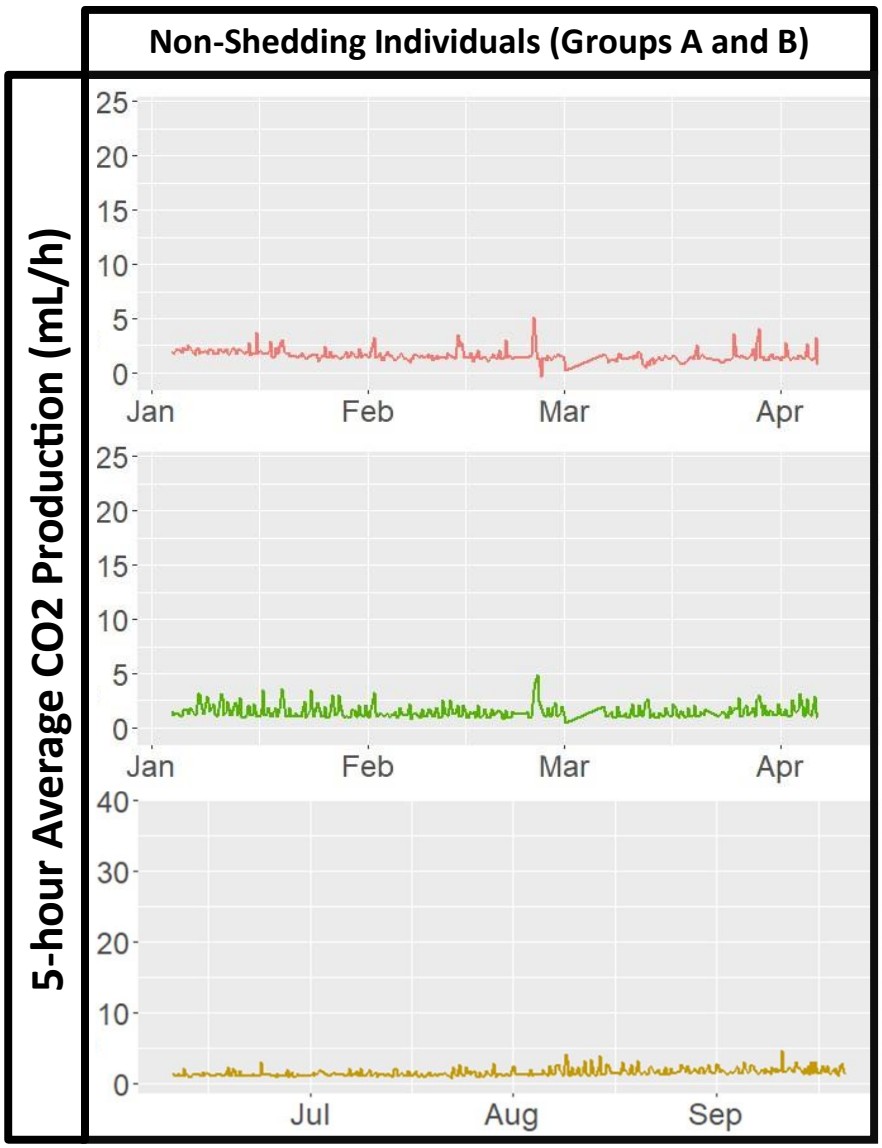

Figure 5: CO_2 Production of snakes that did not shed during trials A (bottom panel) and B (top and middle panels) across the length of data collection. Note that even over a long time scale, baselines remain relatively constant (no metabolic rate depression), and while there is variability in individuals from day to day (mostly from movement within enclosures), circadian rhythms are not apparent on this time scale.

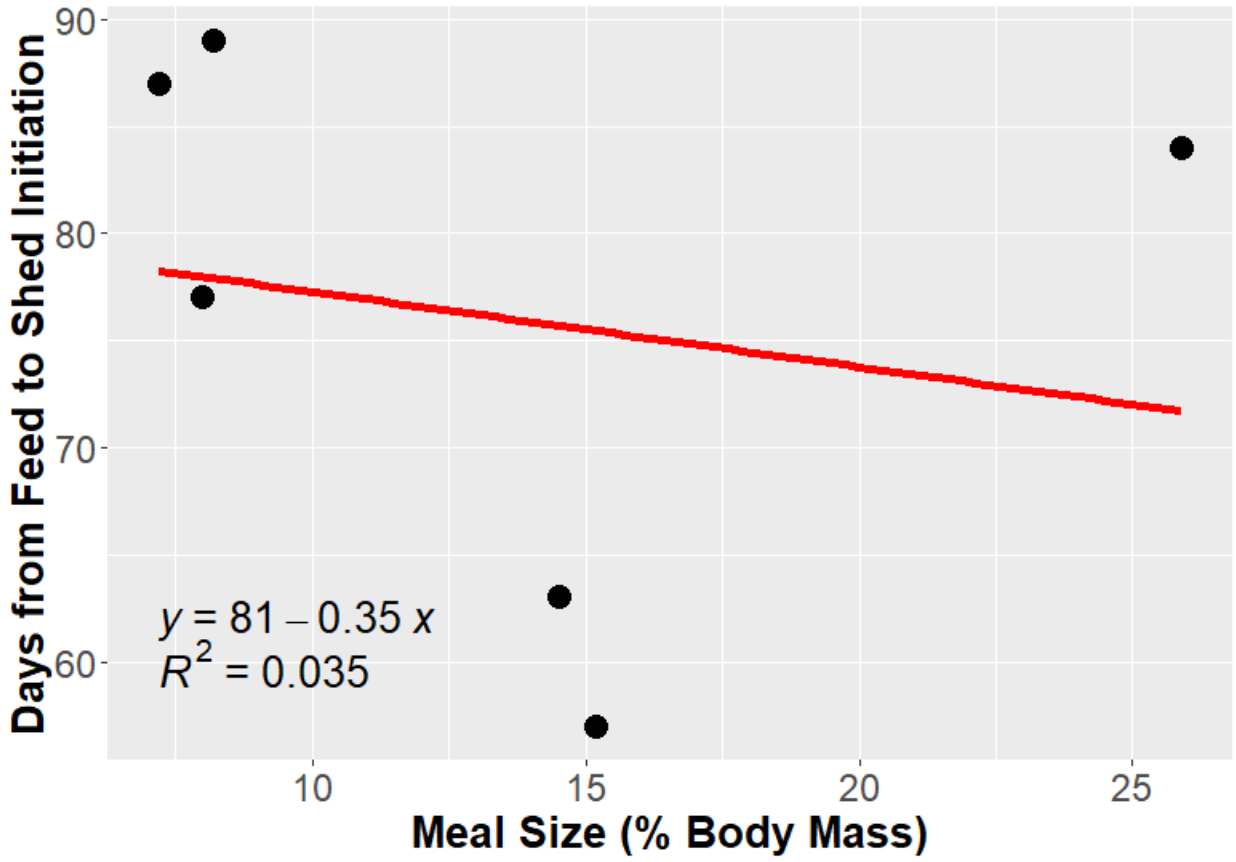

Figure 6: Spread of Meal Sizes in relation to the length of time from feeding to metabolic initiation of shed. Analyses were run with meal size expressed in total grams yielded similar, non-significant results. Note the poor fit of the linear model (P > 0.7) and wide dispersion of data points.

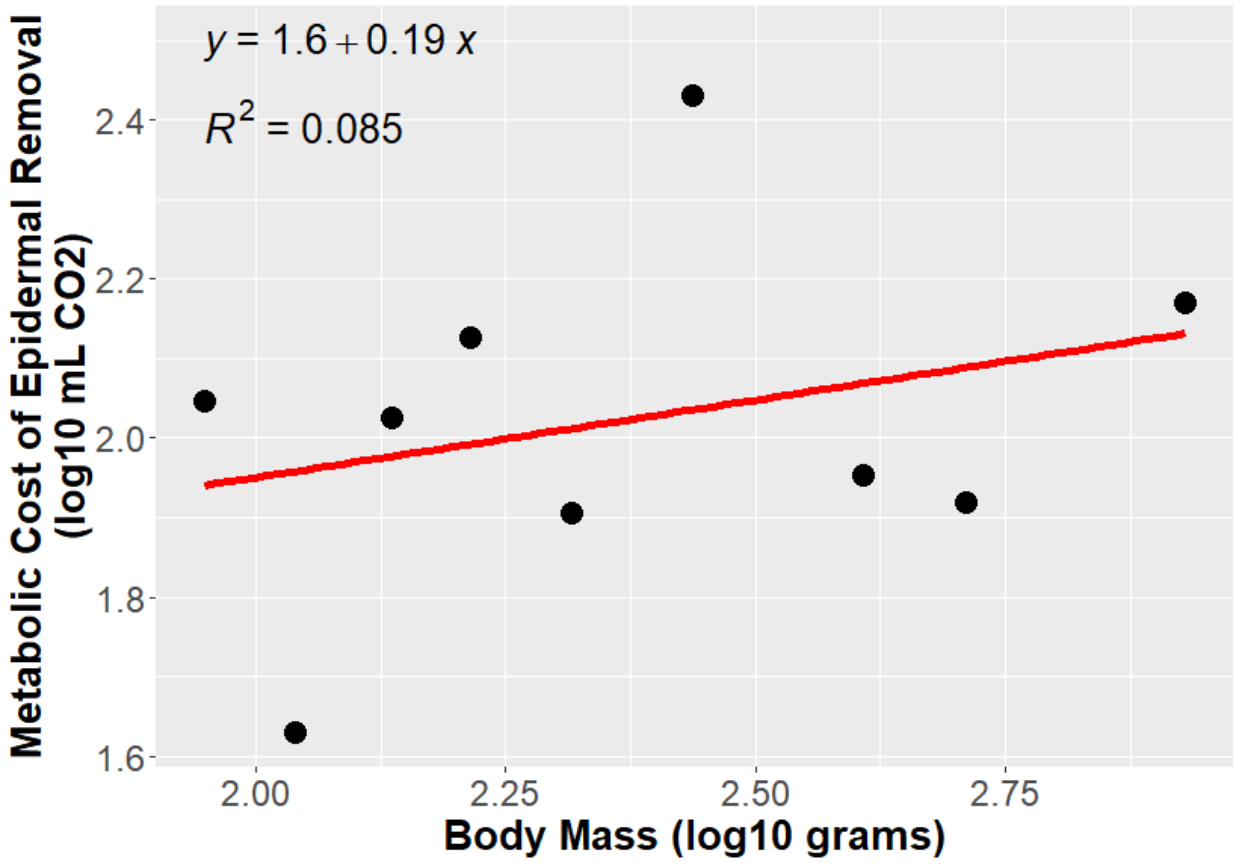

Figure 7: Animal body mass vs mL CO_2 produced during skin removal spikes (E_r). Data have been log transformed and a linear model fit (P > 0.4). Note the shallow slope of the trend line and the outlier value on the bottom left, the removal of that outlier yields a slope of 0. Effort of skin removal is not a function of body mass.

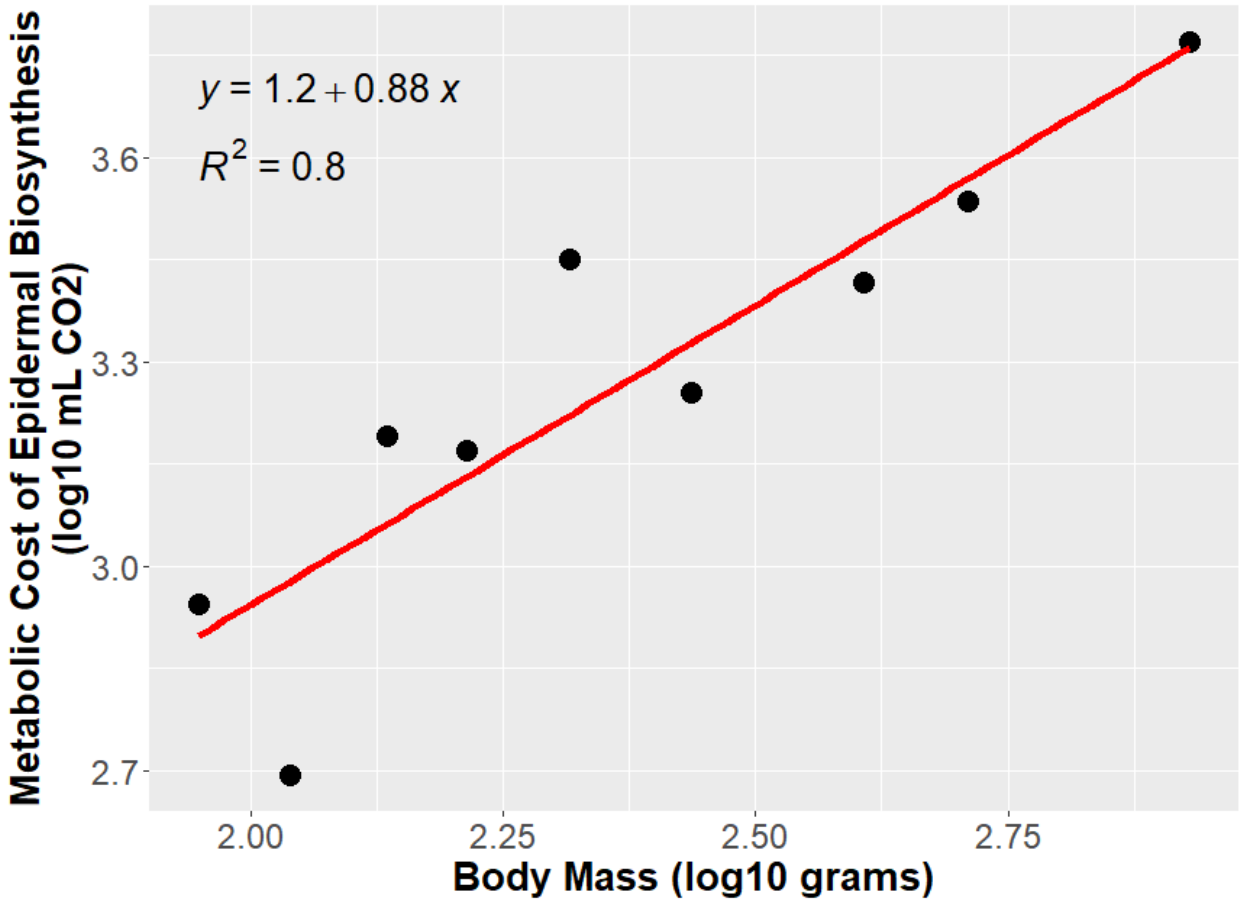

Figure 8: Animal body mass vs. mL CO_2 produced during skin synthesis (E_b). Data have been log transformed and a linear model fit ($P < 0.01$). The equation of the line was used to derive metabolic scaling equation 1.

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Chapter 4: Ecdysis as a Constrained Life-History Trait in Squamates: A Dynamic Energy Budget Individual Based Modelling Exercise in a Simulated Rattlesnake

Abstract:

The semi-frequent replacement of the epidermal layer in squamates (ecdysis; shedding) is a characteristic feature of the group. Recent work has revealed that the process requires significant energetic effort and limits the time individuals have to perform other requisite functions (foraging, reproduction). The degree to which patterns of shed affect whole-organism time-energy budgets remain poorly understood. To explore the importance of patterns and costs of shedding in reptiles, I built a dynamic energy budget (DEB) individual-based model (IBM) to simulate patterns of growth, reproduction, and survival in theoretical populations of Timber Rattlesnakes. By varying shed frequency, I investigated the relative importance of time and mass-energy dynamics in constraining observed frequencies of ecdysis in wild populations. I found evidence that sexual dimorphism in the species may allow resource abundance to constrain shed frequency in adult males. In females, lower shed frequencies than observed in nature produced the highest reproductive output, indicating that additional shed events seen in adult females may serve an explicit reproductive function (beyond simple tissue maintenance). I encourage others to incorporate patterns of ecdysis into their definitions and explorations of reptilian life history strategies and highlight some specific needs in continued empirical research.

Introduction

Ecdysis, the semi-frequent replacement of the epidermis, is a major physiological event seen in all reptiles. In snakes, it requires substantial energetic effort in three distinct forms; the metabolic effort of biosynthesis of new tissue (E_b), energy deposited in the tissue lost to the environment as the old skin is sloughed (E_s), and energy spent to physically remove the old epidermis (E_r; Chapter 3; See Landmann, 1986 or Maderson, 1965 for a review of the renewal process). Shedding in snakes also requires a temporal investment, particularly at the later stages of ecdysis when the spectacle becomes occluded; during ecdysis animals are known to become lethargic, seek out refugia, and refrain from foraging or engaging in ranged movements in the environment (Gibson et al., 1989; Hirth et al., 1969; Kitchell, 1969). Single shed events can take upwards of 4 weeks (Chapters 1, 3; Maderson et al., 1970), with movement and behavior significantly affected in the last 2 weeks of the process (Carnes-Mason and Beaupre, personal observation). The frequency with which individuals in a population engage in epidermal renewal effects the time-energy budgets of snakes, scaling the energetic effort required with increased frequency, and limiting time available for other behaviors during discrete active seasons (i.e., 8 weeks in shed decreases time available to forage – further limiting the energy budget and potentially interfering with reproductive efforts).

The frequency of ecdysis in snakes and other reptiles limits the time and mass-energy budgets of individuals; playing a role in shaping the observed patterns of survival and reproduction that result from an organisms' life history strategy. Across the squamate lineage, groups of snakes seem to display different frequencies of epidermal renewal, indicating that patterns of shed are subject to selective pressure (Cohen, 1975). Renewal frequency may be tied to snake ecotype (aquatic snake families such as *Natricidae* seemingly have higher frequencies of shed than the desert adapted genera such as *Crotalus;* Brown, 1956; Klauber, 1972; Macartney et al., 1990; Stabler, 1939) while active season length (Martin et al., 2021) or

resource-driven growth rates (Wittenberg & Beaupre, 2014) may explain variation among populations of a single species. Unfortunately, my understanding of broad scale patterns of shed frequency is limited because few field studies have reported population-scale patterns of shed frequency. Across those studies, quality of data ranges from direct observations of individuals in shed (Chapter 1 in *C. horridus*; Macartney et al., 1990 in *C. viridis*; both reporting 1-2 sheds per year) to indirect inference of shed frequency from rattle string morphology (Brown, 2016; Martin, 2002; both in *C. horridus;* 1.4 or 1.2 sheds per year respectively).

Recently reported information on the energetic effort, duration, and frequency of shedding in snakes (Chapters 1, 3) indicates that patterns of ecdysis may be an important life history trait (following (Dunham et al., 1989) definition of an allocation-based life history) and raises questions about the selective pressures which shape the observed shed patterns across all reptilian lineages. Selection should favor life history strategies which maximize the efficiency of resource allocations (in both time and energy) ultimately resulting in successful reproduction and increased individual fitness (Dunham et al., 1989). So, I expect that observed shed patterns are the result of competing interactions between mass-energy budgets, time budgets, and reproductive output. The quantification and evaluation of shedding as an important component of reptilian life history strategies requires careful computing of whole-organism time-energy budgets (informed with measurements of the energetic and temporal costs of the various discrete behaviors in which an organism participates; Congdon et al., 1982; Dunham et al., 1989) and the subsequent integration of those data towards answering specific questions.

Computer models can integrate large amounts of data to model ecological and populational questions of interest to an extent that is not feasible using simple mathematical models (Dunham & Overall, 1994). While even simple models can provide insight (Congdon et al., 1982), more complex models demand the separation of often intertwined activity costs and trade-offs (Dunham & Overall, 1994). Using more complex models, I can test my understanding

of the mechanisms which underpin organisms' ecologies and ask questions about the ramifications of life histories at the population or even evolutionary scales (DeAngelis & Grimm, 2014; DeAngelis & Mooij, 2005). Dynamic Energy Budget – Individual Based Models (DEB-IBM) use replicated "individuals" to account for stochastic variation between individuals over time and model the outcome to a variety of ends (Kooijman et al., 2008; Nisbet et al., 2012; Yang et al., 2022). Dynamic energy budget individual-based models can be parameterized with data collected from real populations and used to model time-energy budgets or project individual and populational outcomes in response to alterations of parameters (i.e., how will survivorship be effected if shed frequency is increased?). Such models are a powerful tool for mapping theoretical energy allocation patterns in individuals and populations; facilitating the integration of relevant parameters into a computer model to gain a more complete understanding of the relative impact of ecdysis on snake life histories.

Pit vipers are model organisms for the study of interactions between individual physiology and environmental conditions in reptiles, particularly in investigations of mass-energy budgets and metabolism (Beaupre & Duvall, 1998). With respect to the investigation of ecdysis here, they are suited to the collection of data on the metabolic and temporal effort of the process as it often occurs in isolation of other physiological and behavioral functions (i.e., rattlesnakes can endure months without eating and seek refuge during ecdytic cycles, facilitating the measurement of shed-specific metabolic effort; Chapter 3). While their slow life-histories (slow reproducing, long lived) limit their utility in field studies of how individual patterns of energy allocation and behavior scale to the population level, computer modeling exercises can help to bridge the gap between total energy budget computation and the consequences of energy budget variation at the broader population scale. Among pit vipers, the physiology of the Timber Rattlesnake (*Crotalus horridus*) is perhaps the most well studied. Work from Beaupre and colleagues has detailed the temporal and energetic effort required for the majority of

behaviors and physiological processes in which these animals regularly engage (digestive efficiencies (Beaupre & Zaidan, 2012; Zaidan & Beaupre, 2003), metabolic rates (Beaupre & Zaidan, 2001), reproduction (Gardner-Santana & Beaupre, 2009; Lind et al., 2016; Lind & Beaupre, 2014, 2015; Van Dyke et al., 2012; Van Dyke & Beaupre, 2011), and the partitioning of time in the field (Beaupre, 2008; Beaupre & Douglas, 2009; Lind & Beaupre, 2015)). Their work culminated in two informative models/energetic accounting exercises of time energy budgets in a reptile (Beaupre, 2002; Beaupre et al., 2017) which detailed interactions between body size, metabolic rate, and growth rate and offered insight into observed patterns of sexual maturation and reproduction in the population of interest. However, the previous models were unable to incorporate the effort required for ecdysis (a characteristic and energetically expensive physiological process; (Chapter 3)) or apply their physiological mechanisms towards population scale repercussions.

Here, I endeavored to incorporate shed frequency and the energetic and temporal effort of shed into a model of the mass-energy budget of a generic Timber Rattlesnake. The goal of my modeling exercise was to examine the magnitude of time and energy expenditure exerted because of requisite ecdytic cycles relative to the total time-energy budgets in snakes. Specifically, I was interested in any fitness tradeoffs which may constrain shed frequency, and whether those constraints may manifest as a result of limited time or energy (e.g., are stored resources insufficient for additional sheds? Does the duration of shed prohibit more frequent shedding during limited active seasons?). I scaled the resulting individual based model to a population scale model (in which females produce offspring that join the simulation) and used the output of those simulations to explore whether observed frequencies optimize reproductive output. I discuss possible utility of ecdysis beyond a strict tissue maintenance function with reference to effects shifting life history strategies (time-energy allocation patterns of which ecdysis is a part) have on female fecundity.

General Modeling Approach

The model presented here is a refinement of an earlier approach (Beaupre, 2002) and I encourage the reader to consult that work to better understand my modeling ideology. I have updated a variety of modules (foraging, digestion, reproduction) with Timber Rattlesnake-specific physiological information collected over the last 20 years at my long-term study site in Northwest Arkansas (the "source population"; (Beaupre, 2008; Beaupre & Zaidan, 2012; Lind & Beaupre, 2015; Van Dyke & Beaupre, 2011)). I have altered the structure of the model from a parallel array construct to the object-oriented approach discussed below to improve processing efficiency and facilitate continued scaling and updating of the model for future investigations. Central to this communication, I used recently collected data on ecdysis in the Timber Rattlesnake (Chapters 1, 3) to include the effort and cost of shed and help refine my understanding of its importance. I applied my updated model towards the following specific questions; 1) How does the temporal and energetic effort of ecdysis (scaling with shed frequency) affect resource stores, growth rates, and survival in a static resource environment? 2) Do active season length and/or available food resources potentially explain variation in shed frequency observed between populations? 3) Is shed frequency constrained as a consequence of trade-offs between allocation of time and energy towards reproduction in females? and 4) How do potential fitness costs associated with life histories with increased shed frequencies affect populations under stochastic resource conditions?

Methods

Model Construction

I used an object-oriented approach in R (Rstudio version 4.0.2; R6 Class System; package "R6") to create an individual based model (IBM) of a "typical" Arkansas Ozarks Timber Rattlesnake (primarily informed with data collected from the source population). My model consisted of two broad classes of objects; a singular "Environment" object was populated at the beginning of each simulation (see description below; henceforth "virtualEnvironment"), and a

simulation-dependent number of “Individual” objects (henceforth “eSnakes”). Some relevant values pertaining to the simulated resource environment and the efficiency of energetic conversion (from consumed prey to available energy or from available energy to tissue) did not vary through the simulation or across individuals and so were stored as static values in the global Environment in R (

Table 12). eSnakes were initialized using parameters drawn from long term ecological data (Beaupre, unpublished) and previous research (Table 9). Each eSnake was a distinct R6-class object containing a list of mutable variables ("state variables";

Table 11) which were allowed to change with each iteration of the day loop following mathematical relationships derived from the literature (see appendix for complete list of utilized equations and source materials). I used a dynamic energy budget (DEB) approach as the underlying mechanism of the model (following Beaupre 2002), driving changes in allocation of time towards competing "behaviors" (modeled as functions; outlined below) and altering state variables through time. Female eSnakes within the "population" were allowed to reproduce, adding new individuals to the simulation at the end of each year (day 365 = August 27), ultimately allowing us to produce a population model built upon an underlying individual-based, dynamic energy budget construct. I used the DEB-IBM to model growth and reproduction of individuals through time, with energetic consequences and behavioral tradeoffs occurring because of changes in season, temperature, and the physiological state (i.e., in shed) of the eSnakes.

Under my object-oriented framework, eSnakes and the virtualEnvironment were distinct objects which interacted with one another. In each "day" of the model, all "living" eSnakes (energy stores > 0kJ) in the population engaged in different "behaviors" (characterized as functions) based on state variable values (chiefly sex and sexual maturity, body size, mass of food in gut, and energetic reserves). As the model stepped through days, the virtualEnvironment held information such as day of the year, season, and daily temperature profiles; changing through time on a 365 day cycle. Functions of the model and "behaviors" of eSnakes referenced values held in both object classes (eSnake or virtualEnvironment) and those held in the global environment. The flow of events (Figure 1) each day consisted of season and individual foraging and digestion subroutines, payment of daily metabolic expenditures, seasonal and sex dependent allocation of resources towards growth and reproduction (Beaupre, 2002, 2008; Beaupre & Zaidan, 2001; Lind & Beaupre, 2015; Van Dyke & Beaupre, 2011; Zaidan & Beaupre, 2003), with additional subroutines to evaluate shed

condition of individuals and the temporal and energetic effort required for ecdysis. The key feature of this model, shedding, was parameterized to match observed patterns of shed duration and frequency (Chapter 1) and the energetic effort of ecdysis (Chapter 3).

Initializing Parameters

For the comparisons presented here, populations were initialized with male and/or female neonates (simulation dependent) following the initialization state variable values (

Table 11; from long term observational data; Beaupre unpublished). Simulations started at “day 1”, set here as 28 August (the average date of parturition for Timber rattlesnakes in the source population; day 365=27 August of the next calendar year). All virtualEnvironment parameters (described below) were set in reference to the day within the model based on approximations of active season periods in real snakes, additional relevant values (

Table 12) were saved to the global environment in R.

Storage dynamics

My bioenergetic model tracked a series of mass-energy budget related state variables in simulated eSnakes through their lives. Stored and available energy (in kJ) were central to the structure and function of my model, with available energy driving allocation patterns, growth rates, and reproduction. All eSnakes were "born" with energy reserves equivalent to ~4g of fat (~135kJ; 35-37 kJ/g; Charrondiere et al., 2004) which allowed animals to survive their first winter regardless of foraging success in the late summer of their birth. Each eSnake had a long-term storage pool (LTS) and a short-term storage pool (STS). Short term stores were enriched by the consumption and subsequent digestion of "prey" items. Relevant daily energetic requirements were paid preferentially out of STS. If STS on a given day was insufficient to cover daily costs (no energetic gains in a day, daily expenditures were larger than daily income), the remainder of the energetic debt was paid from LTS (simulating the mobilization of fat reserves in states of fasting). Whenever short-term stores remained at the end of the day (after metabolism had been paid), the remainder of energy was added to LTS and STS was set to 0 (simulating the storage of surplus resources as fat). Resources allocated towards growth were only drawn from STS, while energy for reproduction or metabolism could be supplemented from LTS if daily incomes were insufficient.

Characteristics of the virtualEnvironment

The virtualEnvironment stored values for season (Summer, Autumn, Winter, Spring) and season-dependent temperature profiles (Beaupre, 2002, 2008; Wills & Beaupre, 2000) seen in the source population of Timber Rattlesnakes. Values were set by the following functions;

setSeason: Season of the year was set at the start of each day loop based on the value of the day. Since the simulation starts in the late summer (day 1=August 28), seasonal values

follow Table 10, modified from Beaupre's (2002) original model that based "seasons" on available body temperatures in the environment.

setTemperature: Based on the season (defined above; saved to the virtualEnvironment object), a vector of temperatures was stored as a "temperature" state variable to the virtualEnvironment. The vector of temperatures in this model was only accessed by the metabolism routine (below), drawing a temperature value for each block of time within the day (see metabolism subroutine for details).

Characteristics of eSnakes

In my model, eSnakes were stored in a list (the "population") and were differentiated from one another by a unique 16-digit alpha numeric ID's (generated and assigned at eSnake initialization; package UUID). Each eSnake was represented as a list of state variables (reported in Table 3). State variable values for each eSnake were initialized following Table 3 and then updated and altered by subroutines (detailed below) as the model looped over days. Each eSnake was handled as a distinct object in the model, variations in outcomes (growth rate, resource acquisition, energy budgets, lifespan) between eSnakes were the result of stochastic processes (primarily foraging success and meal size) built into the model.

forage: eSnakes were allowed to forage in any season but winter, provided they were in a physiological state that did not prevent it (i.e., eSnakes in the last stage of shed, gravid females, or eSnakes currently digesting a meal could not forage). The forage function generates a random sized prey item drawn from a uniform distribution ranging from a predetermined minimal prey item size (a proportion of total body size; assumes that large snakes (500g+) will not prey upon very small rodents (<5% body mass)) scaling up to either a maximum gut capacity (using the allometric relationship between SVL and largest meal (grams) taken in a population of *C. horridus* from New York to give a size-class specific maximum meal

size; $Largest\ Meal = \frac{SVL^{2.35}}{235.99}$; small snakes (~29g) will take meals up to 70% of their body mass, while large adult males (~1200g) rarely exceed meals more than 25% of their mass; derived from Clark, 2002 with additional raw data provided by the author) or maximum prey item size (a 500g grey squirrel in this simulation); whichever value is smaller. A second random number ("forage success") was drawn from a uniform distribution between 0 and 1. If the value of "forage success" was less than or equal to the user-determined "mean foraging success" value for the simulation or year, the random sized prey item determined above was "captured" by the animal. Values that exceed the proportional cut off result in a failed foraging attempt for the day. Mean foraging success (MFS) served to determine the proportion of foraging attempts which were successful; a MFS of 0.05 represents a successful foraging event approximately once every 20 attempts.

The forage function also set the length of time (in days) that an eSnake required to digest a meal and saved it to a counter state variable stored to the eSnake ("digestTime"). The magnitude of digestTime dictated the duration over which metabolic rate was elevated as a result of specific dynamic action of digestion (SDA; see below). Duration of digestion scaled with the meal size as a proportion of body size. I classified meals as; small (15% or less of body mass), medium (15-40% of body mass), or large (40% or more of body mass) and used size-class-specific equations to set duration of digestion (derived from Zaidan & Beaupre, 2003; meal size effects the linear relationship between digestion time and body mass). Any digestion duration shorter than three days was rounded up to three days, assuming three days to be the minimum processing time of any meal based on evidence of the required up-regulation of gut function prior to- and down regulation of gut function following- digestion; both of which are involved in the metabolic effort required for the SDA response (Secor, 2009).

Once meal size and digestion time were determined, the forage function then calculated a total CO_2 production (mL CO_2) of SDA based on body size and meal size (following

relationships published by Zaidan & Beaupre, 2003). While in reality the carbon dioxide production attributed to the SDA response is approximately bell shaped through its duration, I took a more simplified approach, modeling it as a uniform expense lasting for the duration of digestion by dividing the total CO_2 production of the response by the length of digestion to produce a daily CO_2 production attributed to SDA. The volume of gas was then converted to kJ (following Gessaman & Nagy, 1988; 27.42 Joules/mL CO_2) to give a daily energetic cost of SDA, a value that was later subtracted from energy stores (see "metabolism").

digest: The energy that eSnakes extracted from consumed prey was added to short term resource pools to pay daily maintenance costs and allocate towards long term storage or other competing functions (growth and reproduction). The Digest function converts prey wet mass (g) to kJ content (assuming 25% dry mass of wet mass food items with an energetic density of 23.311 kJ per gram dry mass Beaupre et al., 2017) and calculates a total "metabolizable energy content" of the prey item based on published conversion efficiencies of wet mass to usable energy for the predator (80%; Beaupre & Zaidan, 2001). The function then adds a proportion of that total energetic content to short term storage on each day that an individual is digesting (Figure 2). Total kJ gained from a meal item is divided by the duration of digestion (in days; see "forage"). The digest function also reduces the digestTime counter (a characteristic of the "individual" object) by one on each day of digestion, eventually reducing digestTime to 0, ending the digest subroutine and allowing individuals to return to foraging on subsequent days if applicable. All energy added to STS pools via the digest subroutine were added to a state variable carrying a running tally of lifetime assimilated energy, total lifetime income was used to construct energy budgets for relevant modeling exercises.

metab: The metabolism subroutine calculates a total daily metabolic cost for the eSnake based on season, seasonally variable temperature profiles, mass-specific scaling relationships, and the relationship between field metabolic rate (FMR) and resting metabolic rate (RMR). First,

an estimate of total daily CO_2 production for the eSnake at rest is produced following mass dependent scaling equations (Winter: Agugliaro, 2011; Spring, Summer, Autumn: Beaupre & Zaidan, 2001). During the active season (Spring-Autumn) RMR values are estimated using blocks of field active body temperatures informed by Beaupre & Zaidan (2001) to account for inter-hour variability in metabolic rate exhibited in real animals throughout the day. During winter, eSnakes are assumed to be at constant temperature and therefore experience static RMR's. Estimates are converted from mL CO_2 to kJ by standard conversion factors (1000 mL/L; 27.42 J/mL CO2; Beaupre & Zaidan, 2001; Gessaman & Nagy, 1988).

Next, an estimate of total active season Field Metabolic Rate is produced (following Beaupre et al., 2017) and converted to daily FMR estimates. However, daily FMR varies with season but FMR measurement techniques such as doubly labeled water produce coarse estimates of FMR's over broad time spans. To account for seasonal variability in FMR, I estimated the proportion of total energy expenditure that occurs in the different phases of the active season (Spring, Summer, Autumn) based on the scaling of RMR values and assumed these proportions to be the same for FMR. A daily FMR estimate was multiplied by the length of the active season (244 days; "active season FMR"), scaled by the seasonal proportion of expenditure (e.g., 90% in summer), and divided by the length of that season (182 days of "summer") to give a season-specific daily FMR estimate. The daily FMR (which includes RMR) was divided by my RMR estimate to give metabolic scope (the magnitude of expected total metabolism above the minimum value (RMR) on any day). If the value of scope was greater than or equal to 1 (i.e., if daily FMR was greater than the estimate of RMR), RMR estimates were multiplied by the scope value to give an estimate of total daily metabolic cost. If values of scope were less than 1 (FMR was less than RMR), daily metabolism was assumed to equal RMR rather than incorporating my rough estimate of FMR to the model under those conditions.

At the end of the metabolism routine, the computed daily metabolic expenditure (RMR or RMR*scope) was subtracted from available energy stores. If daily metabolism was less than short term energy stores, the entirety of metabolic maintenance costs were paid from the short-term pool. If the short-term pool was insufficient to cover daily metabolic costs, the difference in cost (STS – RMR) was taken from long term storage pools to meet the cost (Figure 2). On occasion, the subtraction of metabolic costs from long term stores resulted in a value of LTS less than 0. In these instances, the individual was considered "dead" and was removed from the population and omitted from future daily loops.

growth: eSnakes in my simulation grew through time by increasing in Mass. Changes in mass were then translated to changes in SVL following published relationships between mass and svl from the source population (SVL = 11.73 * Mass ^ 0.324; Beaupre & Zaidan, 2001).

At the start of each day of the simulation, sex-, maturity-, and seasonally-explicit values for the proportion of available short term stores that an individual would devote towards growth were set (see "canGrowRepro" and "alloRules" below). I assumed that juvenile snakes in real populations devote all available resources towards growth, as maximizing body size improves survival and increases fitness (by decreasing time to first reproduction and increasing lifetime reproductive bouts; Beaupre, 2002; Duvall et al., 1992). Once mature, female Timber rattlesnakes in the wild grow slowly, a consequence of shifting allocation priority as excess resources are better spent on reproduction (Beaupre, 2002). Mature males in nature continue to grow (one aspect of the sexual dimorphism seen in some rattlesnakes; Beaupre 2002) into adulthood, with increasing body size conferring a combative advantage and improving access to females in vipers (Andrén, 1982). In general, I modeled growth to capture the central patterns seen in the wild; juvenile and adult male eSnakes devoted all available energy towards growth, while adult female eSnakes limited growth allocations (1.5% of available short term storage

surplus; Beaupre, 2002) in favor of reproduction (Figure 2). Growth stopped for all eSnakes during winter.

On each day that an eSnake had energetic income (i.e., were absorbing energy from a food item), those resources entered STS pools, maintenance costs (metabolism and ecdysis) were paid, and any remaining STS was allocated towards reproduction and growth (Figure 2). The available resources were multiplied by allocation proportions and corrected for the efficiency of converting raw materials to tissue (0.60; Beaupre 2002) to give a new value; growth energy (kJ). Growth energy was converted to the corresponding mass of wet tissue (assuming 75% water and a density of 23.287kJ per gram of dry tissue synthesized), and the wet tissue mass was added to the eSnake's mass. The total energy used to synthesize the new tissue (including energy lost as heat during tissue synthesis; Beaupre & Zaidan, 2012) was subtracted from the short-term resource pools and the eSnake's SVL was updated following mass-svl relationships. It should be noted that my approach potentially counts some portion of the cost of growth twice; once as the metabolic cost of growth detailed here, and again as some portion of the SDA response (outlined under foraging and digestion). The lack of a clear estimate of what proportion of the SDA response is attributable to tissue growth necessitated my approach (Zaidan & Beaupre, 2003). However, since the issue only manifests on days when eSnakes had energetic income and after maintenance costs were paid, the true effect was likely limited and only acted to slow growth rates (as energetic cost of growth in reality is probably less than as modeled). It is unlikely that the artificial inflation of cost of growth affected model outcomes as it could not cause direct mortality (maintenance costs were paid or caused death prior to growth accounting) and did not interfere with the ecdysis routine central to my simulations.

reproduction: In Timber Rattlesnakes, reproductive effort differs between adult males and adult females. For the purposes of this model, male eSnakes were assumed to devote minimal effort towards reproduction. While an oversimplification of reality, male snakes in the

wild exert reproductive effort in the form of mate searching and mate guarding (manifesting as increased metabolic rates associated with increased movement; Aldridge & Duvall, 2002; Duvall et al., 1992) and so total reproductive effort in males is variable, related to body size (as larger males spend more energy to move around the environment), and may depend on environmental/seasonal cues and vary between years based on body condition. In my model, male eSnakes played no role in successful offspring production (I assumed that female eSnakes in suitable reproductive condition had free access to males), but since my sex-specific approach for estimating metabolic scope was drawn from a data set which included mate-searching males, the model incorporated some increased metabolic effort in the mid-summer, attributable to reproductive effort, for adult male eSnakes. However, due to the variability of mate searching effort (occasionally metabolic scopes may have exceeded 12 times RMR; Beaupre et al., 2017; Beaupre, unpublished data) my estimate of male reproductive effort is conservative and uniform across individual eSnakes. As such, male long-term energy stores from this model should be viewed with caution because though they do not include realistic and variable estimates of effort devoted towards reproduction, and so are an overestimate of male snakes' ability to store energy through time (in reality, excessive energy stores are likely allocated towards increased mate searching and courtship). However, I assume that males with low energetic reserves do not engage in reproductive activities in the wild (Beaupre; personal observation; Lind & Beaupre, 2014b, 2015) and so my inclusion of elevated metabolic rates (associated with mate searching periods) may have caused some mortality events as low energy eSnakes still experienced increased costs in summer. As such, mid-summer mortality may have occurred in some simulations as a result of unrealistic mate searching in low energy eSnake males, while high resource males likely have unrealistic long-term stores as reproductive effort in real snakes likely scales accordingly. Future iterations of my model will attempt to better model male investment, but as long as trends in my model output are viewed as relative to other conditions (all males were subject to the same metabolic scope rules) I feel

that the affect of my approach to male reproduction modeling had minimal effects on my findings.

Females are the focus of reproduction in this model, with the energetic condition and body size of eSnakes dictating reproductive frequency and output (Aubret et al., 2002; Gardner-Santana & Beaupre, 2009; Van Dyke & Beaupre, 2011). In timber rattlesnakes, reproduction is biennial at a maximum as temporally disjointed patterns of gametogenesis, copulation, vitellogenesis and parturition coupled with an obligate seasonal diapause (e.g., winter) force multi-season effort to produce a single litter (Aldridge & Duvall, 2002; Duvall et al., 1992). To replicate the natural reproductive patterns of *C. horridus*, I included additional state variables for female eSnakes which tracked vitellogenic status (True/False), length of vitellogenesis (in days), and reproductive resource pools (in kJ). Live bearing viperid snakes are best described as capital breeders (Naulleau & Bonnet, 1996; Van Dyke et al., 2012) allocating stored resources towards reproduction during reproductive events. To mimic reality, I used a series of checks of available resources (capital breeding pools) at phenologically relevant times during the year (i.e., is this female in good "condition"?; will she survive a reproductive attempt if she devotes resources to reproduction now?) and used the results of those checks to determine the course of reproduction in eSnakes. My model includes four distinct reproductive periods/events in adult females, 1) Initiation of primary vitellogenesis (the first day of spring in year A), 2) Initiation of secondary vitellogenesis (the first day of springing in year B), 3) Energetic allocation towards embryo development (metabolic cost of vitellogenesis; spring and summer in year B), and 4) Parturition (the last day of the "year" in year B; August 27th).

I made several key assumptions in my modeling of female reproduction. First, I assumed that there is a threshold of long-term energetic stores necessary to initiate reproduction. Since females in the field will avoid reproducing following years of low resource acquisition (Beaupre personal observation; Lind et al., 2016) and mortality caused by allocation

towards reproduction should be selected against in long-lived, slow-reproducing species, I calculated a threshold value of LTS necessary for an eSnake to become vitellogenic. On the first day of spring, if a female eSnake had enough long term reserves to survive the daily metabolic maintenance costs of a winter (122 days) and produce a minimum clutch size as determined by body size and observations of frequencies of clutch size in wild populations (55% of SVL = body available for embryo development; 4cm = average embryo size; minimum embryo number = available body capacity minus three (assuming effort in pregnancy is only effective if they produce some minimum clutch size; Beaupre 2002)) she was deemed to be able to successfully carry a pregnancy to term, and was therefore labeled as vitellogenic. Second, I assumed that the energetic investment in pregnancy in real animals is minimal during the first summer. During primary vitellogenesis females continue to accrue resources and copulate with males, but do not significantly alter their behavior or physiology relative to non-vitellogenic females. So, to simplify my modelling approach, I handled primary vitellogenesis as a requisite qualifier to producing embryos in the following year (replicating observed biennial reproductive cycles), but did not assign any specific metabolic cost to that portion of the process. Third, females have the ability to abort pregnancies prior to the onset of secondary vitellogenesis when the bulk of nutrient resources are deposited to follicles (Beaupre, personal observation; Figure 2). In my model, at the start of the second spring, female eSnake's resources were again checked (requiring sufficient reserves to produce a minimal clutch and survive a winter), if LTS was insufficient, the pregnancy was aborted, reproductive reserves were shuttled back to long term storage, and the female became non-reproductive ("vitellogenic" state variable set to "False"). Finally, I assumed that at parturition females produce a clutch between the minimum viable clutch size and the maximum that the body cavity could accommodate. My approach to modeling clutch size was based on observations that clutch sizes vary, and clutches in *C. horridus* often include some proportion of non-viable embryos (Beaupre personal observation).

Functionally, the model used body size and long-term stores to assess female condition, dictate reproductive events, and ultimately produce embryos. During secondary vitellogenesis (second summer) each day included an additional metabolic cost of vitellogenesis. The total metabolic cost of vitellogenesis was determined at the initiation of primary vitellogenesis from the amount of energy required to produce a minimal clutch size (but was only subtracted from energetic reserves during secondary vitellogenesis). Daily metabolic cost of vitellogenesis was calculated by dividing total metabolic cost by the length of secondary vitellogenesis (177 days). Each day, the daily cost was paid (and shuttled to a temporary capital breeding pool) from short term stores or supplemented from long-term stores (if short term stores were insufficient; Figure 2). If the energy available for reproduction that day ("ReproEnergy"; a product of STS and allocation proportion towards reproduction) exceeded the daily energetic requirement to produce the minimum number of embryos, all remaining ReproEnergy was shuttled towards the "capital breeding pool" state variable (e.g., on days with more energy than that required to produce the minimum clutch, the capital breeding pool can increase beyond the minimum, allowing females with good foraging success during pregnancy to eventually produce larger clutches than the minimum viable size). At the end of the gestation period, the amount of energy stored for reproduction was assessed, the number of viable embryos that could be generated with that amount of energy was computed, and offspring were added to the population as new individuals (see "populationGrowth" subroutine). If the energy for reproduction was insufficient to produce a minimal clutch size on the date of parturition, the pregnancy was aborted and the stored energy was returned to LTS. By allowing eSnakes to abort pregnancies, I modeled the possibility that at any point in a summer if energy reserves start to become depleted, females in real populations have the ability to abort pregnancies when the energetic effort of carrying embryos to term might result in the death of the mother, preserving future reproductive potential. While criticism may be levied that my approach of shuttling energy to a capital breeding resource pool is similar to "income breeding", I argue that

my approach highlights the complexity of true breeding systems rather than the income/capital dichotomy. Functionally, animals only devote energy in a single lump sum from storage (capital breeding) but tracking the resources that would eventually be allocated towards reproduction as I did allowed us to simulate the way in which females must modulate reproduction (through variable clutch size and abortion if necessary) in a variable resource environment. I encourage the reader to view my approach as "ear-marking" resources for reproduction in healthy females rather than a simulation of income, or even capital, breeding.

sexMaturation: Sexual maturation was based on SVL in my model. Beaupre (2002) demonstrated that size to sexual maturity affected lifetime reproductive success, he found that reproductive patterns when eSnakes matured at 70cm SVL were most similar to patterns of reproductive frequency and lifetime reproductive output observed in the source population. I elected to use a static 70 cm maturation size for all simulations. The sexMaturation function was incorporated at the end of each day loop in my model. If an eSnake grew to 70 cm SVL or more during that day (via the growth function), a state variable for sexual maturity was updated (sexually mature = True) and that eSnake was handled as an adult for all subsequent day loops.

reserveLTS: Allocation of short-term energetic stores towards growth and reproduction is expected to halt when animals are in poor energetic condition. To replicate this phenomenon in my model, I constructed a reserve storage attribute for all eSnakes based on season and body size. At the start of each day, the reserveLTS function calculated a daily resting metabolic rate (following the procedure for RMR outlined in metab; above), multiplied it by a season specific metabolic scope scalar (following Beaupre, 2002; Winter=2, Spring and Fall = 3, Summer=3.6), and then multiplied by the length of the season (except in winter, when no minimum storage was required, all resources are available for survival during winter months). The product of metabolism, scope, and length of season was saved as a state variable for each

eSnake. If LTS fell below this value, growth and reproduction were not allowed, individuals allocated short term stores towards long term storage to replenish safety stores.

alloRules: Allocation towards growth and reproduction was limited by season, sex, and maturation categories. In my model, I assumed that snakes could not allocate energy towards growth or reproduction during the winter and instead conserved energy to pay basic maintenance costs. During the active season (Spring-Autumn), juvenile eSnakes were not allowed to devote energy towards reproduction, all surplus above maintenance costs and maintenance of long-term energy reserves was devoted towards growth. Adult male eSnakes also allocated all energy towards growth during the active season, although some portion of the metabolic effort of mate searching (paid as maintenance cost in this model) is captured via my metabolic scope approach (see reproduction above). Once female eSnakes reached sexual maturity, they devoted the majority of energy towards reproduction, and only minimal effort towards growth (which can increase reproductive output over the long term). Such patterns in females may partially explain the sexual dimorphism seen in *C. horridus* (Beaupre, 2002), and realistically model allocation decision making seen in the wild. Here I used allocation proportions of 98.5% (reproduction) and 1.5% (growth; following Beaupre, 2002) for adult female eSnakes. The alloRules subroutine ran at the beginning of each day for each eSnake, based on a series of checks for sex and sexual maturity and a season-specific proportion for growth and reproductive allocation was saved as a state variable to the eSnake (during the active season for juveniles and adult males: growth =1, repro = 0; adult females: growth=0.015, repro=0.985).

shedRules: Shedding requires energy in three forms (Energy of biosynthesis; Eb, energy of physical sloughing; Er, Energy sequestered in tissue; Es). The shedRules function determined how frequently eSnakes shed and what the energetic effort of shedding was. The function ran each day of the simulation for each eSnake. If the animal was not in shed (as

determined by the value of the eSnake state variable “shedDay”; a counter of days of shed remaining; shedDay=0 for eSnakes not in shed), the function used day in the year and random number draws (depending on desired shed frequency; Table 5) to determine if a specific eSnake should enter a “shed” state (if so, shedDay counter set to 28, behavior and physiology of the eSnake altered accordingly in subsequent loops). Shed frequency, and therefore the triggering conditions to begin a shed varied between simulations and among groups within simulations. I simulated shed frequencies of 0, 1, 1.15 (control), 2, and 3 sheds per year (Table 13) and used a different version of the shedRules function for each shed frequency group. All eSnakes shed a single time following their birth, and then did not begin another shed until the spring following their first winter. Thereafter, shed events were initiated based on the day of the year for 1, 2, and 3 shed groups. The control group, 1.15 sheds per year (based on long term shed data; Chapter 1) shed in the early summer, and then shed a second time in the year pending the outcome of a random number draw (eSnakes entered into a second shed 15% of the time). Once an animal entered shed, the length of the shed and the proportion of time during that shed when eSnakes did not engage in other behaviors (e.g., foraging; simulating observed changes in behavior in snakes with occluded spectacles) were saved to state variables of the eSnake, allowing us to count down the days of shed, calculate daily energetic efforts, and affect the activity budget. At the beginning of each shed event, efforts of shed (Eb, Es, and Er) were calculated based on body size at shed initiation (assuming the total effort to be established as soon as skin synthesis begins; following Chapter 3; with reference to Blem & Zimmerman 1986 for Es). Energetic effort of each portion of the shed was saved as state variables to the eSnake and accessed by the shedCost function (below) to model energy expenditure.

Lack of data on the effect of temperature on the duration and metabolic effort of shed (equations used here were collected at constant 25ºC) prevented consideration of temperature

effects in the shed modules of this iteration of my model. Since body temperature during shed likely effects the total duration of the process (especially the portion of the process when snakes seek shelter) and may influence total energetic effort, my approach in this iteration of the model ignores important variation in shed characteristics. Collection of these vital ecdysis-temperature data is ongoing; they will be the subject of future communications and model iterations.

shedCost: Energy lost during shedding (Eb, Er, and Es; following Chapter 3) occurs at different times. The energy of biosynthesis (Eb) and Es occur continuously as an additional daily metabolic expenditure (tissue costs energy to synthesize and the newly synthesized tissue contains energy in the form of chemical bonds). The energy required to physically remove the skin (Er) occurs only at the time of ecdysis as the individual increases its movement and muscular energy demands and rubs against the environment to remove the old tissue. The daily energetic cost from Eb and Es (stored as state variables by shedRules above) was computed by adding the two calculated costs together and dividing by the length of the shed. The energy per day was then scaled by growth efficiency as energy is lost in the conversion of fat and resources to tissue. Each day, the daily energy of ecdysis was subtracted from STS, or the difference between cost and STS was subtracted from LTS if STS was insufficient. The shedCost function then decreased the counter of shed days remaining. If shed day then equaled 0, the animal "shed" and the Er value was subtracted from available stores as a single lump sum. Total energy of the shed (Eb+Er+Es) was added to a running tally of lifetime shed energy (a state variable of all eSnakes) for computation of lifetime energy budgets and proportions.

Population Scaling

For population level simulations (those which allowed for the growth of population and death of individuals through time to simulate natural mortality), I employed the following functions;

populationGrowth: When each adult female eSnake successfully produced a "litter" of neonates, the offspring were added to the population in the model. All individuals were born on the mean date of parturition (Aug 27th) each year. The populationGrowth function took the number of neonates produced from each female eSnake during the year, compared a random number between 0-1 to a proportion which assigned offspring as male or female (sex ratio set to 50:50 for these simulations), generated a new eSnake object for each neonate, and then appended the offspring to the population (parameterized following initial conditions; Table 9). From that point on, each day looped over all individuals, including the newly added neonates. The neonates were subject to the same resource environments, energetic fluxes, and behavioral decisions and joined the simulation at their birth year until their death.

populationDeath: At the start of each year, all eSnakes in the population were subject to sex and age-class specific death rates. Death probabilities were strictly proportional, a random number between 0-1 was drawn from a uniform distribution for each eSnake, and values above a static cutoff value result in "death" of that eSnake. For this model, I used population wide vital rate values derived from the literature (Brown et al., 2007; Olson et al., 2015) for neonates (<1 year old; 65% survival), juveniles (between 2 and sexual maturity at 70cm SVL; 92% survival), adult males (95% survival), and adult females (83.5% survival). I imposed an age of senescence in my models of 25 years of age, assuming that animals in the wild rarely surpass this age (Beaupre, Personal observation). Any animal that survived to the end of its 25th year in the simulation was removed from the population at the start of the next year and labeled as deceased in the model (no more reproduction or growth).

Modeling Exercises

Sensitivity Analysis for Selection of Mean Foraging Success

As a bioenergetic model, resource availability is a key driver of all interactions in my simulations. Changes in foraging success (MFS) determine energy status of eSnakes and drive

patterns in growth and reproduction (Beaupre, 2002). Since the goal of the simulations presented here was to expand my perception of the relative importance of ecdysis to the energy budgets of rattlesnakes, I performed a sensitivity analysis to select a static Mean Foraging Success for use in my simulations. I wanted to select a value for MFS which resulted in ~50% survival of animals to a realistic age (15; Beaupre personal communication) to enable evaluation of general patterns of survival as other variables were manipulated (i.e., does increased shed frequency effect lifetime energy budgets?). I parameterized the model under control conditions (Table 9) with frequency of ecdysis set to reproduce naturally observed patterns (1.15 sheds per year on average) and varied foraging success. Each run replicated 100 eSnakes of each sex, through 15 years under static food conditions. I used sex-specific patterns of survival (out of 100 individuals) and the accumulation of long-term stores in animals through time to select a suitable MFS for my purposes (Figure 3). Survival was very low in either sex at MFS=0.06 (1/100 males; 8/100 females), and high at MFS=0.10 (64/100 males; 82/100 females). A median value, MFS=0.0875 produced approximately 50% survival in either sex (44/100 males; 59/100 females) with a range of energetic reserves that captured broad patterns in variability in body condition seen in wild populations (variable among individual eSnakes but constrained across the population through time).

While real populations are, in part, limited by annual fluctuations in resource abundance, a static value was necessary for comparison across the multiple simulations presented below. I assumed that a value of MFS=0.0875 was a suitable choice for modeling other processes and set it as the nominal condition in future simulations unless otherwise noted. However, since a foraging success of 0.0875 is not realistic in the source population (assuming a 242 day active season, juvenile eSnakes forage approximately 140 days per year; MFS=0.0875 translates to an unrealistically high ~12 meals per active season), and accumulation of long term stores exceeding 5,000 kJ suggests some unmodelled energetic expenses (e.g., the cost of

locomotion in large snakes, especially mate-searching males), the results of my simulations should be viewed as an exploration of relative effects of patterns of shed as a facet of a life history strategy, rather than an attempt at a complete and realistic accounting of time and energy in a population of snakes.

Impacts of Shed Frequency on Survival, Energetic Reserves, and Growth Trajectory at Static MFS

Central to my investigation is the relative weight of shed frequency (and its accompanying energetic and temporal effort) on the energy budgets of individuals. I expected increasing shed frequency to decrease the amount of energy available for growth, thereby decreasing growth rates (as measured by annual change in SVL). As a mandatory cost, I also expected that energy spent on shedding would decrease available resource pools (as measured by accumulation of long-term stores). Using the static MFS value determined above, I looked at the effect that increasing shed frequencies (0, 1, 1.15 (control), 2, 3) had on patterns of growth and storage accumulation through time. I modeled 100 eSnakes of each sex (M, F) with each shed frequency through 15 years and assessed patterns in SVL and LTS in survivors as well as proportional survival to 15 years in each group.

As shed frequency increased, proportional survival decreased in both sexes. However, the majority of mortality events occurred in juveniles, prior to attaining adult body size (Figure 4). In adult eSnakes, survival was nearly static in females (with the exception of 3x shedders), but adult mortality continued through adulthood in males with shed frequencies >0. I attribute the difference in survival between the sexes to the transition in allocation patterns at sexual maturation. Adult female eSnakes plateaued in body size at maturity as they allocated the majority of available energy towards reproduction rather than growth (observed in wild populations, simulated in my model). The decrease in growth allocation in mature female eSnakes limited total body sizes in my simulations, leading to a relative energetic savings of daily metabolism and cost of shed (both functions of body mass) when compared to large adult

males. My patterns suggest that energetics may play a role in constraining shed frequency in male rattlesnakes because eSnakes exhibiting more frequent ecdysis than observed in nature suffered decreased survival.

Patterns of long-term storage accumulation under varied shed frequency show a similar pattern to those reported for survival. eSnakes that shed more frequently had fewer energetic reserves (Figure 5), driving the patterns of survival discussed above. In my simulation, increasing shed frequency led to decreased resource pools (for allocation towards growth and reproduction) which in turn increased the frequency of "starvation" in those higher shed frequency groups. In the most extreme example, males that shed three times per year, no individuals survived more than 12 years. Conversely, in hypothetical eSnakes that never shed, males experienced exponential increases in stored energy (an impossible scenario in reality). Non-shedding female eSnakes had the highest rates of LTS accumulation among females, but over a 15-year period, storage did not increase exponentially. I attribute this difference between sexes to the way I chose to model reproduction. In my model, female eSnakes make large allocations towards reproduction, while male reproductive investment was likely underestimated by my approach (modeled an increase in summer metabolic scope; not a direct allocation from STS pools). Males in nature experience increasing costs of locomotion and move more during the active season when in good health (Lind et al., 2016), manifesting as increased spending which might temper the unrealistic LTS accumulation seen in my model. While continued development of my modeling approach for male reproductive effort may improve the relative realism of LTS accumulation in my simulations, the relative patterns (high shed groups have decreased storage accumulation and survival) would likely remain the same.

I found that increasing shed frequency altered time to adult body size (70cm; four or five years on average; potentially delaying start of reproduction) in my simulated animals, but that asymptotic body size did not vary among groups within sexes (Figure 6). Growth rate of

juvenile female eSnakes was slightly limited as a trade-off with shed frequency, but no change was visible after adult body sizes were attained. Increasing male shed frequency decreased average simulated body size through time, but the effect diminished as eSnakes approached asymptotic body sizes (as limited by bioenergetic tradeoffs; Beaupre, 2002). I hypothesize that the observed patterns are the result of trade-offs between foraging, digestion, growth, and ecdysis. In my model, growth can only occur on days when an eSnake is digesting (Figure 2). While energetic effort of ecdysis is split evenly across the 28 days of the process, eSnakes could not forage during the last two weeks of ecdysis and so could only digest meals that were acquired within the first half of the shed. Therefore, eSnakes could only digest and grow in a portion of their active season (which decreased with increasing shed frequency as a simple function of days in shed per year). Largely similar growth rates among varying shed frequencies suggest that growth is primarily regulated by foraging success, with cost of shed only limiting the daily resource pool during ecdysis. However, shed energy does seem to have an influence on survival in juvenile size classes.

My simulation illustrates an important aspect of ecdysis. Shed frequency and the associated temporal and energetic efforts in natural populations are likely constrained by available resource pools, particularly in large males. My simulations may partially explain why animals shed once per year in my source population, with a second or even third shed occurring in some individuals in some years. It seems unlikely that a shed frequency of three sheds per year could be sustained from my simulations, but since my model was parameterized from a source population which endures frequent food bottlenecks (Beaupre, 2008; McCue et al., 2012) parameterizing with data from other populations may yield different outcomes.

Effect of Shed Frequency, Active Season Length, and Mean Foraging Success on Lifetime Energy Budgets.

Shed frequency affected both time and energy budgets by increasing total metabolic effort and limiting the time available for foraging. Across latitudes, Timber Rattlesnakes have

been reported to shed more frequently in regions with longer active seasons (e.g., southern populations; Martin et al., 2021). While often assumed to be linked to increased growth rates in larger southern animals, I hypothesized that shed frequency is shaped by time available to forage and the frequency of resource acquisition rather than strictly growth (Chapter 1). Animals in such populations (increased active season length or resource acquisition) likely experience larger energy budgets and therefore are not subject to mortality imposed by the higher costs of more frequent ecdysis.

To assess the relative influence of time and energy as forces which impose constraints on shed frequency, I simulated male rattlesnakes through 15 years with varying shed frequency and manipulated active season ("AS") length and resource availability ("MFS"). I simulated snakes through time with static shed frequencies of one, two, or three sheds per year under combinations of mean foraging success ("control" MFS=0.0875; "increased" MFS=0.1075) and active season length ("control" Active Season = 246 days (late March – mid-December); "extended" Active season =306 days (Late February to Mid-January)). I tracked total metabolizable energy available to individual eSnakes (total usable STS gained from all successful foraging events and subsequent digestion) and total energy devoted towards ecdysis (the sum of all E_b, E_r, and E_s for each shed event) throughout their lives (Figure 7).

In general, total energy budgets decreased within MFS and AS groups as shed frequency increased, indicating that the shed related decrease in time available to forage (2 weeks per shed event) affects lifetime resource acquisition. In eSnakes that shed three times per year, survival was very low (2 of 3000 MFS=control, AS=control; 7 of 3000 MFS=control, AS=extended; 111 of 3000 MFS=high, AS=control). I only report energy budgets from eSnakes that survived the entire 15-year duration, but the survival data are relevant here, suggesting that only small eSnakes with minimal energy budgets and mass scaled metabolism and efforts of shed were able to persist under the extreme limitations of three sheds per year. My data

suggest that increased active season length had a greater effect on total energy budgets than increased foraging success under my parameterized conditions (as evidenced by larger total area of pie charts; Figure 7). In three-shedding eSnakes, the effect was opposite to that of total energy budget size, with increased food availability leading to larger total energy budgets in high frequency shedding eSnakes. However, in the other two groups, there was an apparent inflection point between two and three sheds per year, with higher shed frequencies leading to smaller energy budgets (and presumably smaller eSnakes). Also of note is the observation that total lifetime proportion of energy devoted to ecdysis scaled with shed frequency (once per year = ~3%, twice per year = ~6%, and three times per year = ~9%), but rarely exceeded 11% of lifetime energy. I interpret this result as a limit in allocation, with animals allocating more than ~11% of their resources towards shedding dying before the age of 15, even with increased resource availability or active season length.

Animals with longer active seasons and/or higher resource availability shed more frequently in natural populations. My simulation suggests that increased foraging success results in larger lifetime resource pools, permitting increased shed frequency and survivorship at those increased frequencies. While longer active seasons did increase lifetime budgets (relative to control active season length), it appears that the limits on time for foraging imposed by the shed cycle are problematic for eSnakes if the resource environment remains the same. As parameterized, it appears that time and energy budgets may act to constrain ecdysis frequency since eSnakes that allocated more than ~11% of their total energy budget towards shedding did not persist until the end of the simulation as a result of unbalanced energy budgets. In summation, shedding is an important aspect of the life history strategy of snakes. Shed frequency is likely limited by time-energy tradeoffs, shaping the frequencies I observe in wild populations and potentially explaining the variation observed between populations with different resource abundance or active season length.

Effects on Female Fecundity

Preliminary simulations with the present iteration of the model indicated that survivorship and fecundity of adult female eSnakes drives population trends in my simulated Timber Rattlesnake. As such, the effect of varying shed frequency on female reproductive output was a suitable indicator of large-scale repercussions of natural shed patterns. Additionally, in a population of timber rattlesnakes, shed frequency in females has been found to be related to reproductive condition, with gravid females shedding only once per active season, while early vitellogenic and non-reproductive females often shed a second time per active season, cooccurring with observations of male-female courtship activities (Chapter 1). To address the possibility that shed frequency may be constrained by patterns of reproductive output and total lifetime offspring production, I ran a series of simulations, under static food availability (MFS=0.0875) with differing shed frequency (0, 1, 1.15 (control), 2, or 3 times per year) in female eSnakes. I parameterized the timing of shed events following natural observations of shed timing in Timber Rattlesnakes (Table 13; extrapolating based on observations or evenly dividing available time for higher than observed shed frequencies). I simulated females from birth to age 15. Survival through the entire simulation was shed frequency dependent (see previous simulations; above), but here I ran large enough samples of eSnakes to garner 500 surviving individuals from each group. Here I report age to first reproduction, lifetime offspring production, and number of reproductive events per group. Results were averaged from 500 individuals surviving through age 15 from each shed frequency group.

Shed frequency interacted with fecundity in female eSnakes. At higher shed frequencies, eSnakes experienced a delayed start to reproduction and tended to reproduce fewer times in 15 years, reducing lifetime offspring production (Figure 8). However, because survival was lower in more frequent shedding eSnakes under constant resource availability, the fitness cost to female eSnakes that shed more frequently than observed in my source

population (2 and 3 shed groups relative to the 1.15 shed per year control) was greater than through consideration of fecundity alone. For eSnakes shedding three times per year, survivorship dropped to ~25% with those that survive having their first reproductive bout 1-2 years later than other eSnakes, missing at least one additional reproductive bout during their lifetime, and reducing total neonate production by ~30% (relative to 0, 1, and 1.15 shed per year groups). Increasing shed frequency decreased the energy budget available for reproduction (both as an implicit energetic expense and indirectly limiting total energy budgets by decreasing the time available for foraging) indicating that observed shed frequencies may be constrained, in part, by reproduction. While limited energy did not prevent animals from reaching mature body sizes (all animals converge on adult body size in their fourth or fifth year, though growth is slowed in higher shed groups; simulation above), it seemed to effect fecundity by delaying the start of reproduction and limiting reproductive frequency thereafter (i.e., tri-ennial reproduction was more frequent than bi-ennial in higher shed groups). While there was overlap in the variation of reproductive frequency between groups (Figure 9), histograms of frequency tended to shift/skew left as shed frequency increased.

If I consider changes in survivorship and fecundity together between the one and two shed groups, I could imagine seeing a group of 500 females producing 4,500 neonates over 15 years in the one shed group, but only 1,900 in the two shed group (ignoring the output of females that do not survive the 15 years for simplicity, assuming survivorships of 66 and 30 percent (one and two sheds respectively), and average lifetime neonate production of 13.8 (one shed) or 12.7 (two shed)). Why then do non-gravid females in real populations shed more often (sometimes twice per year) and risk decreasing energy available for reproduction but gravid females shed only once per year (Chapter 1)? It has been suggested that ecdysis serves a reproductive function in rattlesnakes (Chapter 1; Martin et al., 2021) with males mate-guarding females during the late summer mating season and only copulating or giving up on courtship

after the female has shed (Aldridge & Duvall, 2002). My output offers support for the suggested reproductive utility of ecdysis. Skin shedding is an obligate and substantial energetic expenditure for snakes, but frequency of ecdysis (above one seemingly mandatory shed per year; Klauber, 1972) appears to be a plastic trait. If less frequent shedding improves reproductive output, it seems reasonable that if reproductive females shed more often than the minimum, then that excess energetic investment likely improves reproductive success in some way. Following detailed work in garter snakes (Lemaster & Mason, 2003; Mason & Parker, 2010; Parker & Mason, 2012), coupled with my observations from this modeling exercise, I suggest that the shed patterns observed in real female timber rattlesnakes may serve a vital function in courtship and reproduction that should only be favored if the benefit to fitness outweighs the energetic costs of increased shedding (e.g., shedding more and increasing risk but attracting a mate is better than surviving and not attracting a mate at all). The conditions which trigger shed events in snakes remain a mystery and so I have opted to model shed frequency and occurrence free of causation. But it seems feasible given my findings that the increased ecdytic frequency seen in reproductive females may be causally related to courtship in Timber Rattlesnakes. Empirical work should follow up on my findings, working to demonstrate reproductive utility of ecdysis in the field.

Affects at the Population Scale with Varying Shed Frequency and Resource Environments

The slow life histories of rattlesnake species (long-lived, slow reproducing) make the study of population trends difficult, with a single researchers' 25-year career spanning the lifespan of a solitary cohort. However, energetic allocation strategies which vary among populations and species must be guided by selective pressures. Therefore, allocation strategies carry fitness costs that are not easily studied in the field (Dunham et al., 1989). Relevant to the modeling exercise here, I wanted to evaluate the relative effect of the resource environment and shed frequencies on populations. I used my model to project simulated

populations through time using age-specific survival probabilities from the literature (W. S. Brown et al., 2007; Olson et al., 2015) and boom-bust variations in resource abundance drawn from a normal distribution around a mean foraging success to evaluate population scale outcomes of varying shed frequency.

I modeled two resource availability patterns ("Control" = 0.0875 +/- 0.02 (SD); "High" = 0.1075 +/- 0.02 (SD)) in populations (initial population; 30 male neonates, 30 female neonates) with static shed frequencies of 0, 1.15 ("control"), or 3 sheds per year. MFS varied by year, but year-specific values were the same across simulations pending resource abundance category (high or control). I tracked and plotted total number of animals still "alive" at the end of each year of the simulation over 100 years (Figure 10). There was a clear effect of both food resources and shed frequency. I considered the "control" SF under "control" MFS as my control, producing semi-stable populations through time (since the MFS of 0.0875 was chosen based on a shed frequency of 1.15 sheds per year). In general, increasing shed frequency above control lowered survival and lead to population collapse under either food condition in less than 100 years in animals that shed three times per year. Populations without shedding incorporated into the model (SF=0, either MFS) and the population with control shed frequency under increased food grew exponentially, with the rate of increase differing with food and shed frequency. While these results are relative, they demonstrate that shed frequency and resources affect allocable energy and therefore survivorship. The model output indicates that the increased energy demand of more frequent shedding seen in some populations of Timber Rattlesnakes (Martin et al., 2021) can be offset by the resource environment. More data on the availability of resources in the environment are needed to improve the accuracy of the population aspect of this model and its utility in evaluating the relative importance of competing selective pressures. However, for the time being, my model demonstrates that shedding is an important life history characteristic in snakes and is shaped by resource abundance and

individual mass-energy and time budgets all of which carry fitness costs warranting further investigation.

Conclusion

My modeling exercises indicate that shedding is intricately interrelated with the time-energy budgets of snakes. Because of the principle of allocation in a constrained energy budget, any bioenergetic cost associated with ecdysis is interrelated with the patterns of survival, growth, reproductive output and frequency, and lifetime reproductive success that result from the snake's life history strategy. As such, patterns of shed are shaped by the interplay between the survival and fitness outcomes as organisms test their life history strategy against fluctuating environmental conditions, with suboptimal strategies driving extirpation. It seems clear that shed frequency is somewhat plastic, but the forces which produce minimum shed frequencies and the conditions which illicit shed events from snakes are still poorly understood. Mechanistically, the frequency of shedding affects energy budgets both directly (as a requisite expenditure) and indirectly (by limiting time available for other activities and reducing the total energy budget). More empirical data are needed on natural patterns of shed in wild populations, but ecdysis frequency and timing may have important implications for the clade, especially in the face of anthropogenic climate change and the accompanying changes in active season length, resource availability, and total energy budgets.

The simulations presented here were subject to decreased survival at increased shed frequencies, and outcomes depended upon the resource environment (hard set in most simulations to decrease ambiguity of model output). As such, it is impossible to completely disentangle shedding, survival, and resource abundance and so my results should be viewed as relative with respect to shed frequency. However, my output strongly suggests that shedding is an important aspect of reptilian life history strategies and urge its inclusion in future modelling exercises. Additionally, I acknowledge that in real populations, outcomes may be more complex

as individuals may alter shed frequency, encounter prey with variable success, or alter their allocation strategy with respect to those variables. Nonetheless, it seems apparent that shedding is a vital, but understudied aspect of reptilian life history, worthy of additional research focus.

Tables and Figures

Table 9: Simulation initialization parameters. Constant values and neonate initialization values.

Variable	Initial Value
Duration of Shed Events	28 days
Proportion of Shed with Occluded Spectacles	50%
Neonate Survivorship (annual)	65%
Juvenile Survivorship (annual)	92%
Adult Male Survivorship (annual)	95%
Adult Female Survivorship (annual)	83.5%
Mass at Birth	25g
SVL at Birth	33.28cm
Long Term Storage at Birth	135 kJ

Table 10: Environmental variables showing the division of days and body temperature.

	Summer	Fall	Winter	Autumn
Days (Julian)	1-33; 216-265	34-64	65-184	185-215
Temperature Profiles	32, 30, 28, 25, 28, 32	25, 22, 18, 16, 20, 23	10, 10, 10, 10, 10, 10	25, 20, 17, 17, 22, 23

Table 11: State Variables of eSnakes tracked throughout simulations. Note that some variables were specific to female individuals. Each variable is updated with a different frequency, and the currency used to store the information is displayed.

Sex	Variable	Update Frequency	Currency
Male and Female	Food in Gut	Daily	g wet mass, rodent
	Mass	Daily (During Growth)	g
	SVL	Daily (During Growth)	cm
	Sexual Maturity	Daily (end of day)	True / False
	Long Term Stores	Daily	kJ
	Short Term Stores	Daily	kJ
	Safety Stores	Seasonally	kJ
	Digesting Time (remaining)	At meal Capture, daily during digestion	days
	Proportion of Allocation to Growth	Seasonally, with sexual maturity	Proportion between 0-1
	Proportion of Allocation to Reproduction	Seasonally, with sexual maturity	Proportion between 0-1
	Shed Day (remaining)	Daily during Shed cycles	days
	Er (energy of skin removal)	Daily at end of Shed Cycle	kJ
	Eb (energy of skin biosynthesis)	Daily during Shed cycles	kJ
	Es (energy sequestered in skin tissue)	Daily during Shed cycles	kJ
	Specific Dynamic Action	At meal Acquisition	kJ
	Birth Year	At Birth	numeral
	Shed Energy (Cummulative)	At end of shed event	kJ
	Total Metabolizable Energy	Daily during digestion	kJ
Female Only	Vitellogenic	Seasonally	True / False
	Reproductive Stores	Daily	kJ
	Metabolic Cost of Vitellogenesis (daily proportion)	Seasonally, at initiation of vitellogenesis	kJ
	Minimum Energy for Viable Clutch	Seasonally, at initiation of vitellogenesis	kJ
	Lifetime neonates produced	Annually	Number offspring
	Reproductive Count	Annually	Number of completed cycles

Table 12: Variables saved to the global environment. These values affected all eSnakes in the same way regardless of sex, size, or time period and so were saved in the readily 155ccessible global environment.

Variable	Static Value
Mean Foraging Success	*Simulation Specific
Largest Available Meal Size	500g
Smallest Acceptable Meal Size	5% eSnake Body Mass
Conversion Efficiency (consumed tissue to metabolizable energy)	80%
Growth Efficiency (metabolizable energy to tissue)	60%
Size to Sexual Maturity	70cm SVL

Table 13: Conditions used to initiate sheds for the shed within the year (rows) in each of the relevant shed frequency groups used in simulations (0, 1, 1.15 (control), 2, or 3 shed per year).

Shed Event	0	1	1.15	2	3
1		June 10 (day 279	Between May 10-June 10 (days 259-279) pending draw from random distribution	May 20 (day 269)	April 1 (day 216)
2			Between July 11 and July 31 (days 322-342) in 15% of individuals pending a random number draw.	July 21 (day 332)	May 27 (day 276)
3					July 25 (day 336)

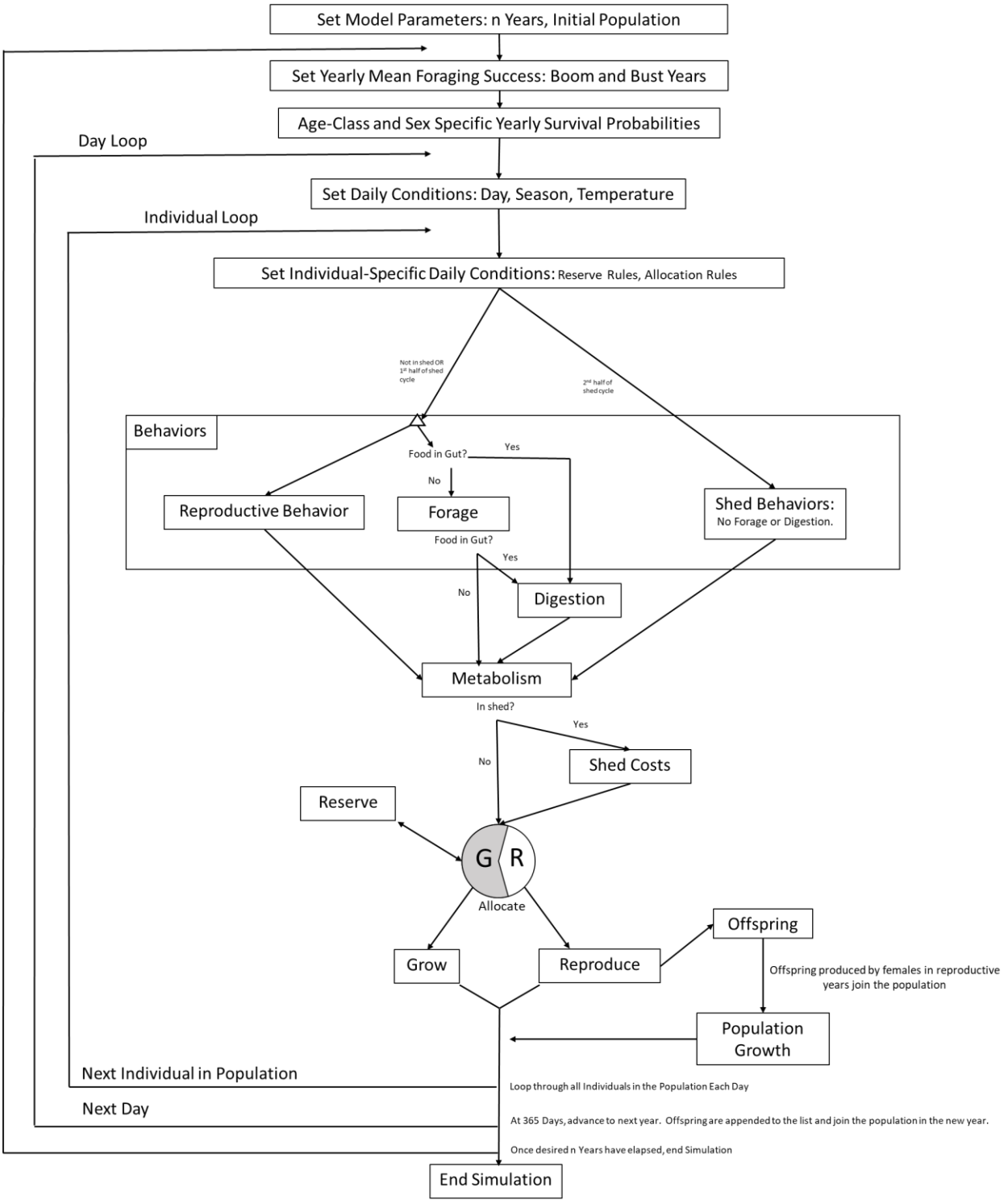

Figure 1: Flow Chart of Model Structure. Note that some aspects of the model (population growth and age-specific annual mortality) were only incorporated in population level simulations.

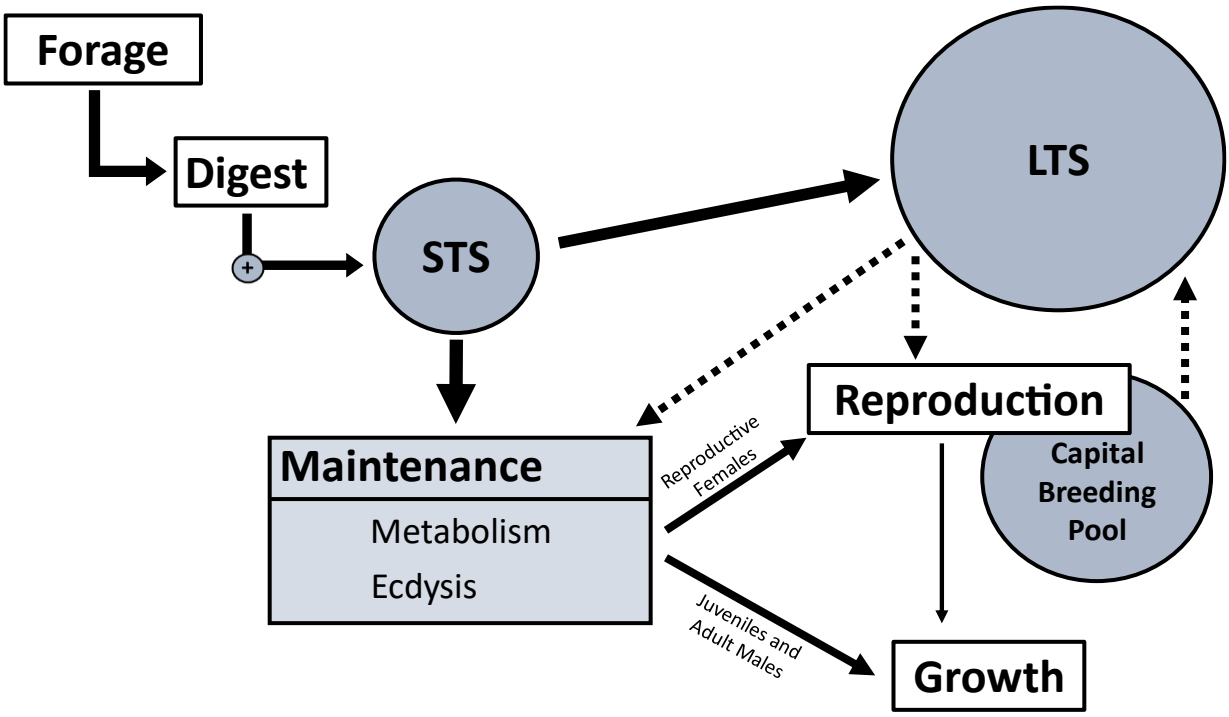

Figure 2: Flow chart of energy allocation in the model. eSnakes only gain energy through digestion of prey items following successful foraging events. On each day when digestion occurs, energy is added to the short term storage pool (STS). Short term storage must be used to pay maintenance costs before all other costs. If short term pools are insufficient, maintenance costs can be supplemented from long term stores (LTS; dashed arrow). Short term stores remaining after maintenance costs are paid are forwarded towards reproduction (females only) and growth. In reproductive females energy is allocated towards reproduction and sequestered in the capital breeding pool. The capital breeding pool is increased daily during pregnancy (daily cost of reproduction) coming from STS remainder after maintenance when applicable but being supplemented from LTS when STS is insufficient. At some periodic checks, female eSnakes may abort a pregnancy, in those instances, the capital breeding pool is shuttled back to LTS and the female can try again at the next reproductive cycle. After maintenance (males and juveniles) or reproduction (adult females), the remaining surplus from STS for the day is allocated towards growth. Energy is only devoted to growth when digestion is occurring and other costs leave additional energy for growth. On days when no energy is gained (from digestion) or when maintenance and reproduction costs exceed STS, growth does not occur. Note that line weight flowing from STS > Maintenance > Reproduction > Growth reflects relative magnitude of remaining STS. Juveniles and Adult males generally have a larger STS remainder available for growth than reproductive females. In real snakes, long term fat stores would be mobilized and added to STS to pay costs, my model functionally operates the same (as mobilization of long term stores subjects the energetic content therein to a decay to replicate the inefficiency of that energy conversion), but for ease of accounting, energy flows directly from LTS to supplement costs; energy never flows directly from LTS to STS pools but is attributed to specific costs as needed.

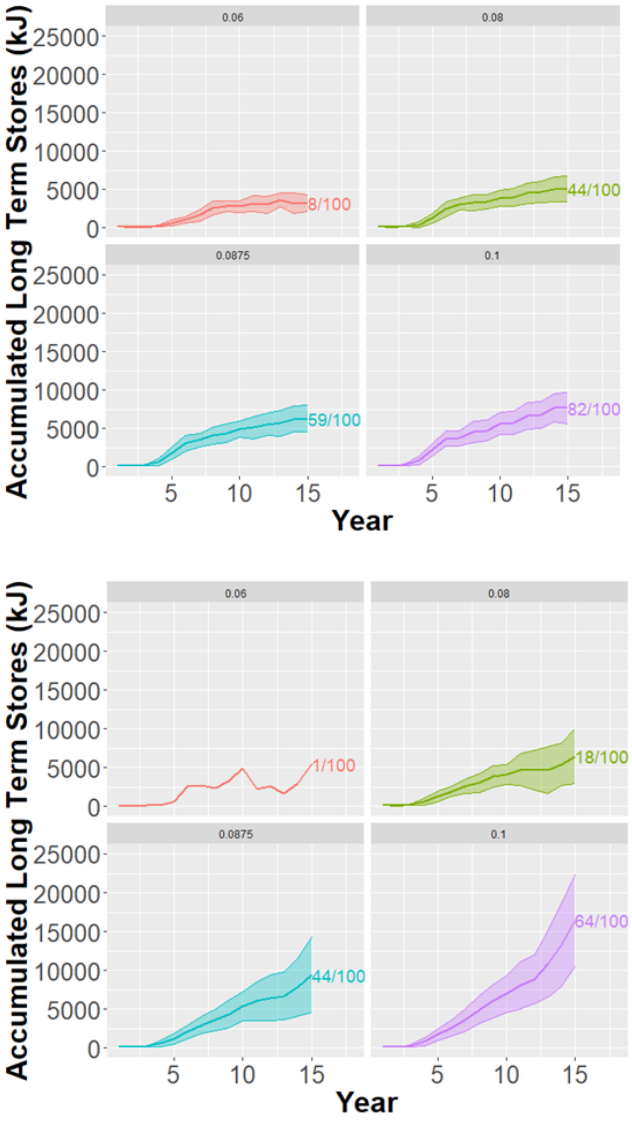

Figure 3: Accumulation of long-term storage and relative survival through 15 years in female (top) and male (bottom) individuals at different mean foraging success (faceted by color; red=0.06, green=0.08, blue=0.0875, purple=0.1). Note that the ribbon around each line denotes 1 SD (not 95% CI).

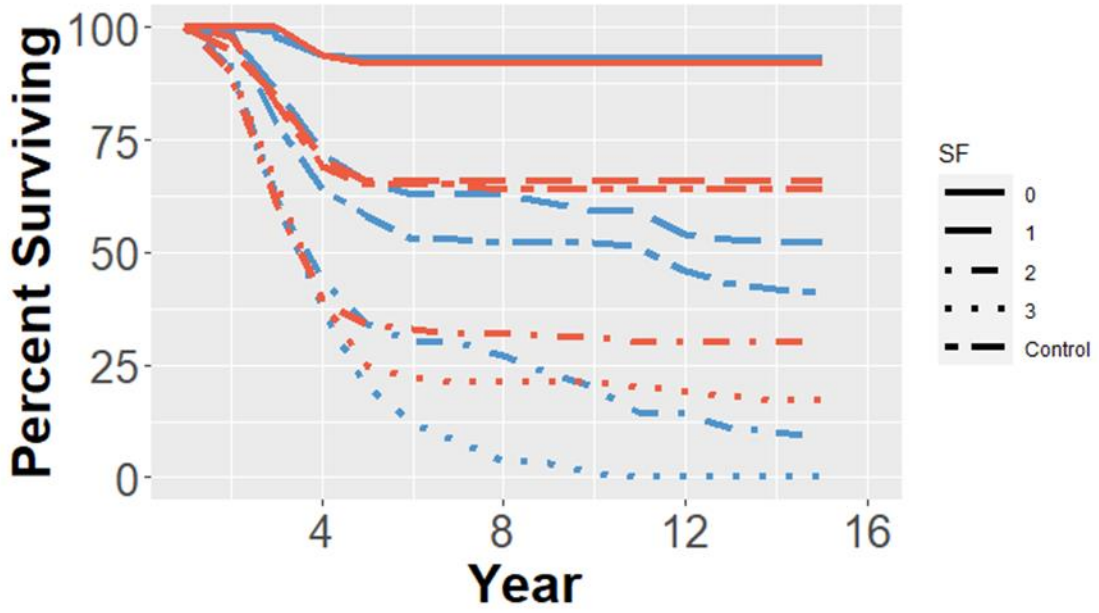

Figure 4: Survival (in percent surviving) at 0.0875 MFS in males (blue) and females (red) of varying shed frequencies ("SF"; denoted by line type; control=1.15 sheds per year). Survival drops most between years 1 and 4 (commonly the age at which individuals reach sexual maturity in my model) in all groups. In some groups (primarily males) survival continues to decrease into adulthood.

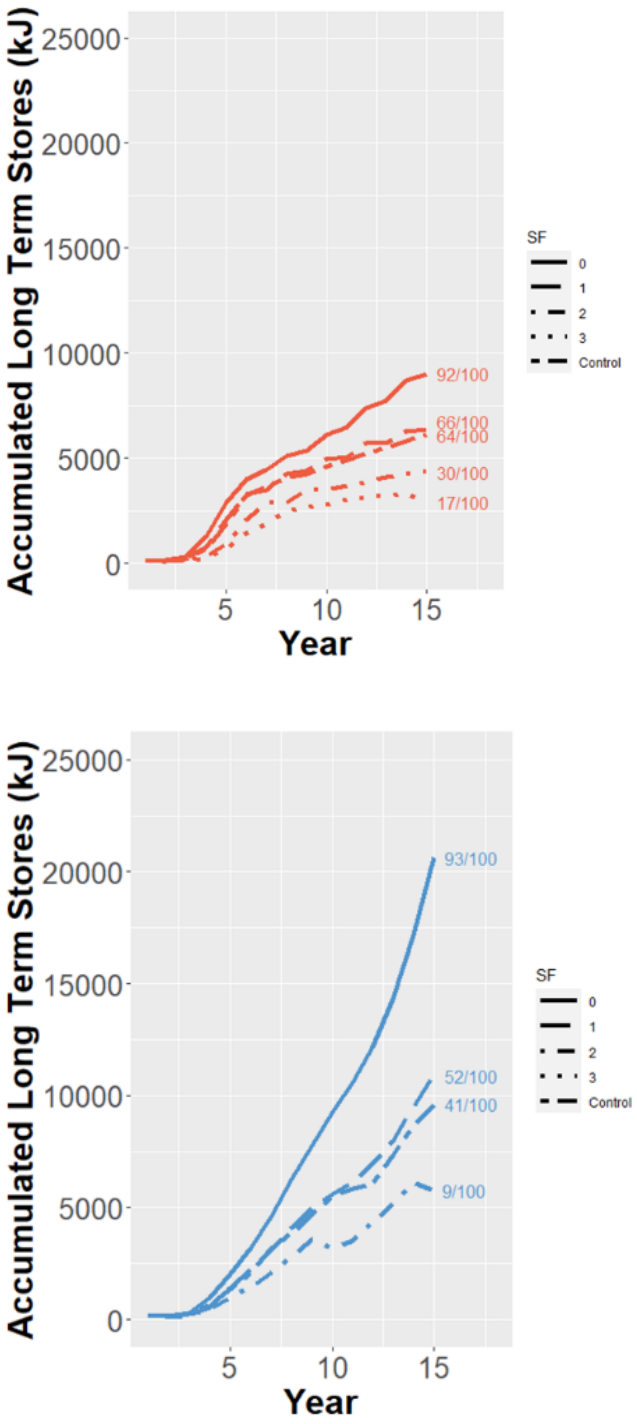

Figure 5: Average LTS accumulation of individuals surviving 15 years in males (blue) and females (red) with different shed frequencies. Numbers show the proportional survival rates of 100 animals at the end of the 15 year simulation. Line types denotes shed frequency. Survival and average accumulated storage decrease with increasing shed frequency, indicating that decreased storage as a result of increased shed expenditure drives mortality.

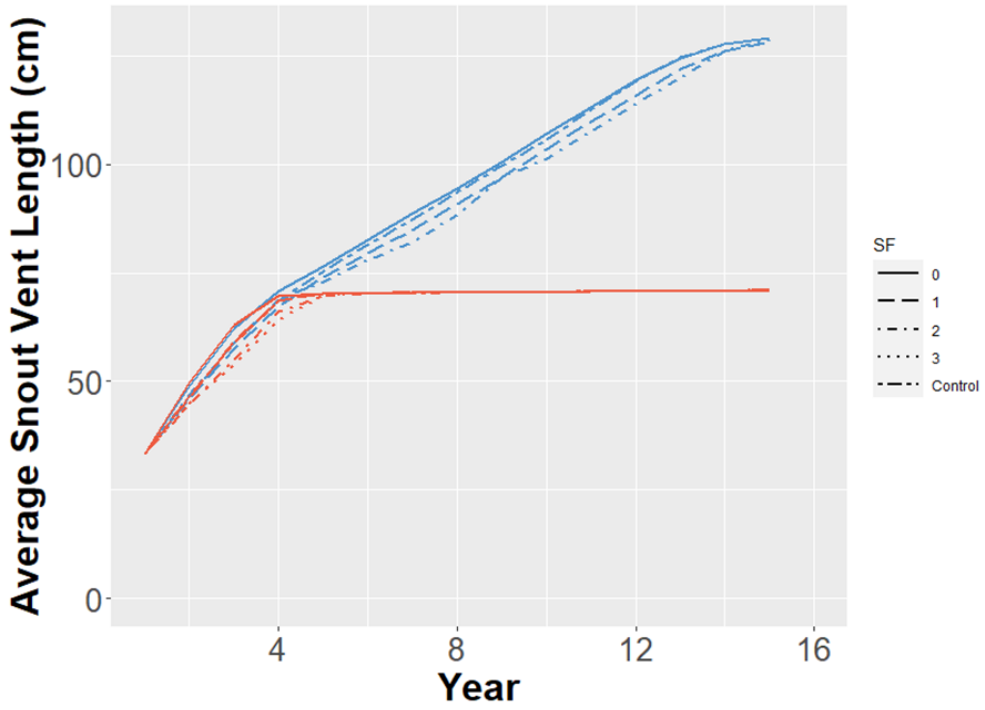

Figure 6: Growth rates to asymptotic body sizes by sex (blue=males, red=females) under varying shed frequency (line type) over 15 years. SVL's are based on average length at each time point across individuals that survive the length of the simulation. Females reach adult body size between ages 4 and 5 depending on shed frequency. Males with increased shed frequency may be slightly smaller than their counter parts, but asymptotic body size (as limited by energy availability and consumption and processing rates) remains similar among groups, forcing the conversion of all animals to a body size slightly above 125cm.

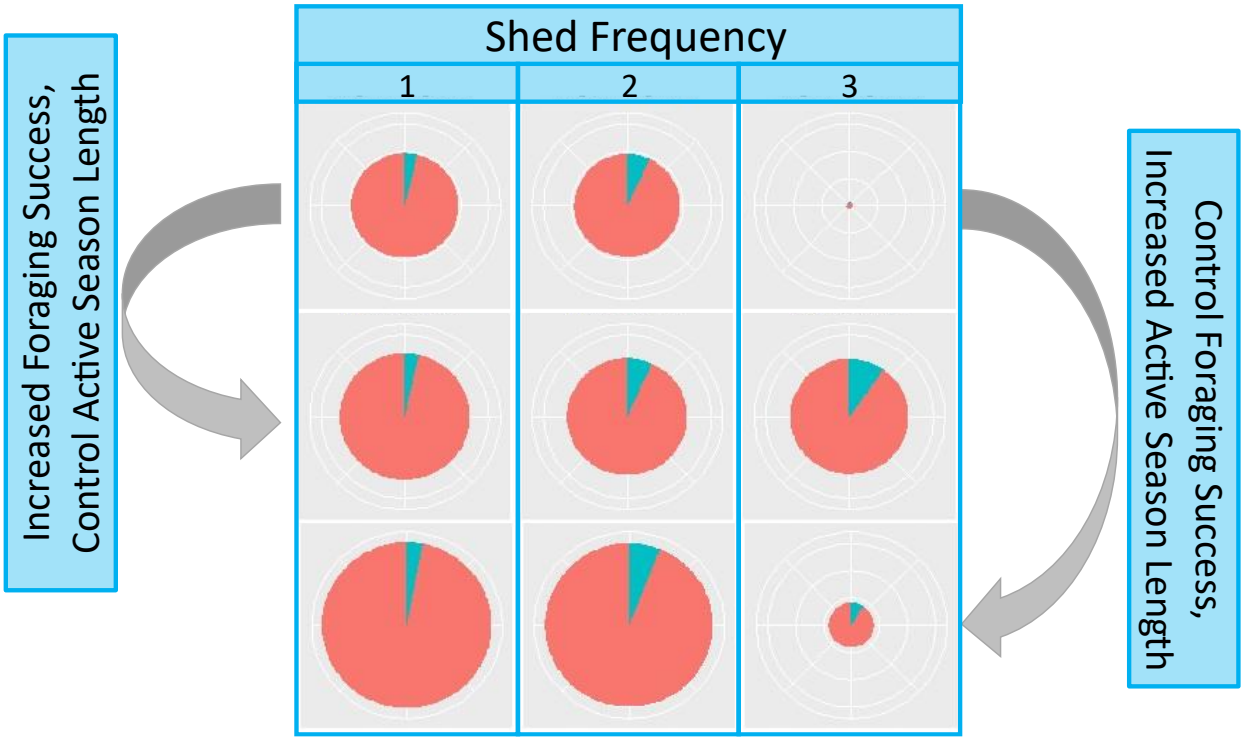

Figure 7: Variation in total energy budgets (area of pies) and the proportion of that budget devoted towards ecdysis (teal portion) in animals that survived 15 years (survival decreased with increasing shed frequency but is not reflected here) with different shed frequencies and in different resource environments or under alternate active season lengths. Note that increasing shed frequency decreases total energy budget area slightly, but that increased food availability, and to a lesser extent, increased active season lengths increased total energy budget size. However, proportion of total energy budget remained similar within shed frequency groups. Since values are drawn from surviving animals, these patterns should be viewed as reflective of successful allocation strategies only, increased allocation towards ecdysis (above 11% of total energy budget) was associated with mortality.

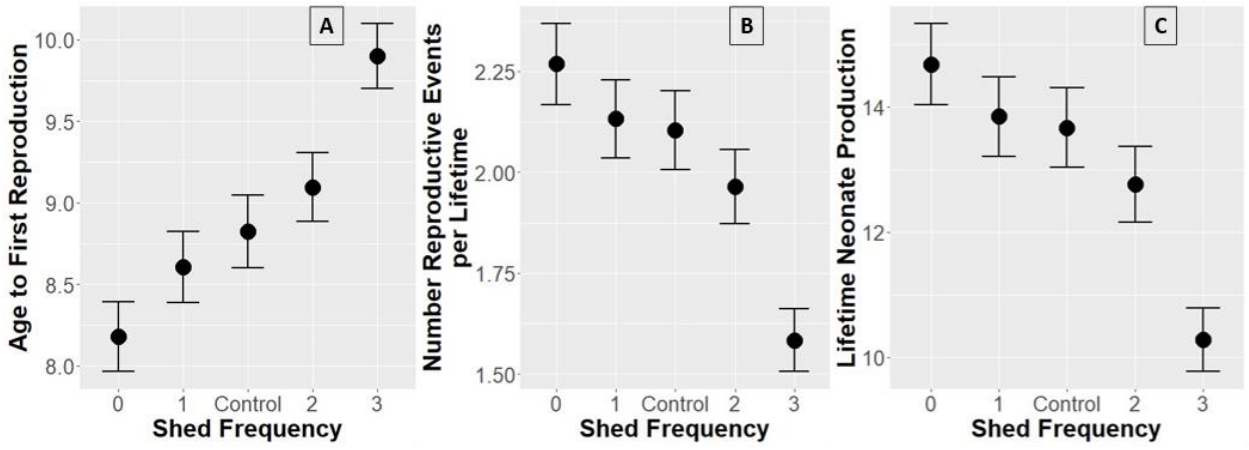

Figure 8: Age to first reproduction in years (Panel A), Number of reproductive bouts (Panel B), and Total number of offspring produced (Panel C) in females under varying shed frequencies. Values are averages of 500 individuals and do not reflect the decrease in survivorship seen at increasing shed frequencies (previous simulations). Error bars show +/- 1.96 SE.

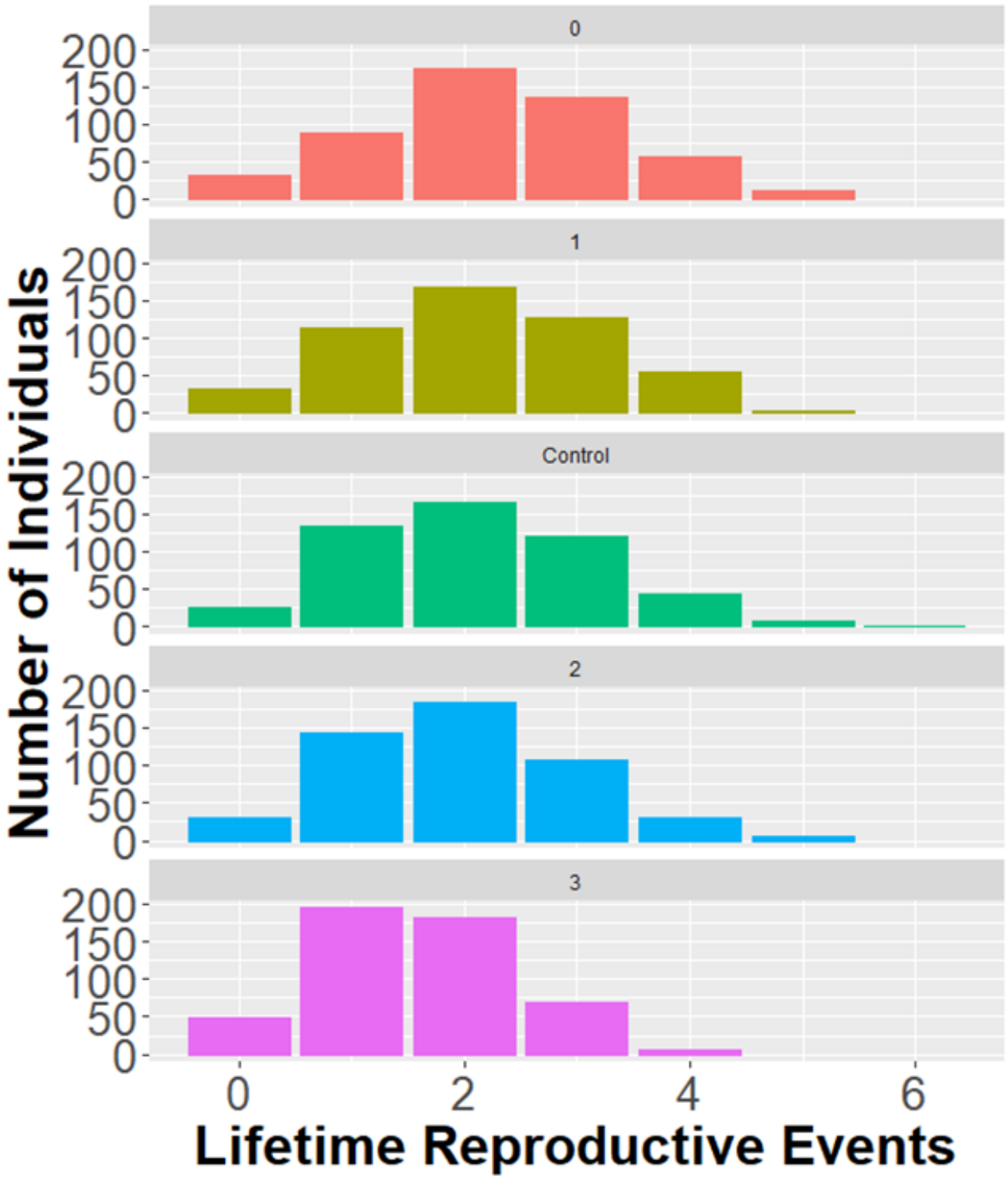

Figure 9: Histograms of number of reproductive events (0-6) for each of 500 females in simulations of varying shed frequencies (denoted by color and panel; 0, 1, 1.15, 2, or 3 sheds from top to bottom). Bar height reflects the number of animals in the simulation exhibiting that reproductive frequency. Note that while broadly similar, some lower shedding groups were able to reach higher reproductive frequencies, while high shed animals (three sheds) tended to have more 0 and 1 reproductive event females.

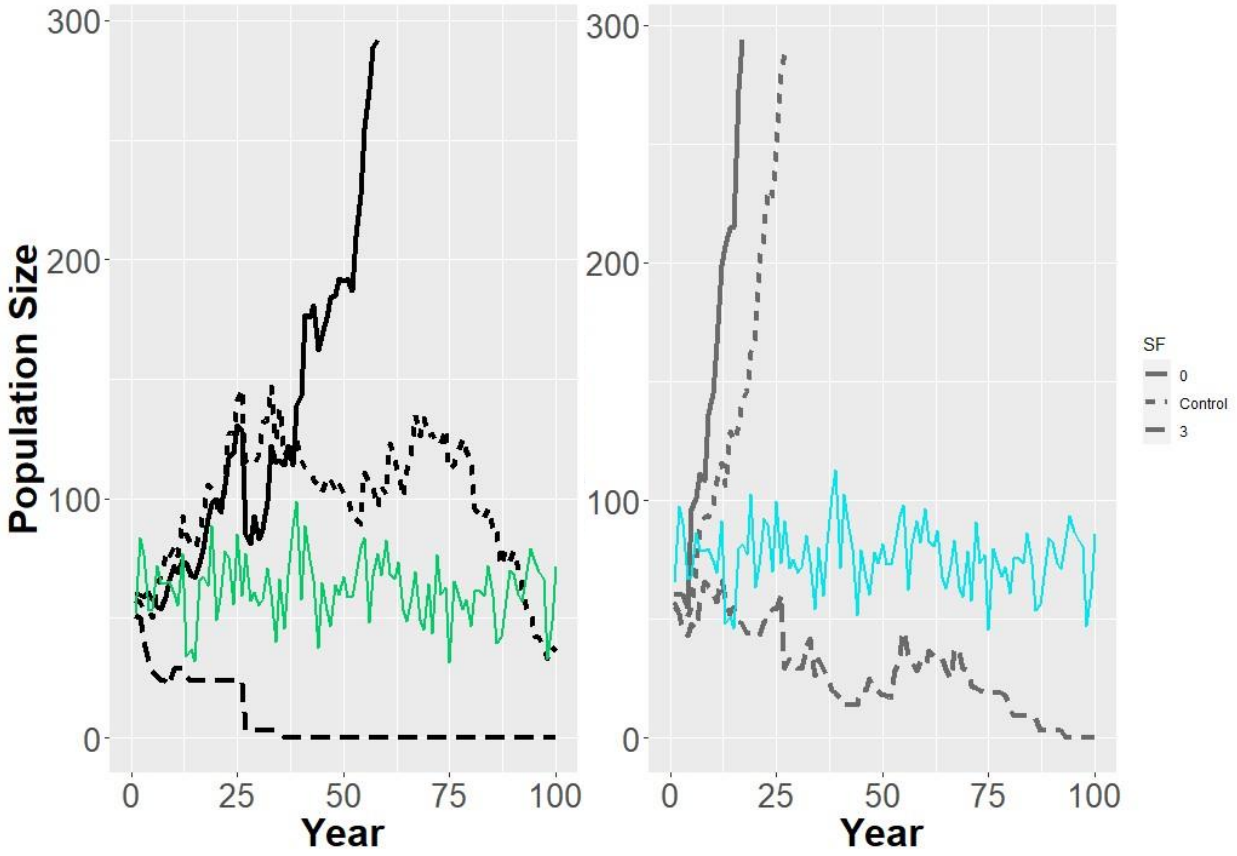

Figure 10: Population trends (in number of individuals in the population) as they vary with resource availability (control=green, left; elevated=blue, right) and shed frequency (line type). Colored lines show resource abundance during the model, the lines are relative to each other and show general trends in increases and decreases in resources through year but are unscaled relative to the larger figure. Animals under control resources (left) or high resources (right) with shed frequencies of 0 (solid line), control (1.15; dashed line), or 3 (long dashed line) sheds per year. Both foraging success and shed frequency affected population trends through time. Note that lines that do not continue to extend along the x-axis reflect populations growing exponentially.

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Appendix:

Table 1:Mass scaling and temperature dependent mass-scaling equations used to inform the DEB-IBM. W=body mass, T=body temperature, D=meal size. For some equations, multiple forms were used depending on specific variables (e.g., meal size or time blocks). Source material is referenced for each equation.

Type	Group	Equation	Units	Source
Digest time	small meal	(22.25 * LogW + 60.95) / 24	Days	Zaidan and Beaupre, 2003
Digest time	medium meal	(116.14 * LogW + 6.88) / 24	Days	Zaidan and Beaupre, 2003
Digest time	large meal	(150.33 * LogW - 34.10) / 24	Days	Zaidan and Beaupre, 2003
Specific Dynamic Action		$12.158*W^{0.160}*(D^{1.030})$	mL CO2	Zaidan and Beaupre, 2003
Metabolism	Winter	$24 * (0.7546) * (W^{1.0457}) * (10^{((-1.0768T) + (0.1091T^2) - (0.00317T^3))})$	mL CO2 per day	Agugliero diss.
Metabolism	active season block 1: 1500-1800	$4*(0.00107W^{0.825})*(10^{0.0569T})$ Temps: Spring:25 Summer:32 Autumn:25	mL CO2 per time block	Beaupre and Zaidan, 2001
Metabolism	active season block 2: 1900-2300	$5*(0.00091W^{0.799})*(10^{0.0628T})$ Temps: Spring:20 Summer:30 Autumn:22	mL CO2 per time block	Beaupre and Zaidan, 2001
Metabolism	active season block 3: 0000-0300	$4*(0.00095W^{0.741})*(10^{0.0680T})$ Temps: Spring:17 Summer:28 Autumn:18	mL CO2 per time block	Beaupre and Zaidan, 2001
Metabolism	active season block 4: 0400-0700	$4*(0.00120W^{0.727})*(10^{0.0643T})$ Temps: Spring:17 Summer:25 Autumn:16	mL CO2 per time block	Beaupre and Zaidan, 2001
Metabolism	active season block 5: 0800-1100	$4*(0.00124W^{0.777})*(10^{0.0590T})$ Temps: Spring:22 Summer:28 Autumn:20	mL CO2 per time block	Beaupre and Zaidan, 2001

Metabolism	active season block 6: 1200-1400	$3*(0.00128W^{0.787})*(10^{0.0650T})$ Temps: Spring:23 Summer:32 Autumn: 23	mL CO2 per time block	Beaupre and Zaidan, 2001
Mass-SVL relationship	Mass to SVL	$11.73*W^{0.324}$	cm	Beaupre and Zaidan, 2001
Ecdysis	E_b	$(15.2175*W^{0.88}) * (0.02742)$	kJ	Chapter 3
Ecdysis	E_r	$(36.4*W^{0.19}) * (0.02742)$	kJ	Chapter 3
Ecdysis	E_s	$0.258*W^{0.8795}$	kJ	Adapted from Blem and Zimmerman, 1986

A base version of the DEBIBM can be accessed at:

https://github.com/mdcarnes/DissertationDEBIBM

Conclusion

Ecdysis is important for reptiles. It requires significant metabolic and temporal effort and occurs with variable frequency, but the forces which dictate its frequency, at least in part, remain a mystery. I found that even basic data on the process are lacking. Without a clear understanding of natural patterns of frequency and timing or measurements of the effort involved, we cannot begin to address larger questions about the evolutionary processes which have shaped the observed patterns of shed or even define what is “typical” within a population. I also found repeated evidence (through multiple lines) that implicates reproduction in rattlesnakes as a potentially causal correlate of ecdysis. Natural patterns of shed indicate that shed frequency in females is related to reproductive condition, and that the timing of some shed events may correlate to reproductive events. While the mechanisms that initiate shedding remain elusive, the total cost of the process is of significant magnitude to warrant consideration in reptile time-energy budgets. My data and the result of my simulations suggest that shedding may be related to the resource environment (and associated energy budgets) in males, but that in females, frequency of shed may have been selected to facilitate copulation and mate attraction as shed frequencies less than those observed in wild population should produce higher fitness given my measurements of cost and effort.

Most groups of reptiles rely on some degree of chemical signaling to communicate with conspecifics for reproduction (Mason & Parker, 2010). Effective signaling molecules are often byproducts of other body processes and as such are energetically inexpensive to produce (Duvall, 1986) while their relative concentrations may convey information about the quality of an animal as a potential mate (Duvall, 1986; Mason & Parker, 2010; Shine et al., 2003). Within reptiles, squamates exhibit a diverse array of semiochemical producing glands and strategies. Many lizards produce waxy secretions from femoral and follicular glands on their ventral surfaces; these compounds play a role in dictating an individuals’ response to a conspecific (i.e.,

mating with attractive individuals, aggressive behaviors towards rivals; Alberts, 1991, 1993; García-Roa et al., 2017; Martín & López, 2000, 2006). Although snakes and lizards evolved from a common ancestor, it is now believed that that common ancestor lacked any sort of specialized ventral pores (García-Roa et al., 2017). The evolution of femoral and follicular pores in lizards has occurred multiple times, but no snakes produce signaling molecules in this manner (García-Roa et al., 2017). Snakes instead rely on cloacal gland emissions, the buildup of semiochemicals in certain cell types in the dermis, and the permeation of signaling lipids across certain areas of the integument (Garstka & Crews, 1981; Maderson, 1986; Mason & Parker, 2010). However, it has been demonstrated that snakes are more likely to trail integument secretions over cloacal ones, adding support to the popular conception that cloacal secretions are mostly used for defense while skin secretions perform the majority of conspecific signaling functions (Andrén, 1982; Andren, 1986; Noble & Clausen, 1936). Much of the work done in the last 20 years illuminating chemical ecology in garter snakes (Mason, 1992; Mason & Parker, 2010) has focused on the molecules present on the integument which play a large role in the reproduction of these species. Additional reports have demonstrated the reproductive utility of shed events (*Crotalus*: Radcliffe & Murphy, 1984; *Python*: Ramesh & Bhupathy, 2013) in other species and genera, suggesting that integumental chemical signaling plays a role in a wide variety of snakes. Because the integument of snakes is thickly keratinized and only allows limited diffusion of lipids, and because snakes lack any additional glands for the direct secretion of pheromones to the outside environment, snakes release chemical signals passively across thin portions of the skin and actively by shedding (Garstka & Crews, 1986; Maderson, 1986). While signaling molecules do not require significant effort to produce (Duvall, 1986), the process of replacing the entire integument is energetically expensive, making reproductive signaling in snakes a potentially expensive component of the time-energy budget. As such, we would expect snakes to optimize reproductive output while simultaneously limiting energetic investment in the replacement of the integument. Since snakes inhabit a range of environments

that pose various challenges to the ways animals spend time and energy (i.e., temperature variation, differential resource availability, environmental seasonality, population densities), the ways species reproduce, chemically signal, and shed will vary. For instance, we might see that some groups (such as natricines) shed frequently but the energetic investment in their integument is decreased (aquatic snakes might produce less lipid rich, more permeable coverings), while desert adapted species might produce expensive, water-tight skins but limit shedding to the most reproductively advantageous times of year or to time periods when water is most readily available. My data suggest that *Crotalus horridus* in NW Arkansas follow the later pattern, limiting shed frequency in a low resource environment and potentially optimizing reproductive success by producing sheds during reproductive windows. Further exploration into the shed frequency, timing, and energetic cost of shed events in different groups may reveal a previously unstudied aspect of life history diversity in snakes.

The integral and interrelated role of ecdysis in Timber Rattlesnakes suggests that patterns of shed in other species may also be subject to selection from similar or alternate constraints. Variations in patterns of shed across species, environments, and populations are likely indicative of the biophysical conditions that force trade-offs in the expenditure of time and energy. Since shedding is energetically costly, can occupy a significant portion of an animals' active season, likely plays a major role in annual water flux (unstudied but assumed based on the integument's importance in regulating rates of water loss and observations that sloughing is a source of instantaneous water loss), and may be vital to successful reproduction, I expect that populations and species have been forced to optimize allocation strategies in the face of limiting resources (time, energy, water, reproductive partners, temperature) and that patterns of shed result from those tradeoffs. If such constraints have persisted over sufficient time scales, comparative studies of shed patterns may reveal interesting evolutionary patterns. Do snakes with high shed frequencies in food rich environments show similar reproductive utility for some

shed events? Do desert adapted forms always limit shed frequency to improve reproductive output and limit energetic expense and water loss? Can behavioral changes (such as thermoregulation) alter rates of shed to limit effects on the time-budget and relax constraints on shedding frequency? Do certain constraints result in a loss of function for some shed events (e.g., if shed events are constrained to non-reproductive times of year by annual patterns of resource abundance (i.e., food and water) is semiochemicals signaling achieved in alternate ways or removed from the courtship rituals entirely)? Do shed events have additional, undiscovered utility in other groups? The data reported in this dissertation are a starting point for a great variety of future studies into a previously unrecognized facet of squamate biology and continued research should further illuminate the role of shedding as a characteristic life-history trait in reptiles.

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Expiration Date: December 12, 2022

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol #**20049**, *Behavioral, Physiological and thermoregulatory monitoring of the top predators in the Ozark Ecosystem*.

In granting its approval, the IACUC has approved only the information provided. Should there by any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond November 12, 2022 you must submit a new protocol. The IACUC may not approve a study for more than three years at a time.

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To: Steven J. Beaupre
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