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PHENOTYPIC PLASTICITY OF RATTLESNAKE TROPHIC MORPHOLOGY

PHENOTYPIC PLASTICITY OF RATTLESNAKE TROPHIC MOPRHOLOGY

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

By

Matthew Thomas Smith
North Dakota State University
Bachelor of Science in Zoology, 2002
North Dakota State University
Master of Science in Biology, 2006

December 2012
University of Arkansas

ABSTRACT

The trophic morphology of gape-limited predators constrains the shape and size of prey items they can ingest. Trophic morphology consists of any morphological feature that is involved in the handling and ingestion of food. Diet has a profound effect on the morphology of many gape-limited predators. Identifying how prey type and resource level affect the morphology of different populations is an essential step in understanding the mechanisms contributing to patterns of morphological diversity. Species interactions (Chapter 1) induce plasticity in morphology that can lead to increased fitness, morphological divergence, and eventually speciation.

In Chapter 2, a laboratory study tested the effects of defensive strikes on snake trophic morphology. In conjunction with morphological measurements, the metabolic costs of replacing venom were quantified. Control and milked snakes had baseline metabolic rate and morphology quantified before treatment manipulation. Milked snakes showed no significant difference in metabolic rate after expending venom than. Trophic morphology was not significantly different after snakes struck defensively. Venom expenditure does not impose significant increases to metabolic rate or changes in trophic morphology.

In Chapter 3, I quantified the effects of starvation on trophic morphology and its allometric relationship with body size. Starvation periods of short (100 days) and long (200 days) intervals were compared to control snakes. Shorter periods of starvation had no effect on morphology or its relationship with body size, but longer periods resulted in a significant shift in the relationship between head and body size. Results showed extended periods of starvation can significantly alter the allometry of trophic morphology in snakes.

In Chapter 4, I studied the effects of prey size and resource level on trophic morphology. Juvenile snakes were raised for 480 days on varying biomasses and sizes of whole and homogenized rodents. Snakes exhibited significant differences in trophic morphology due to differences in prey size. Larger prey items induced relatively broader and deeper head shapes. Resource level and population also showed varied influences on the shape and size of trophic morphology. The results of this study provide insight into the plasticity in snake trophic morphology and possible ecological causes for observed patterns of variation.

This dissertation is approved for recommendation
to the Graduate Council

Dissertation Director:

_____
Dr. Steven J. Beaupre

Dissertation Committee:

_____
Dr. William J. Etges

_____
Dr. Gary R. Huxel

_____
Dr. Giovanni Petris

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ACKNOWLEDGEMENTS

I thank Dr. Steve Beaupre for enduring six long years of my constant pestering and shenanigans. Thank you for taking a chance on me, supporting me, and giving me the opportunity to grow as a scientist. I have greatly benefited from your wisdom, experience, and insight as both a person and a professional. I truly believe that I am walking away from the University of Arkansas as a more complete and skilled scientist and that I have been provided all the tools I need to succeed. I cannot begin to quantify everything that your guidance has given me, nor can I convey how truly touched and grateful I am. I know there were times when you probably questioned your choice, but I have never regretted a day. I look forward to continued collaborations and hope that my career is a monument to all you have given me.

I thank my dissertation committee (Drs. William Etges, Gary Huxel, and Giovanni Petris) for their input and guidance over my time at the University of Arkansas. My project, dissertation, knowledge, and ability have all significantly benefited from your role on my committee. Each of you has provided me with a unique insight into the world of science as well as presenting me with a set of challenges and benchmarks so that I could maximize my potential. Thank you for everything you have done for me.

I thank my friends and colleagues Drs. Michael Collyer, Mark Davis, and Trevor Krabbenhoft for the years of comradery and scientific discussions. I have learned more from the three of you than a lifetime of degrees could ever instill. It has been a true pleasure being your friend, colleague, and fellow science enthusiast. I look forward to years of collaboration, discourse, good times, and of course the occasional beverage.

I thank the past and present members of the Beaupre lab who have made my doctoral experience truly enjoyable: Rod Wittenberg, James Van Dyke, Lara Douglas, Joe Agugliaro,

Marshall McCue, Jason Ortega, Craig Lind, and Brenna Levine. All of you have provided periods of great scientific debate, discussion, and insight along with enjoyable times of relaxation and fun in the field. Rod and Joe made my first years in Arkansas very enjoyable. The many trips to the field and adventures exploring for new sites will never be forgotten. The professional and person conversations had over beers at Grubs helped keep me focused and entertained. Joe, thank you for the great *viridis* collection trip of 2008, the field beard challenge, and exposure to some great music. I thank Jason for being just a great guy in general. You have always been the first to lend a helping hand, an ear to listen to my ideas and complaints, and a shoulder to lean on. I have enjoyed our time together and look forward to continued collaboration. I thank Craig for making me feel short and pushing me to be a better scientist and enjoy life more. Van, Marshall, and Lara, thank you for all of the great lab meetings and paper discussions. I thank all of the graduate students in the department (too numerous to name) that contributed to my experience here. I look forward to continuing the friendships and professional connections I have made here.

I thank my family for all their love and support. My parents and all five of my siblings have always been there for me in my times of need and provided me with so many great memories. I know that much of who I am today is because of the experiences they have provided me as I have grown up. I am truly blessed to have such a great family.

I thank Carrye for all of the love and support over an incredibly stressful period. Meeting you was one of the greatest aspects to my time here. We met during the most chaotic times of my career and I know you had to endure a lot of stress and turmoil. For that I thank you and can only hope to one day return the favor.

DEDICATION

I dedicate this dissertation and really the rest of my life to my father. I would be so much less of a person without your guidance. You were the greatest man I have ever known, and your life provides continual inspiration to become the best person I can. I could spend the rest of my dissertation detailing how much you have impacted my life and how grateful I am for all of the love and support. Since your passing, I have been a ship lost at sea and not a second goes by that I don't think of you and wish that you were still with us. My deepest and greatest regret is that you could not be here to share this moment with me. I hope that I have made you proud and that I have not let you down. I will live every day for the rest of my life with you in my heart and with the desire to make you proud of your son. You are my coach, my father, and my hero; and I dedicate this dissertation and all my future accomplishments to you.

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INTRODUCTION

The shape and size of the trophic morphology (any structural feature used in prey acquisition) of predators exhibit a great deal of variation. Predators have displayed cases of morphological convergence in divergent taxa and morphological divergence within a single species. Both examples are interpreted as evidence of matching trophic form and function to prey items. Studies examining how dietary habits affect trophic morphology within and among taxa provide crucial information towards understanding the overall patterns of morphological diversity and predator design. Understanding genetic and environmental factors that influence trophic morphology, along with the direction and degree of morphological changes associated with each factor has become the central goal of ecomorphology and an important concept in sympatric speciation. Trophic polymorphisms serve as a model for studying the ecological and evolutionary factors of morphological variation, convergence, and divergence.

Gape-limited predators are a broad group of taxa ideally suited for studies of ecomorphology and trophic polymorphisms. These predators swallow their prey whole and gape size sets the upper limit to the size and shape of prey they can swallow. Such limitations suggest prey choice and resource level should significantly influence the size and shape of trophic morphology. Patterns of morphological variation and divergence as a result of prey size, prey type, resource level, and habitat are well documented in such gape-limited predators as fish, birds, and amphibians. Unfortunately, there has been relatively little work done on snakes. Snakes are model organisms for studies on trophic polymorphisms and phenotypic plasticity for several reasons: 1.) unlike some other gape-limited predators, snakes only use their trophic morphology for feeding, not in other activities such as mating or combat; 2.) snakes are often a dominant predator in their communities and indicators of ecosystem health; 3.) snakes are easily

kept in captivity in large numbers without extreme resource demands. The goal of this dissertation is to quantify the magnitude and direction of morphological change in relation to prey size, resource level, ecological context, and geographic location. I conducted a series of laboratory experiments to investigate the influence of starvation, population, prey size, resource level, and defensive strikes on the trophic morphology of the Prairie Rattlesnake (*Crotalus viridis viridis).*

Chapter 1 reviews previous work on the plasticity of trophic structures, inducible morphological changes due to species interactions, and the few studies that have specifically studied snakes. The literature review provides the context and framework for the study objectives of the subsequent chapters and defines pertinent terminology. My research documented the effects of defensive strikes and envenomation on metabolic rate and morphology (Chapter 2), changes in the allometric relationship between trophic morphology and body size due to periods of starvation (Chapter 3), and litter differences in the morphological response to prey size and resource level (Chapter 4). The results and conclusions discussed within this dissertation represent one of the most complete investigations on genetic and environmental influences of trophic morphology in a snake species.

CHAPTER 1: A REVIEW OF PHENOTYPIC PLASTICITY, GAPE-LIMITED PREDATORS, AND PLASTIC RESPONSES IN TROPHIC MORPHOLOGY

Introduction:

Trophic or resource-based polymorphisms, defined by the presence of multiple morphotypes within a single population, are common among invertebrates and vertebrates (Wimberger, 1994; Andersson et al., 2007). The presence of polymorphisms and plasticity in morphology provide potential for intraspecific niche differentiation and possibly speciation (Mayr, 1963). Polymorphisms can be manifested through morphology, physiology, growth, demography, color, behavior, or life history characteristics (Skúlason and Smith, 1995; Smith and Skúlason, 1996; Miner et al., 2005; Whitman and Agrawal, 2009). Phenotypic differences related to trophic morphology are likely more common than currently understood and may evolutionarily play a significantly larger role than first thought (Smith and Skúlason, 1996; Maerz et al., 2006).

Trophic polymorphisms occur when: 1.) organisms use only their mouth to subdue, capture, and process its prey; 2.) there are two or more food types or suites of food that require different modes of capture and/or processing; 3.) sufficient behavioral flexibility is present, allowing the predator to take advantage of multiple prey types; 4.) prey populations are temporally stable (relative to the development of the polymorphism); and 5.) ecological communities in which polymorphic species persist are relatively species-poor or characterized by empty niches (Wimberger, 1994; Smith and Skúlason, 1996; Andersson et al., 2007). Polymorphisms can result from genetic and environmental factors or a genotype by environment interaction (Skúlason and Smith, 1995; Padilla, 2001). In addition, diverse food resources are often accompanied by trade-offs in the efficiency of use. Trade-offs in turn creates possible

trophic niche separation, and many populations are distributed among habitats where resource availability can change dramatically (Maerz et al., 2006; Whitman and Agrawal, 2009).

The expression of one phenotype over another in the same environmental conditions often suggests an advantage in overall fitness of the more common phenotype (West-Eberhard, 1989, 2003). Organisms exhibiting polymorphisms provide an excellent avenue for investigating the mechanistic, functional, and evolutionary basis for phenotypic variation (Hoffman and Pfennig, 1999). Plasticity can alter a variety of direct and indirect interactions between the individual and their environment, therefore plasticity should affect many ecological processes including population dynamics, community dynamics, and ecosystem function (Miner et al., 2005).

Snakes are model organisms for investigations of trophic polymorphism for two primary reasons. First, snakes are gape limited predators (Arnold, 1993), whose trophic structures (e.g. the jaws) are not used in any other activities (i.e.: mating or combat). Snakes cannot physically reduce their prey and therefore must ingest it whole (Shine, 1991). The shape and size of trophic morphology dictates the type and size of prey that can be ingested (Forsman, 1996a; Shine, 2002). Second, of all the reptilian gape-limited predators, snakes are more commonly a dominant predator in their community. Their body condition, growth rate, and population dynamics can also be indicators of ecosystem health (Beaupre and Douglas, 2009).

Previous research into the adaptive phenotypic plasticity in trophic morphology of snakes has had varied results (Schuett et al., 2005). While some species and/or populations have shown moderate levels of morphological change due to diet changes (Queral-Regil and King, 1998; Aubret et al., 2004), others have been unresponsive (Bonnet et al., 2001; Schuett et al., 2005).

In this chapter, the underlying concepts of trophic polymorphism, including phenotypic plasticity, inducible defenses, inducible offenses, whether plasticity is adaptive, and the evolutionary importance of plasticity are reviewed and discussed within the context of provided examples and previous literature. Existing studies on snake trophic morphology are discussed in detail, concluding with possible areas of improvement and questions that remain unanswered.

Phenotypic Plasticity Overview

What is Phenotypic Plasticity

Phenotypic plasticity is broadly defined as the environment-dependent expression of a phenotype (Stearns, 1989; DeWitt and Scheiner, 2004; Miner et al., 2005; see Box 1 in Whitman and Agrawal, 2009); where alternative alleles or their products respond differently to environmental cues. While a majority of the definitions of phenotypic plasticity appear equal, the details of the definition can have a significant impact (Whitman and Agrawal, 2009). For the purpose of this manuscript, we define phenotypic plasticity as the capacity of a single genotype to produce a range of phenotypes in response to environmental variation (Fordyce, 2006).

Phenotypic plasticity allows organisms to respond to environmental shifts within the lifetime of a single individual (Miner et al., 2005; Pigliucci, 2005). Environmental modification of a phenotype is common in quantitative traits of organisms that inhabit heterogeneous environments (Via and Lande, 1985). Plasticity can be passive or anticipatory, instantaneous or delayed, discrete or continuous, reversible or permanent, adaptive or non-adaptive, expressed in an individual or trans-generation (West-Eberhard, 1989; Via et al., 1995; DeWitt et al., 1998; Uller, 2008; Badyaev and Oh, 2008; Whitman and Agrawal, 2009).

The numerous definitions of plasticity have created some question and controversy when determining what types of traits are indeed plastic (DeWitt and Scheiner, 2004). There are two

distinct classes of traits that are considered plastic. Strict definition of phenotypic plasticity limits plastic traits to those with a developmental basis. Developmental pathways are differently expressed under specific environmental cues (Frankino and Raff, 2004). Behavioral and physiological responses are often less considered when discussing phenotypic plasticity (Sih, 2004). Behavioral and physiological responses differ from developmental processes in their speed and reversibility (Houston and McNamara, 1992). Developmental processes are generally slow and irreversible, while behavioral and physiological traits change rapidly and are easily reversible (Sih, 2004). Regardless of the trait, an underlying biochemical-physiological process is at work; therefore any plastic trait represents altered physiology (for review see Whitman and Anathakrishan, 2009).

The first study of phenotypic plasticity was conducted by Woltereck (1909), which showed that *Daphnia* developed a 'helmet' when raised in the presence of a predator. Schmalhausen (1949) investigated stabilizing selection and developmental reaction norms which incorporated allometry, ontogeny, and plasticity. It was proposed that phenotypically plastic traits can become genetically fixed over time, referred to as genetic assimilation (Schmalhausen, 1949; Waddington, 1953). Bradshaw (1965) stated there must be a correlation between how fast a character develops and the degree to which this structure could be modified by the environment. Bradshaw's (1965) early work suggested: 1.) observed patterns of variation for plasticity imply that it should be considered a trait in itself and that it should be under genetic control; 2.) plasticity for one trait was independent of the plasticity for other traits; 3.) a correlation exists between heterozygosity and plasticity; 4.) selection can act not only on the trait that is plastic, but plasticity itself.

Two classes of genetic components influence the presence and extent of phenotypic plasticity (Via et al., 1995; Pigliucci, 2001). First, alleles that are expressed in several environments with varying effects on the phenotype make up a component called allelic sensitivity (de Jong, 1990). Second, regulatory loci (switches) turn other genes on or off in different environments (Scheiner et al., 1991). The two components differentiate between the trait mean and plasticity of the trait (Schlichting and Pigliucci, 1993). Via et al. (1995) suggested these two components could be viewed as 'plasticity genes' and both plasticity and phenotypic means are controlled by these 'plasticity genes' and therefore both determine the function of the character state.

There is clear genetic variation in plasticity (Windig et al., 2004). Plasticity can be broken down into a series of stages (Windig et al., 2004). Environmental cues are the essential, first stage in plasticity. If organisms cannot obtain cues about their environment, plasticity will not develop (Bradshaw, 1973). An adaptive advantage can be inferred from how accurately the cue can predict the future environment and how accurately the organism can detect that cue (Windig et al., 2004). Next, environmental cues are propagated into signals that are perceived by the internal environment of the organism (Schmitt et al., 2003). Detection of environmental cues serves no purpose without the ability to transmit the signal to the needed pathway. Propagated signals must be transported and at times stored before being translated into phenotypic change. Lastly is the formation of the actual phenotype. The signal is interpreted and appropriate developmental pathways are initiated (Windig et al., 2004).

From the perception of environmental cues, translation of the signal, and induction of the phenotype lie dozens of steps and pathways that are influenced by hundreds of genes and an infinite number of environmental and physiological factors (Whitman and Agrawal, 2009).

Phenotypic modification can be enacted by a genotype via transcription, translation, enzymes, hormones, morphogen regulation, morphogenesis, apoptosis, neural control, or any combination of these processes (Miura, 2005; Emlen et al., 2007). Selection can act anywhere along this chain (Windig et al., 2004).

When is Phenotypic Plasticity Favored

Investigations of the evolutionary aspects of phenotypic plasticity fall into one of three categories (Berrigan and Scheiner, 2004). First, under what conditions does natural selection favor plasticity over fixed strategies? Second, what form will plasticity assume, discrete or continuous plasticity, the shapes of the reaction norms, and the time scales of plastic responses? Third, what are the evolutionary dynamics of plasticity? Questions concerning the evolutionary dynamics of plasticity focus on: the genetic basis of plasticity, selective forces that influenced the evolution of plasticity, the population structure of the organism in question, and the population characteristics that will impact the evolution of plasticity (DeWitt and Scheiner, 2004).

If environments were unchanging, phenotypic plasticity would not be favorable when compared to fixed strategies (Relyea, 2002; Pigliucci, 2005; Whitman and Agrawal, 2009). Phenotypic plasticity should be favored when it results in higher fitness than those employing a fixed strategy across all environments (Berrigan and Scheiner, 2004). For plasticity to be favored there must be a variable environment, temporal and spatial heterogeneity can both contribute to environmental variable and favor different types of plasticity (Levins, 1963; Scheiner, 1993), and cues need to be reliable (DeWitt and Langerhans, 2004). The reliability of cues can be related to patterns of within generation heterogeneity or lag between the time a trait becomes fixed during development and when selection occurs (de Jong, 1990; Moran, 1992). The less reliable the cue,

the more likely selection will favor a fixed strategy. Another important factor for cue reliability is the developmental delay, or how fast an organism can respond to a change in the environment (Padilla and Adolph, 1996).

Models suggest: that plasticity is more likely to evolve under temporal heterogeneity versus spatial (Moran, 1992), when environmental cues slowly harm individuals instead of being rapid, destructive events (Järemo et al., 1999). A multitude of other environmental, genetic, and gene by environment interaction factors all influence if plasticity is favored and what type of plasticity will evolve (for a review see: Whitman and Agrawal, 2009). In summary, plasticity evolution should be favored by environmental variation, strong differential selection in alternative environments, accurate environmental cues, high fitness benefits, low fitness costs of plasticity, certain combinations of population parameters, and heritable genetic variance for plasticity (Stearns, 1989; Pigliucci, 2001; Relyea, 2002; Scheiner, 2002; Schmitt et al., 2003; Berrigan and Scheiner, 2004; Doughty and Reznick, 2004; Miner et al., 2005; Pigliucci, 2005).

Costs and Benefits of Phenotypic Plasticity

Costs of plasticity come in three forms: costs of maintenance, production, and information (Relyea, 2002). The influence of plasticity costs depend on the reliability of cues (DeWitt et al., 1998; Berrigan and Scheiner, 2004; Pigliucci, 2005). Plasticity costs reduce the slope of reaction norms. Costs and limits to plasticity will influence the circumstance in which it will be favored (DeWitt et al., 1998; Relyea, 2002). Costs include maintenance costs (energetic costs of sensory and regulatory mechanisms), production costs (costs of inducible structures), information costs (process of acquiring information), developmental stability (phenotypic instability), and genetic costs or inertia (linkage, pleiotropy, and epistasis) (DeWitt et al., 1998; Relyea, 2002; Berrigan and Scheiner, 2004). Limits to plasticity include information reliability, lag-time, developmental

range, and epiphenotype problem (add-on phenotypes may not be as effective as integrated phenotypes) (DeWitt et al., 1998).

In spite of the costs, plasticity has clearly demonstrated its possible benefits (Whitman and Agrawal, 2009). Benefits of plasticity stem from allowing individuals to match a changing environment (DeWitt and Langerhans, 2004). Plasticity is also beneficial in a self-reinforcing process, referred to as a self-induced adaptive plasticity (Swallow et al., 2005). Self-induced plasticity involves processes such as increasing muscle mass or heart function through exercise, the gradual process of detoxifying new food sources (Whitman and Agrawal, 2009). Since plasticity is often beneficial in multiple environments, it often widens niche breadth, geographic range, aids in dispersal and colonization (Schlichting, 2004; Pigliucci et al., 2006), and helps evolutionary transitions (Aubret et al., 2007). Ultimately, the greatest benefit plasticity provides is that it generates adaptive genetic change (see below). Plasticity may provide populations the ability to jump maladaptive valleys and reach new fitness peaks in the adaptive landscape (Price, 2006). Plasticity may also protect hidden genetic variation from being eliminated, allowing it to later be expressed (Schlichting, 2004).

Is Phenotypic Plasticity Adaptive?

Under many circumstances, phenotypic plasticity is clearly adaptive (Agrawal, 2001; West-Eberhard, 2003). In other cases, plasticity is non-adaptive, but complex environmental interactions often make the evaluation of its adaptiveness difficult (Pigliucci, 2005). Plasticities are often under opposing selective pressures and have trade-offs (Fordyce, 2001; Sih, 2004). There are several factors that make it difficult to evaluate whether a plastic trait is adaptive or not. First, many traits are altered by a given environment and many are often not recorded or studied to include in the cost-benefit analysis (Relyea, 2004). Second, genetic and environmental

correlations are themselves plastic to the environment, with the adaptive nature changing from season to season (Pigliucci, 2005). Third, not all benefits or costs are bestowed or incurred by the current generation, and the next generation is rarely analyzed (Agrawal, 2001). Fourth, most studies about the adaptive nature of plasticity are done under unnatural conditions (lab, greenhouse, etc.) and may not reflect the true relationships in a much more complex natural setting (Ghalambor et al., 2007). Finally, the term adaptive is generally applied to past selection and the history of a population may not be accurately assessed (Doughty and Reznick, 2004). Ghalambor et al., (2007) stated that only plasticity that places populations close enough to new optimum for directional selection to act can be considered adaptive, but this type of plasticity is most likely the product of past selection on variation that was initially non-adaptive.

Whether plasticity is adaptive or not, its importance should not diminish. All environmentally induced changes place an individual in a different selective regime and provide evolutionary potential. The evolution of many currently adaptive traits may have been stimulated by detrimental plastic responses in the past (West-Eberhard, 2003). Examples of such instances include diapause, alternative morphologies, and sociality (Emlen et al., 2006).

The Importance of Phenotypic Plasticity

Current evolutionary theory explaining life on earth tends to be "genocentric", with phenotypic plasticity often considered 'noise' (Whitman and Agrawal, 2009). More recent shifts in scientific thinking about phenotypic plasticity have recognized its potentially adaptive nature in a variety of circumstances. Incorporating phenotypic plasticity into evolutionary models enables researchers gain insights not otherwise possible and creates a better overall model of evolution.

In mutation-driven evolution models, the environment acts after phenotypic variation exists, selecting among genetically determined phenotypes. When phenotypic plasticity is added

to the model, the environment plays a dual role, not only selecting among variation, but also creating it (West-Eberhard, 2003). Adding phenotypic plasticity to evolutionary models shows great improvements over those that are mutation based. Mutations are rare, occur in an individual, and are most commonly deleterious, as opposed to phenotypic plasticity which is much more common and a single environment could produce numerous individuals with similar phenotypes (Whitman and Agrawal, 2009). Mutations are random, environmentally induced phenotypic plasticity is highly directional, allowing new environments to rapidly produce and select among new phenotypes (Badyaev, 2005). Altered or new environments not only reveal hidden genetic variation, but create novel phenotypic traits and regulation pathways through plastic response. Recurrence of environmental conditions produces large numbers of individuals that vary in numerous genetic, phenotypic, and environmental characteristics, providing fertile ground for selection to act (West-Eberhard, 2005). This process is believed to have led to many cases of adaptive phenotypic plasticity and even speciation events, giving phenotypic plasticity a much larger role in evolutionary theory (West-Eberhard, 2003; Fordyce, 2006).

Genocentric evolution theories also emphasize between-generation adaptations by populations. Phenotypic plasticity allows for the incorporation of within and between generation responses by an individual and populations (Pigliucci, 2001). The addition of phenotypic plasticity results in a more comprehensive model than those based on mutation and allelic substitutions alone. Good examples of how phenotypic plasticity provides more easily modeled explanations in evolution are aposematism (Sword, 2002), exaggerated morphologies (Emlen and Nijhout, 2000), response to exercise (Swallow et al., 2005), and alternative mating strategies (Pfennig, 2007).

Phenotypic plasticity allows researchers to include stress as an ecological and evolutionary concept (Badyaev, 2005). Most individuals are under constant environmentally induced stress, yet it is rarely considered in evolutionary models. Stress may have been responsible for the evolution of such traits as stress proteins, homeostasis, acclimation, immune response, and learning (Emlen et al., 2006). In addition, modeling consequences of stress plasticity from the interaction between humans, the environment, and populations may improve the predictive power of evolutionary models (Relyea, 2003).

Phenotypic plasticity allows for the merger of genes and the environment. Arguments of nature versus nurture become irrelevant, as genes and gene activities can never be separated from the direct environment (Sultan, 2007). Basic processes such as transcription are all affected by the environment in which they take place, such that most traits be necessity represent a gene-by-environment interaction.

Speciation through Phenotypic Plasticity

Claims that phenotypic plasticity can stimulate selection and evolution and even result in speciation are using met with the accusation of Lamarckian evolution (Pigliucci et al., 2006). Contrary to those arguments, phenotypic plasticity can result in environmentally induced changes to become absorbed into the genome of a population, and that process occurs via traditional Mendelian processes (West-Eberhard, 2003; Schlichting, 2004). One such pathway is detailed by Whitman and Agrawal (2009). 1.) A trait originates via phenotypic plasticity. This could be due to any type of plasticity and may be beneficial or detrimental. 2.) Phenotypic accommodation whereby an individual adaptively alters its physiology, behavior, or morphology to accommodate for the newly originated plastic trait (West-Eberhard, 2003). 3.) Genetic accommodation allows for a repeated environmental induced novel trait to be repeatedly tested

among genetically variable individuals. Genetic accommodation can shift the overall fitness value of the induced phenotype. Natural selection can act to improve regulation, form, and side effects of the novel traits by selecting certain alleles and/or gene combinations. 4.) Natural selection alters the frequency of genes and their combinations involved in the expression of plasticity. These changes in plasticity gene frequency alter the capacity and shape of reaction norms, also called the Baldwin Effect (Crispo, 2007). Baldwin proposed that a beneficial learned behavior acquired during an individual's lifetime contributed to the fitness of that individual, and thus evolution favored the increased capacity to acquire and perform that behavior in the population (Baldwin, 1886). Baldwin (1986) was the first to link plasticity to evolution, and that environmentally induced traits can evolve. The theory was similar to Lamarckism due to how acquired beneficial traits induced by the environment could become more genetically determined (Whitman and Agrawal, 2009), but natural selection was acting on the capacity for plastic responses not the acquisition of such traits. Baldwin's notion was that plasticity influenced whether an individual will survive in a new environment (natural selection), thus dictating the course of evolution (Crispo, 2007). Baldwin also stated that heritable variations in plasticity could direct phenotypic evolution (Baldwin, 1986; Crispo, 2007). 5.) The initially environmental induced trait becomes genetically fixed or obligatory within the population. A once facultative trait within the population is now constitutively expressed, also known as genetic assimilation (Schmalhausen, 1949; Waddington, 1953; Crispo, 2007). Genetic assimilation is important because it provides the mechanism in which environmental induction of a trait can initiate evolutionary change (Pigliucci and Murren, 2003). 6.) Speciation through differences in reproductive strategies, timing, or isolation. The environmentally induced trait results in an increase in assortative mating. Continual natural selection, genetic drift, and mutation of the

population can increase habitat, mating, and genetic divergence from other populations leading to eventual speciation (Whitman and Agrawal, 2009). This process/pathway (or others like it) is Mendelian and Darwinian because it relies on preexisting genetic variation and natural selection, and could be as important as mutation for producing biodiversity (Schlichting and Pigliucci, 1998; West-Eberhard, 2003).

Reciprocal Phenotypic Plasticity

Organismal plastic responses are commonly observed in interactions with other species (Agrawal, 2001). A phenotypic change in one individual may induce a change in a second individual, which in turn induces a further change in the first individual, in a continuous, reciprocal, phenotypic plasticity loop (Agrawal, 2001; Fordyce 2006). Interactions such as predation, competition, and mutualism are commonly observed to produce plastic responses that are often adaptive (Tollrian and Harvell, 1999; Agrawal, 2001; Pigliucci, 2001; DeWitt and Scheiner, 2004; Kishida et al., 2006; Mougi et al., 2010). Phenotypic changes in interacting organisms often cause substantial shifts in some environmental factors mediating their interaction (Kishida et al., 2006). Biological interactions are temporally and spatially variable ecological processes, and therefore can produce heterogeneous environments (Kishida et al., 2006). Inducible defenses and offenses are common examples of the adaptive nature of phenotypic plasticity involving species interactions.

Inducible Defenses

Inducible defenses are phenotypic changes that are directly induced by biotic agents (Harvell, 1990; Harvell, 1992; Tollrian and Harvell, 1999). Inducible defenses measurably diminish the effects of predators or attacks by other species (for review see: Tollrian and Harvell, 1999). Examples of inducible defenses are found in a wide variety of taxa under the selective pressures

of predation, parasitism, herbivory, and competition (Dodson, 1989; Harvell, 1990, Tollrian and Harvell, 1999). Four common factors have emerged as conditions that favor the evolution of inducible defenses (Tollrian and Harvell, 1999; Trussell and Nicklin, 2002): 1.) the selective pressure of the inducing biotic agent is variable and unpredictable, but at times strong. If a biotic agent is constantly present, then the defense strategy should become fixed; 2.) a reliable cue is present that conveys the proximity of the biotic agent and/or threat; 3.) the induced defense is effective and benefits the organism; 4.) there is a trade-off between the benefit that accompanies the defense and the cost that is incurred from its development. If there is no trade-off, the trait should become fixed.

Both constitutive and inducible defenses against herbivory have evolved in plants (Järemo et al., 1999). Hundreds of plant species have inducible defenses to past and present herbivory, displaying a wide range of responses (Karban et al., 1999). Morphological structures such as thorns, spines, and bristles serve as deterrents to large herbivores; while chemical defenses are typically employed to deal with smaller herbivores (Berenbaum and Zangerl, 1999). Examples of morphological changes in plants in response to herbivory include: increases in spine length in African *Acacia* species (Young et al., 2003), increased number of spines in *Acacia tortilis* (Gowda, 1997), *Opuntia stricta* (Myers and Bazley, 1991), and *Rubus fruticosus* (Bazley et al., 1991), hardening in algae (DeMott, 1995), and return to a different growth form (Bryant, 1981). Chemical responses to herbivory include: production of toxins such as furanocourmarins in wild parsnips (Berenbaum and Zangerl, 1999), production of volatile compounds to attract carnivores (Dicke, 1999), and extracellular compounds produced by cyanobacteria that decrease the grazing activity in *Daphnia* (Dicke, 1999).

Examples in invertebrate taxa are just as prevalent (Tollrian and Harvell, 1999). Rotifers respond to chemical cues from various predators, changing morphology and forming spines (Gilbert, 1999). Ciliated protozoa changes cell shape in response to predators (Kuhlmann et al., 1999). Zooplankton change their migration behavior in the presence of predators and select water depths that significantly differ from those when predators are absent (De Meester et al., 1999). Water fleas (*Daphnia*) undergo a variety of morphological, behavioral, and life-history changes in response to predators (Dodson, 1989; Tollrian and Dodson, 1999). *Daphnia* develop neck teeth in the presence of *Chaoborus* larvae (Dodson, 1989), a crown of thorns when chemical cues of *Triops* predators are present (Petrusek et al., 2009), large 'helmets' on top of their head during portions of the year that predators are at their highest densities (Wolterek, 1909), and changes in offspring size and delaying maturity in response to predation levels (Dodson, 1989; Spitze, 1992; for a review see Tollrian and Dodson, 1999). Other examples are present in bryozoans (Harvell, 1992), gastropods (Appleton and Palmer, 1988), snails (Trussell and Nicklin, 2002), and barnacles (Lively, 1986).

Examples of inducible defenses in vertebrates have generally been limited to aquatic environments and/or species that undergo metamorphosis. Crucian carp (*Carassius carassisus*) develop deeper bodies in the presence of piscivorous fish (Bronmark et al., 1999). Deeper bodied fish are less prone to predation and exhibit an increased survival rate (Bronmark et al., 1999). Hylid tadpoles develop deep, short tails in the presence of predators compared to those raised in the absence of predators (McCollum and Van Buskirk, 1996). *Rana temporaria* tadpoles showed varying morphological and behavioral responses to invertebrate and vertebrate predator species (Van Buskirk, 2001). Anuran tadpoles often decrease activity and foraging habits in the presence of predator chemical cues (Van Buskirk and McCollum, 2000). Tadpoles

of *Rana pirica* had bulgier body shapes in the presence of the larval salamander *Hynobius retardatus*, than those tadpoles reared in the absence of predators (Kishida and Nishimura, 2004).

Inducible Offenses

Just as prey species can have plastic responses to predators and form inducible defenses, so can predators have plastic response to prey species in the form of inducible offenses (Padilla, 2001). Far less attention has been paid to plastic responses by predators, but resource-based or trophic polymorphisms are likely more common and evolutionary significant than originally thought (Smith and Skúlason, 1996). Most cases of inducible feeding morphologies are shifts in trophic structure over the lifetime of a single individual and are considered to be use-induced (Padilla, 2001). Use-induced morphologies stem from direct feedback during feeding that stimulates the trophic morphology to undergo modification to accommodate the demands of feeding on particular prey items (Padilla, 2001). Inducible offenses can manifest as changes to morphology, color, behavior, or life-history traits (Wimberger, 1994; Smith and Skúlason, 1996). Trophic polymorphism is an association between trophic phenotype and diet among members of a single population (phenotypically variable population), specifically trophic polymorphism are differences in trophic structure or behavior, while resource polymorphisms are a subset that are correlated with differences in diets (Maerz et al., 2006).

A majority of the work on trophic polymorphism has focused on discrete, alternative phenotypes (Wimberger, 1994; Smith and Skúlason, 1996; Padilla, 2001), but potentially more common are those polymorphisms that show a continuous distribution (Svanback and Eklov, 2002; Maerz et al., 2006). Continuous variation in trophic morphology is most often associated with the use-induce principle and is often referred to as phenotypic modulation (Smith-Gill, 1983). Historically, the variation in a continuous trait was believed to be developmental noise

(Maerz et al., 2006), but more recently such variation has revealed evolutionary and ecologically relevant patterns (Skúlason and Smith, 1995; Svanback and Eklov, 2002).

Examples of trophic polymorphisms are present in a large number of vertebrate taxa (Wimberger, 1994). The unifying characteristics of trophic polymorphisms are: 1.) Organisms use only their trophic structures (mouth and any other structures required to feed) to subdue, capture, and ingest prey items. 2.) There are two or more prey 'types' or suites of prey items available. 3.) There is sufficient behavioral and morphological flexibility to take advantage of the multiple prey types present. 4.) Prey populations are temporally stable. 5.) The predator occupies an environment that is relatively species-poor and may have empty niches (Wimberger, 1994). In cases where all five of these characteristics are present, trophic polymorphism may result in morphological divergence and eventually speciation (Pigliucci et al., 1997; West-Eberhard, 2003).

Trophic Polymorphism in Fish

Fish provide the most cases of trophic polymorphism in any vertebrate taxa. Freshwater and anadromous species provide the majority examples (Smith and Skúlason, 1996). Whitefish (*Coregonus* and *Prosopium* spp.) are some of the better known examples of morphological polymorphisms (Wimberger, 1994). Whitefish species occupy temperate lakes in the Holarctic where typically have two or more forms of a single species can be found (Svardson, 1979). Sympatric forms vary in resource use and morphology (Smith and Skúlason, 1996), and can generally be lumped into benthic versus limnetic forms. Whitefish morphs usually differ in body shape, mouth position, gill raker abundance and gill raker size (Wimberger, 1994; Smith and Skúlason, 1996; Kahilainen and Ostebye, 2006). Fish feeding on zooplankton have longer and more numerous gill rakers and more fusiform body shapes; while those fish feeding on

stationary, benthic prey have fewer and short gill rakers, and a deeper, rounder body shape (Webb, 1984). The differences in morphology involve a large number of characters, but all of them are important in food capture and each character is canalized to varying degrees (Bernatchez et al., 1996; Pigeon et al., 1997; Lu and Bernatchez, 1999).

Artic charr (*Salvelinus alpinus*) also commonly exhibit trophic polymorphisms. Charr are a circumpolar salmonid often displaying two to four sympatric resource morphs of a single species (Wimberger, 1994; Smith and Skúlason, 1996; Imre et al., 2001; Andersson, 2003; Andersson et al., 2005). Charr morphs are characterized by differences in gill rakers, pyloric caecae, color, body proportions, and life-history characters all correlated to differences in diet (Johnson, 1980; Wimberger, 1994; Smith and Skúlason, 1996; Andersson et al., 2005). Dwarf forms are a mid-water fish that feeds on zooplankton and are characterized by an increased number of gill rakers, more pointed snout, a larger eye, and smaller body size (Johnson, 1980). The 'normal' charr morph feeds on benthic organisms. At its most diverse, the charr's eco-morphs within a single lake consist of a small benthivore, large benthivore, planktivore, and a piscivore (Skúlason et al., 1989; Wimberger, 1994). Charr populations vary in number of trophic morphs, degree of reproductive isolation, and degree of genetic control on trophic morphology in each drainage that has been examined (Dynes et al., 1999; Saint-Laurent et al., 2003).

Goodeids (*Ilyodon* spp.) are riverine fish in Mexico that occur in a narrow-mouthed and broad-mouthed form (Turner and Grosse, 1980; Wimberger, 1994). Narrow-mouthed morphs are characterized by round jaws, narrow gapes, and several rows of teeth, while broad-mouthed forms have a square jaw, broad, horizontal gape, and reduced or absent inner rows of teeth (Turner and Grosse, 1980). Broods of known parentage were raised in the laboratory; where all but one homomorphic crosses resulted in progeny of the opposite morph as the parents and no

progeny exhibited the degree of differentiation between morphs observed in the wild (Grudzien and Turner, 1984), suggesting an environmental (food) component to morphology.

Threespine sticklebacks (*Gasterosteus aculeatus*) occur in limnetic and benthic morphs in lakes in British Columbia (McPhail, 1984). Morphs feed on distinctly different prey items and occupy different habitats (Wimberger, 1994). Limnetic morphs have a shallow body, longer head and snout, and a longer upper jaw and gill rakers than benthic morphs. Limnetic morphs feed on zooplankton while benthic morphs feed on benthic invertebrates. Morphology and gut contents are highly correlated (Schluter and McPhail, 1992). More recent work on stickleback populations has shown a mix of underlying genetic and plastic responses, with some polymorphisms having a genetic basis, while other traits exhibit true phenotypic polymorphisms (Schluter, 1996; Reush et al., 2001; Hendry et al., 2002; Sharpe et al., 2008).

Bluegill sunfish (*Lepomis marcochirus*) and pumpkinseed sunfish (*Lepomis gibbosus*) co-occur and occupy distinct niches in North American lakes (Smith and Skúlason, 1996). Both species have multiple trophic morphs occurs in some lakes. Both species have a shallow- and open-water morph; with shallow-water morphs having deeper body and longer fins than the open-water morph (Mittelbach et al., 1992; Robinson et al., 1993; Smith and Skúlason, 1996; Mittelbach et al., 1999). When both species occur in the same lake, a majority of adults comprise the open-water morph that feed on plankton, while pumpkinseed sunfish are primarily shallow-water morphs that feed on snails. Removal of a species and/or drastic changes in frequency induces a trophic polymorphism in the remaining (or more common) species, such that it has both shallow- and open-water morphs that feed on the two prey types (Mittelbach et al., 1992; Robinson et al., 1993; Skúlason and Smith, 1995; Smith and Skúlason, 1996; Mittelbach et

al., 1999). The morphological differences are subtle and continuous, and went unnoticed in numerous ecological studies (Smith and Skúlason, 1996).

Variation in gape-size, allometry, and ontogenetic growth rate in Eurasian perch (*Perca fluviatilis*) and pike (*Esox lucis*) have been correlated to differences in prey availability and habitat (Magnhagen and Heibo, 2001; Svanback and Eklov, 2002). Genetic and environmental factors, including resource level (Marcil et al., 2006), influence body shape in Atlantic cod (*Gadus morhua*). Perhaps the best known species that exhibit trophic polymorphisms are the cichlids (Smith and Skúlason, 1996). Cichlid fish in the lakes of the African rift and Central America exhibit multiple trophic morphs. In Mexico, *Cichlasoma minckleyi* exhibit a vegetarian and carnivore morph (Kornfield et al., 1982). The vegetarian morph has a narrow head, long intestinal tract, and small papilliform pharyngeal jaw dentition. The carnivorous morph feeds on snails and is characterized by shorter intestines, a wide head, stout jaws, and large molariform pharyngeal teeth (Kornfield et al., 1982; Smith and Skúlason, 1996). The scale-eating cichlid (*Perissodus microlepis*) in Lake Tanganyika exhibits left or right handedness in jaw morphology depending on what side of the prey they remove scales from (Hori, 1993). The majority of cichlid morphs interbreed and feeding segregation is most pronounced when resources are limited (Hoogerhoud, 1986; Smith and Skúlason, 1996), suggesting phenotypic plasticity explains the diversity in trophic morphology of many cichlid species (Hori, 1993; Takahashi and Hori, 1994, 1998; Stewart and Albertson, 2010).

Trophic Polymorphisms in Amphibians and Birds

Amphibians are characterized by both inducible defenses and offenses. Tiger salamanders (*Ambystoma trigrinum*) occur in two larval morphs (Pfennig, 1990). A larger ‘cannibalistic’ morph has a broader head, enlarged vomerine teeth, and a wider mouth (Maret and Collins,

1997; Hoffman and Pfennig, 1999; Johnson et al., 2003). Typical morphs of larval salamanders feed on zooplankton and other invertebrates, while cannibal morphs feed on conspecifics (Smith and Skúlason, 1996). Density experiments have shown that the frequency of cannibal morphs is positively correlated with population densities (Collins and Cheek, 1983). Larvae of the salamander *Hynobius retardatus* exhibit similar trophic polymorphisms, with a normal-headed morph and a broad-headed, cannibal morph (Nishihara, 1996; Michimae and Wakahara, 2002). Unlike the tiger salamander, the broad-headed morph of *Hynobius retardatus*, while cannibalistic at times, most likely evolved to eat large, tough prey like con- and heterospecifics in conjunction with a cold-adapted feeding strategy to allow them to feed on prey items partially covered in ice (Michimae and Wakahara, 2002). Spadefoot toad larvae cannibal morphs are larger and develop more rapidly than the smaller, normal morph (Pfennig, 1990). Cannibal morphs are more frequent in pools that contain larger prey items such as fairy shrimp and conspecifics (Pfennig, 1990; Wimberger, 1994; Skúlason and Smith, 1995; Smith and Skúlason, 1996). Empirical evidence supports both a density dependence aspect and a physiological aspect to the induction of the cannibal morph (Wimberger, 1994). Cannibal morphs can ingest larger prey items than normal morphs, but ingestion of fairy shrimp and conspecifics also increases levels of thyroid hormone, that increases rates of metamorphosis and growth (Pfennig, 1992). Eastern red-backed salamanders (*Plethodon cinereus*) exhibit morphological differences associated with diet (Maerz et al., 2006). Morphological differences between habitats suggested that variation in morphology at the population level may accentuate variation at larger spatial scales and influence the diversification of species (Maerz et al., 2006).

Trophic polymorphisms in birds have been characterized by differences in morphology, behavior, or a combination of the two (Smith and Skúlason, 1996). Hook-billed kites

(*Chondroheirax unicantus*) exhibit bill polymorphisms with a large-billed morph with a deeper, larger, and more hooked bill than the small-billed morph (Smith and Temple, 1982). The frequency of the large-billed morph was correlated to the abundance of large snails (Wimberger, 1994). The black-bellied seed cracker (*Pyrenestes estrinus*) exhibit large- and small-billed morphs (Smith, 1987). The two morphs are not correlated with sex, size, age, or geography and mate at random. During periods of food limitation, competition between the two morphs is intensified and reproductive divergence could occur in response to resource phenology or diversity (Smith, 1990). Bill differences in the Oystercatcher (*Haematapus ostralegus*) are correlated to food handling behavior (Sutherland, 1987; Wimberger, 1994; Smith and Skúlason, 1996). Birds with more pointed bills fed on mussels by inserting their bill between the valves ('Stabbers'), while birds with blunt bills fed by breaking mussel shells open by pounding ('Hammerers") (Northon-Griffith, 1967). Behavioral polymorphisms in feeding behavior have also been documented in bird species, such as the Cocos Island finch (*Pinaroloxias inornata*) (Werner and Sherry, 1987) and the Pacific Reef heron (*Egretta sacra*) (Mock, 1981; Rohwer, 1990).

Trophic Polymorphisms in Snakes

Previous research into the phenotypic plasticity in trophic morphology of snakes has had varied results (Schuett et al., 2005). Some species and/or populations exhibit moderate levels of morphological change due to use-induced diet changes (Queral-Regil and King, 1998; Aubret et al., 2004, while others do not (Bonnet et al., 2001). Experimental manipulations of prey species, along with field observations of diet and morphology have both led to varied results.

Camilleri and Shine (1990) examined the stomach contents and trophic measurements in preserved collections of four species of snakes. Analyses revealed that diet and trophic

morphology did not always differ between the sexes. In three out of the four species, males and females had different trophic morphology in response to ecological divergence in diets (Camilleri and Shine, 1990). Forsman (1996a) investigated the impact of food level on trophic morphology. *Vipera berus* were raised on different biomasses of food and tested for altered head and body size. Snakes raised on increased amounts of food grew larger, but the allometric relationships between head and body size did not change (Forsman, 1996a). Forsman (1996a) concluded that geographic variation in trophic morphology was not simply due to differing resource levels, but more likely due to prey type and/or genetic differences. Queral-Regil and King (1998) found that water snakes (*Nerodia sipedon*) fed larger prey items had longer jaws after correcting for body size. Elongation of the jaw relative to the body would allow snakes to ingest larger prey and was most likely due to increases in mechanical stress during swallowing (Queral-Regil and King, 1998). Gaboon vipers (*Bitis gabonica*) reared on different food biomasses were found to have significantly different trophic morphology (Bonnet et al., 2001). The different allometric relationship between growth rates was only noticed for some of the trophic measurements, while others were unaffected (Bonnet et al., 2001). Field observations of population differences in sexual size and shape dimorphism in *Thamnophis sirtalis* suggested snakes in different habitats exhibited different trophic morphology (Krause et al., 2003). Female *Thamnophis sirtalis* showed plastic responses to increases in prey size in their relative head sizes, while males showed less of a response (Krause et al., 2003).

In the eastern tiger snake, *Notechis scutatus*, experimental manipulation of prey type demonstrated population differences in plasticity (Aubret et al., 2004). Snakes were exposed to either small or large prey items. Those exposed to larger prey items from one population were able to adjust their head size, while those from the other population in the same treatment were

not. Aubret et al.'s (2004) study suggests that the ability of snakes to adjust trophic morphology may not only be influenced by the environment, but also genetic differences within a species. A follow-up study examined the plastic response of multiple populations of mainland and island tiger snakes *(Notechis scutatus*) to differing prey sizes (Aubret and Shine, 2009). Populations that had more recently colonized island exhibited greater plasticity in trophic morphology, increasing the relative size of their trophic morphology for larger prey items. Populations that had colonized island more than 9000 years ago showed reduced plasticity, but always exhibited larger trophic morphology relative to body size. Results suggested that island snakes were under selective pressure for increased trophic morphology size, and that populations responded initially through phenotypic plasticity that is later genetically canalized (Aubret and Shine, 2009).

A study on the *Boa constrictor* found that increased prey size did not significantly affect trophic morphology measurements (Schuett et al., 2005). Treatments were matched for prey biomass and only differed in prey size. Snakes were fed either weanling mice or small rats for 58 weeks, measurements of head size (rostrum-occipital length and mandible length) did not significantly differ between treatments, but other body size variables significantly differed between males and females. Studies of preserved specimens of *Agkistrodon piscivorous* showed that dietary divergence between males and females resulted in an increase in the size of trophic structures in male snakes (Vincent et al., 2004). Male snakes consumed relatively taller prey and were characterized by longer quadrate bones (Vincent et al., 2004). Population studies on four Australian snake species showed that the introduction of an invasive prey species had significant effects on morphology (Phillips and Shine, 2004). The arrival of the toxic cane toad (*Bufo marinus*) in Australia was correlated with reduction in gape size and increase in body length. Two species of snakes (*Pseudechis porphyriacus* and *Dendrelaphis punctulatus*) that were

considered to be higher risk to cane toad toxicity showed significant changes in trophic morphology and body size (Phillips and Shine, 2004). Another two species of snakes (*Hemiaspis signata* and *Tropidonphis mairil*) considered to be low-risk (either too small to ingest cane toads or able to tolerate the toxins) exhibited no consistent patterns of change since the introduction of cane toads (Phillips and Shine, 2004).

Is Snake Trophic Morphology Adaptive?

The matching of form to function has been generally interpreted as evidence for the adaptive nature of trophic morphology (Pough and Groves, 1983; Schluter and McPhail, 1992). Broad comparisons of trophic morphology agree with the hypothesis that plasticity is adaptive in nature, as it is difficult to understand the diversity and patterns of morphology without invoking adaptation (Forsman and Shine, 1997); however, adaptive inferences are weaker when investigating differences at the species or population level. Differences in the relative size and shape of trophic morphology may result from processes unrelated to adaptation (Forsman and Shine, 1997; Aubret, 2012).

Historically, natural selection has been invoked to explain differences in the size and shape of trophic morphology among species (Forsman, 1991, 1996a; Shine, 1991; Grudzien et al., 1992; Skúlason and Smith, 1995; Maerz et al., 2006; Aubret, 2012). In snakes, it is assumed that larger trophic morphology (or more accommodating shape) allows for the consumption of larger prey items (Arnold, 1983, 1993; Shine, 1991; Forsman and Lindell, 1993; Shine et al., 1998; Aubret et al., 2004). Adaptation, however, has many underlying assumptions that need to be met to differentiate from non-adaptive hypotheses (Forsman and Shine, 1997; Aubret, 2012).

There are at least three alternative hypotheses that could explain observed patterns in snake trophic morphology and not invoke adaptation. 1.) Head size divergence among

populations may be correlated with geographic differences in overall body size combined with allometry of measured trophic structures (Lande, 1979; Blouin and Brown, 2000). 2.) Head size relative to body size may diverge in populations due to stochastic processes such as genetic drift or founder effects (Lande, 1979; Forsman and Shine, 1997; Aubret, 2012), and. 3.) Differences in ecological conditions (food supply, thermal patterns, etc.) may induce differences in developmental pathways and give rise to trophic differences not related to diet (Blouin and Loeb, 1991; Shine and Harlow, 1996; Forsman and Shine, 1997).

Investigations into the nature of trophic morphology in snakes have suggested both adaptive and non-adaptive hypotheses. Forsman and Shine (1997) found that trophic morphology in snakes has not evolved along the path of least resistance (Schluter, 1994). The simplest way for trophic morphology to change would be with a change in overall body size. Deviations in the allometric relationship between body and head size suggest that evolution of trophic morphology is adaptive (Forsman and Shine, 1997). Aubret (2012) concluded that variation in the size of trophic morphology evolved through primarily nonadaptive processes. Adult trophic morphology and body size in island tiger snakes *(Notechis scutatus*) resulted from selection on growth rate on neonate snakes and resource availability throughout ontogeny (Aubret, 2012).

In order to understand possible changes in trophic morphology, structures that determine gape-size and are directly involved in prey-handling must be quantified. Detailed description of the processes involved in prey ingestion can be found in Cundall (1987). Many cranial elements are involved in both prey capture and prey consumption. The braincase, supratemporal, quadrate, mandible, and mandibular symphysis form a ring that encloses the prey item (Arnold, 1983). Structures that directly influence gape size and swallowing ability are: length of the maxilla, number of teeth, length of the dentary, number of teeth on the dentary, length of the pre-articular,

length of the quadrate, length of the supratemporal, length of the palatine, number of teeth on the palatine, length of the pterygoid, length of the ectopterygoid, total length of the pterygoid and palatine, and total length of the lower jaw (Camilleri and Shine, 1990). The size of these structures is not the only determinant of function; the articulations between structures and the elasticity of them also affect gape size and swallowing ability (Camilleri and Shine, 1990).

The functional definition of prey size can also be debated. The shape and size of prey items affects swallowing performance. Feeding on large prey items requires extensive mobility of the cranial elements (Cundall, 1987). Increases in handling time have a three-fold effect on the snake. Snakes are vulnerable during swallowing, increased swallowing effort increases the amount of energy needed to consume that prey item, and increased time swallowing decreases the amount of time available for other life history processes (Vincent et al., 2006). Trophic structures may not only be under selective pressures for size, but also the shape of prey items.

Growth rate may alter shape-size relationships and hence alter trophic morphology. Growth rates can affect the relationship between size and shape in two ways: (1) shape may scale non-isometrically, therefore growth rate variation can cause size and/or shape variation; (2) growth rate can independently affect shape regardless of the correlation between growth rate and size, i.e. rate at which an organism reaches a certain size determines its shape (Blouin and Loeb, 1991). Relative head size (a proxy for trophic morphology, gape size) varies extensively in snakes (Forsman, 1996b). The varying effects of growth rate on shape and size (collectively referred to as form) must then be taken into account when assessing trophic changes and their relation to prey type and size.

Criticisms and Future Directions

Previous research investigating trophic morphology in snakes has examined effects of food size and resource level. Much of the previous research that manipulated diets in the laboratory used small sample size and/or individual litters (Bonnet et al., 2001; Schuett et al., 2005). Changes in trophic morphology could be due to sexual dimorphism, growth rate effects on morphology, adaptive phenotypic plasticity (it could also be non-adaptive), or a genetic factor (genetic drift), so future studies need to be designed to account for all of these factors.

Previous studies also incorporated two other sources of error into their experimental design. First, diet treatments were consistently made up of different prey types (frogs vs. mice; rats vs. mice) and these differences in prey type result in differences in nutritional content (Forsman, 1996a; Bonnet et al., 2001; Aubret et al., 2004) and specific dynamic action (SDA). Specific dynamic action is the increased metabolic rate an organism experiences due to the ingestion and digestion of a meal (Secor and Faulkner, 2002; Zaidan and Beaupre, 2003). Nutritional and energetic discrepancies in diet have broad physiological impacts which would most likely impact morphological responses. Previous studies also did not qualify prey size differences; trophic adaptation is commonly thought of in discrete phenotypes, but there may be continuous responses that produce minor morphological differences over small ranges of prey sizes (Maerz et al., 2006).

The majority of work on trophic morphology has relied upon linear measurements (Bonnet et al., 2001; Schuett et al., 2005; Maerz et al., 2006). What linear measurements fail to detect, is that trophic morphology can differ in two ways. Morphological changes can occur through adaptations in size or shape. Linear measurements can only detect differences in size. Trophic morphology involves multiple structures and components working in conjunction, and

different results will result from univariate or discrete analyses (Wimberger, 1994; Maerz et al., 2006).

Recent developments in the field of geometric morphometrics provide a solution to the reliance on linear measurements. Geometric morphometrics (GM) analyses are used to consider the geometrically stable spatial arrangement of anatomical features of organisms, providing statistical (Rohlf, 1999) and visual advantages over traditional approaches based on linear distance measures (e.g., Caldecutt and Adams 1998, Rüber and Adams 2001, Adams and Rohlf 2000). Variation in landmark data includes variation associated with shape, size, position, and orientation. All non-shape variation can be mathematically removed, and shape can be analyzed as the size-free departure of anatomical landmarks from a mean form. Geometric morphometric methods provide the opportunity to better examine variation and its sources by increasing statistical power while lowering bias and error rates (Collyer et al., 2005).

Given the advancements in shape analysis and potentially confounding design of previous studies, several methodological and design improvements are recommended for future research. First, quantifying differences in both prey size and resource level. Many previous studies confounded these two variables by offering a high resource treatment larger prey items than a low resource treatment. Future studies should design treatments such that both size and biomass of prey items are carefully controlled. Second, sample sizes should be sufficiently large to increase statistical power. Spurious results from small sample sizes may lead to erroneous conclusions of treatment effects on trophic morphology. Third, expand study design to include multiple populations or litters. Even though possible genetic differences add an additional level of complexity to study designs, the genetic influences over trophic morphology and its plasticity are just as important as the environmental influences. Lastly, expand morphometric

measurements beyond the use of simple, linear measurements. Geometric morphometrics provides a more complete and detailed quantification of trophic morphology and current advances have made its use quite manageable (Adams and Rohlf, 2000). There have been numerous examples of trophic polymorphisms that have gone unnoticed over successive studies due to the methods used to quantify morphology (Smith and Skúlason, 1996). Many other morphological traits show varying abilities to significantly detect morphological differences when used in univariate and multivariate analyses (Maerz et al., 2006). By expanding study design and modifying how morphology is quantified, insights into the patterns of plasticity and morphological divergence can be improved.

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CHAPTER 2: METABOLIC AND MORPHOLOGICAL RESPONES TO VENOM EXPENDITURE BY PRAIRIE RATTLESNAKES (*Crotalus viridis viridis*)

Abstract

Snakes demonstrate a great deal of variation in the amount of venom injected in both predatory and defensive strikes. The current leading hypothesis is that snakes consciously meter the amount of venom they inject. An underlying assumption in the evolution venom metering is that the production of venom is energetically costly. To date there has been very little research that has quantified the metabolic costs associated with venom production. We used open-flow respirometry to test for significant differences between control and milked prairie rattlesnakes (*Crotalus v. viridis)*. Although several previous studies demonstrated high production costs of venom, we found that milked snakes did not have significantly higher metabolic rates than control snakes. The pattern of metabolic deviation from baseline measurements was similar for both treatments, and on average snakes that are milked only exhibited a 1.1% increase over baseline compared to a 2.5% increase in control snakes. Our data suggest that venom is not energetically costly to produce and that perhaps other costs associated with venom or physical factors can better explain the variability in venom expenditure.

Introduction

The possession of venom is a widespread trait among many different groups of organisms. Venomous species have been identified in cnidarians (Kardong, 1996), arachnids and other arthropods, several families of snakes (Johnson, 1956; Mackessy, 2009), a family of lizards (Mackessy, 2009), and many fish genera (Smith and Wheeler, 2006); in fact nearly 50% of all phyla in the animal kingdom contain at least one venomous species (Pintor et al., 2010). As a

group, reptiles contain the largest vertebrate species that are venomous, and many of those species produce some of the largest quantities of potent venoms (Mackessy, 2009).

Venomous organisms have shown a high degree of variability in the amount of venom expelled during both predatory and defensive strikes (Hayes, 1995). Factors such as snake size, prey size, prey species, bite context (predatory vs. defensive), along with several others have shown to influence the amount of venom expelled during a strike (Hayes 1995, Hayes et al., 2002; Young et al., 2002, Hayes, 2008; Young, 2008). The variability in venom expenditure has been the subject of intense study (see Hayes et al., 2002 for a review).

Venom is a complex mixture of peptides with a wide array of effects on the predator and prey species that are envenomated (Mackessy, 2009, 2010). Snake venom is primarily used to immobilize prey items and even begin the digestion process, while acting secondarily to deter predators (Pintor et al., 2011). Venom composition is known to vary with factors such as phylogenetic relationship (Mackessy, 2009), age (Mackessy, 1985; Haight and Tschinkel, 2003; Mackessy et al., 2003), diet (Barlow et al., 2009), time (Willemse et al., 1979; Williams and White, 1992), and geography (Mackessy, 2010).

In snakes, the venom apparatus consists of a pair of specialized glands that are located bilaterally deep and medial to the upper labial scales, just posterior to the nostrils and behind/below the eyes (Mackessy, 2009). These glands are referred to as venom or Duvernoy's glands. Anterior to the main gland is a smaller, accessory gland. The two glands are joined by a short duct that is lined with simple columnar secretory cells (Mackessy, 1991). There is a primary duct leading to an accessory gland, and a secondary duct connects the glands to the base of the hollow fang (Kardong, 1980, 1982). The venom gland has a series of associated muscles, and in vipers these are the compressor glandulae muscles which insert directly on the capsule of

the venom gland (Kardong and Lavin-Murcio, 1993). Following venom expenditure, there is a sudden up-regulation of RNA synthesis in the venom gland tissue (Rotenberg et al., 1971; Yamanouye et al., 1997; Kerchove et al., 2004), the RNA up-regulation lasts for four to nine days, whereas protein synthesis lasts longer, 30-50 days (Luna et al., 2009).

A primary assumption regarding the evolution of venom metering is that venom is energetically costly to produce (Hayes, 2008). Theoretically high production costs may dictate a tradeoff between the energetic expenditure associated with venom injection and possible fitness benefits from injecting venom. The balance of venom expenditure and energetic costs of production have been cited as the explanation of differential expenditure of venom in several taxa (Hayes, 1995; Hayes et al., 1995; Malli, 1999; Nisani et al., 2007). The energy allocated to venom synthesis should represent an ecologically significant portion of the snake's energy budget and conservation of that energy would be evolutionarily advantageous (Hayes et al., 2002; Young et al., 2002). A subtext of the energy assumption is that snakes need to retain a certain volume of venom at all times. The ability to perform multiple envenomations over a relatively short period of time is ecologically and/or evolutionary beneficial to a snake (Hayes et al., 1995).

The idea of venom metering, while not being supported fully in the scientific literature, has found its way into technical and popular literature and mainstream culture (Young, 2008). The venom metering hypothesis is centered on a snake's ability to intrinsically control the amount of venom that is expended during predatory and defensive strikes. Under this hypothesis, snakes would alter the amount of venom that is injected with each strike depending on the target species, size, or behavioral context of the strike (Young et al., 2002). A conscious

decision-making process of snakes is based on the principle that venom expenditure is optimized for some energetic or ecologically related factor.

An alternative to the venom metering hypothesis is the pressure-balance hypothesis, which is based on the physical properties, first principles, and extrinsic factors involved in a snake strike (Young 2008). At the center of this concept is the motive pressure on the venom gland, the pressure on the distal chamber on the venom delivery system, and peripheral resistance at the fang orifice (Young et al., 2000). The motive pressure on the venom gland is a result of the contraction of the extrinsic venom gland musculature. The differential tension on the fang sheath is associated with the erectile status of the fangs. Compression of the fang sheath depends on the physical interaction and momentum of the snake's head and the inertia of the target species. The peripheral resistance will impact the amount of venom that is expelled from the orifice and how much infiltrates the tissue. The density and homogeneity of the tissue that the fangs puncture will also have pronounced impacts on the end result of the strike (Young et al., 2000, 2002; Young, 2008). The inescapable laws of physics, fluid dynamics, and properties of tissues (both snake and target) combine to result in the passive response of venom expenditure.

The numerous studies of snake venom allocation have focused on amount of venom injected (Kochva, 1960; Schaeffer et al., 1972; Hayes 1995, Young and Zahn, 2001), sexual dimorphism in venom (Furtado et al., 2006), venom composition (Willemse et al., 1979; Williams and White, 1992; Mackessy et al., 2003; Mackessy, 2008, 2009, 2010; Pintor et al., 2011), histological composition (Carneiro et al., 1991; Mackessy, 1991), physiological activity/pathways of venom production (Rotenberg et al., 1971; Oron et al., 1978; Kerchove et al., 2004; Luna et al., 2009); but only a few studies have examined the metabolic and

morphological ‘costs’ associated with venom expenditure (but see McCue, 2006; Pintor et al., 2010).

The assumed high costs of venom may have tremendous impacts on the evolution, ecology, and behavior of venomous species across taxa, yet only a handful of studies have evaluated the costs associated with venom production. Empirical evidence on the costs associated with venom and venom production may help elucidate the widespread evolution of venom (Pintor et al., 2010). McCue (2006) found that pit vipers showed an 11% increase in metabolic expenditure following venom extraction. A study on the common death adder (*Acanthophis antarcticus*) showed that metabolic rate was elevated for six days following venom expenditure, and that metabolic rate peaked 12 hours following extraction, increasing 69% over baseline measurements (Pintor et al., 2010). Averaged over the same 72 hour interval as the McCue (2006) study, death adders showed a 21% increase in metabolic rate over resting levels (Pintor et al., 2010).

Pintor et al. (2010) compared energetic estimates of venom production to the costs of other necessary functions (digestion and shedding/ecdysis). While venom production did significantly increase metabolic rate, the energetic costs were relatively small when compared to other vital functions. Venom production costs averaged 28.63 $J/g^{0.75}$ body mass, while digestion and ecdysis were significantly higher (109.85 $J/g^{0.75}$ and 482.29$J/g^{0.75}$ respectively) (Pintor et al., 2010).

To date, evidence points to venom being significantly costly to produce, but well within the range of other physiological processes. High costs associated with venom production have been the focal aspect of cost-benefit analysis that may explain the selective advantage of venom

metering. Therefore, the increased metabolic rates during venom production have been cited in support of the venom metering hypothesis.

Unfortunately all of the previous studies have examined only a few species (4) and used very small samples sizes (n = 7, 7, 8, 8). There is significant variation within species, populations, and even individuals in measures of resting metabolic rate, field metabolic rate, and annual energy budgets (Beaupre, 2008). Snakes have shown to become acclimated in metabolic chambers (see Figure 1); hence using small sample sizes forces individual snakes to be put into multiple treatments. Limited sample sizes also make estimation of the relationship between metabolic rate and body size, or other variables difficult to assess. A relationship based on a small range of body sizes and only 7-8 data points may provide an inaccurate estimation of allometric scaling factors. If those relationships are then used to predict energy expenditures or budgets based on body size, energetic costs may be inaccurate (McCue, 2006; Pintor et al., 2010).

While many authors agree that the envenomation system makes up a significant portion of the snake's trophic morphology, with estimates of up to 15% of total head volume (McCue, 2006), no one has looked at any changes in that morphology due to envenomation. Many studies have examined the kinematics of snake strikes or the morphology of the venom glands themselves (Kardong 1980, 1982, 1986, 2003; Kardong et al., 1986, 1997; Carneiro et al., 1991; Janoo and Gasc, 1992; Kardong and Lavin-Murcio, 1993; Smith et al., 2002; Mackessy and Baxter, 2006). Yet, to date no one has examined trophic morphology changes due to predatory or defensive strikes and envenomation.

Variation in trophic morphology has been ascribed to differences in populations/regions (Smith and Collyer, 2008), resource level (Forsman, 1996), prey type (Krause et al., 2003;

Aubret et al., 2004), sex (Camilleri and Shine, 1990; Vincent et al., 2004), and evolutionary histories (Aubret and Shine, 2009). If defensive strikes alter the trophic morphology of snakes, previous results could be an artifact of differences in aggression or number of strikes. To accurately assess the presence/absence of significant trophic morphology differences, all aspects affecting said morphology must be accounted for. Investigations into the effect of defensive strikes and envenomation on trophic morphology may have resounding impacts on previous studies.

The current debate over venom control and the limited amount of research on metabolic costs of venom production were the motivation for the current study. Our investigation focused on the three main objectives. 1.) To quantify the metabolic costs associated with defensive strikes and envenomation in *Crotalus v. viridis*. 2.) To assess if metabolic costs associated with venom production are detectible and could account for a significant part of an annual energy budget. 3.) Quantify possible changes in trophic morphology due to defensive strikes and envenomation. All three objectives test previous results and have the potential to shape future directions of venom and trophic morphology research.

Methods and Materials

Study Species

The Prairie rattlesnake (*Crotalus v. viridis*) was selected because of its large distribution and prior work on trophic morphology. The snakes used in this study were captured from sites in western North and South Dakota in the summers of 2007 and 2008 as part of a larger study on trophic morphology. All juvenile snakes used in this study were born in the laboratory, while the adults were wild-caught. All snakes had been in the laboratory for an extended period of time (roughly 3 years) before entering this experiment. Snakes were maintained in plastic containers

of varying sizes (14-31 liters), given water *ad libitum*. All containers were housed under a 12L:12D photoperiod at 24° C (+/- 1° C). Snakes were fed pre-killed, thawed rodents every 2-3 weeks, meals were roughly 15%-25% of their total body mass.

We used 63 individual snakes in this study, separated into 9 runs of 7 individuals each. We used both juvenile and adult snakes of both sexes, ranging in body mass from 37.3 g to 573.02 g. All snakes were deemed to be in good body condition and post-absorptive prior to entering venom trials. Snakes had not eaten or expended venom for at least 14 days prior to entering this study.

Experimental Design

Experimental design was specifically chosen to improve on previous studies, carefully controlling for variation in metabolic rate, treatment effects, and potential confounding factors. Snakes were randomly assigned to one of two treatments and then divided into 9 separate runs (7 snakes each, dictated by respirometry equipment limits). Snakes were measured for body mass (+/- 0.01 g) and SVL to the nearest 0.1 cm using a squeeze box (Quinn and Jones, 1974). Digital images were taken of the dorsal and right lateral aspects of the head using a Cannon ® PowerShot S3 IS 6.0 mega pixel camera. All digital images were taken at specially built camera table setups (one for the dorsal and one for the lateral aspects), that controlled camera and snake positioning and provided a number of rulers for scale (Smith, 2006).

After photographs were taken, resting metabolic rate (RMR) was measured for 48-96 hours to obtain a baseline of metabolic expenditure for each snake. After initial measurements of RMR, snakes were removed from their metabolic chambers and weighed and measured again. Control treatment snakes were harassed (sudden movements and waving our hands in front of the snake), but were not allowed to strike. Control treatment snakes were re-photographed (dorsal

and right lateral) and then placed back into respirometry chambers. Snakes in the milked treatment were harassed, but then presented with a 30ml wide-mouth jar covered with dual layers of Parafilm™. The milked treatment snakes were allowed to repeatedly strike the Parafilm™ covered jars, strikes mimicked defensive strikes against a potential predator. Snakes struck the jar forcefully, such that the impact should be proportional to that of a snake striking mammalian predator. Each snake was forced to strike 3-4 times, regardless of how much venom was expelled.

Milked snakes were re-photographed (dorsal and right lateral) and returned to respirometry chambers. All snakes had CO_2 production measured for 96-104 hours following treatment. Venom wet mass was determined by subtracting the weight of the jar prior to the snake strike to the final mass of the jar and venom. All venom mass measurements were made with an analytical scale to the nearest 0.1 mg.

Metabolic Measurements

Metabolic measurements were recorded as the amount of CO_2 produced. All measurements were made using the Sable System TR-3 open-flow respirometry system (for greater detail see: Beaupre and Zaidan, 2001). Measurements of CO_2 production were obtained from the LI-COR LI 6251 infrared gas analyzer (LI-COR, Lincoln, NE). Dry, CO_2 free air was produced by the Whatman FT-IR 75-45 purge gas generator (Whatman, Haverhill, MA). Positive pressure was used to push the dry, CO_2 free air into a Sable Systems MF-8 manifold (Sable Systems, Las Vegas, NV), which divided the air flow into 8 separate channels/lines. One of those air channels was designated for baseline measurements, the other seven lines were attached to respirometry chambers.

The manifold and respirometry chambers were located inside of a Percival I-30 BL environmental chamber. The Percival chamber controlled air temperature, which was monitored frequently using a thermocouple, within +/- 0.8°C of targeted temperature. Metabolic measurements were conducted at 24 °C and 12L:12D photoperiod, the same as their captive environment.

Baseline and excurrent air flow (the other seven airflow lines) were subsampled through an eight channel multiplexer (Sable Systems Respirometry Multiplexer V.2.0) via negative pressure. Subsample flow rates were always less than 50% of incurrent flow rates. Automated, computer controlled multiplexing (DATACAN V, Sable Systems) allowed for sequential measurements of seven snakes and one baseline. The setup was configured such that each snake was measured for 6.7 minutes for each hour time block, while the baseline was measured for the first and last 3.3 minutes of each hour block. During the recording of subsampled air, CO_2 levels (ppm) were recorded every five seconds.

Excurrent flow rates were measured for each line at the beginning and end of each run. Air flow did not significantly change during each 24 hour measurement block. Flow rates were corrected for standard temperature and pressure (STP, 0°C, and 760 mm Hg) by controller. Corrected flow rates were variable, depending on the mass of the snake, but ranged from 275-675 ml/min.

Before entering the gas analyzer, subsampled air was pulled through two 30-ml drying tubes. Each tube was filled with recharged Drierite to sequester any moisture in the subsampled air (White et al., 2006). The gas analyzer was spanned using 500 ppm compressed CO_2 (+/- 2% analytical accuracy, [Scott Specialty Gases, Plumsteadville, PA]), before every run, and zeroed (using baseline air readings) before each 24 hour measurement block.

Prior to data collection, snakes were allowed to acclimate for 2-4 hours in the respirometry chambers, allowing snakes to come to thermal equilibrium with the environmental chamber and avoid elevated metabolic readings due to stress from placement into the chambers. This 2-4 hour acclimation time was only used at the onset of the block recording (before recording the first 24 hour block of pre-treatment runs only), but not when switching between 24 hour recordings within a given treatment period or for post treatment recordings during which snakes were presumed undisturbed.

Data Processing and Analysis

Metabolic data was processed using Expedata version 1.2.5 (Sable Systems). Baseline adjustments were made to every record for each snake for each hour of the block. Expedata identified the contiguous 50% of baseline samples that exhibited the lowest average ppm CO_2. Each hour's dataset was then rotated to adjust for any baseline drift. Hourly CO_2 averages were calculated using the last 55 samples of fractional excurrent [CO_2] recorded per snake each hour. Hourly production of CO_2 was calculated using the following equation:

$$VCO_2 = (F_e - F_i)*(FR)*(60)$$

Where: VCO_2 = rate of CO_2 production (ml/h), F_e = average excurrent fractional [CO_2], F_i= average incurrent fractional [CO_2], FR = STP adjusted flow rate.

A baseline measurement of RMR (VCO_2) was calculated for each snake during the 'Pre' treatment phase. The first 24 hours of baseline data was thrown out due to an extended period of increased activity (see Figure 1). Average RMR in ml of CO_2/hour was then estimated by averaging the next 24 to 72 hourly records for each snake (only 2 of the 9 runs were run for 96 hours for baseline, all other runs were run for 48 hours). Using an average RMR value allows for

easier comparison to previous studies and also accounts for the variation in RMR due to time of day and individual variation.

Post treatment metabolic data were adjusted for baseline drift and hourly averages were calculated as stated above. Each post treatment hourly value for VCO_2 had the individual's RMR value subtracted from it. Difference in metabolic rate (DMR) is positive if post treatment metabolic rate was higher than RMR during any given hour and negative if post treatment values were lower than average RMR. The values for DMR were calculated for every snake for each hour of post treatment data. Cumulative DMR (CDMR) values were calculated by summing each hourly value, for example the CDMR value for hour 12 would be the DMR values of hours 1-12 summed together. If venom production does significantly increase metabolic rate, milked snakes should have consistently positive DMR values and CDMR values should increase to a point and then plateau as the snake returns to baseline RMR values (Figure 2). All values for DMR and CDMR were calculated using Microsoft Excel 2003 (Microsoft Corporation).

Repeated measures ANCOVA was used to test for significant differences between control and milked snakes. The values for CDMR at six hour intervals were used, for a total of 17 measurements of cumulative difference in metabolic rate for each individual. The values for hours: 1, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90, and 96 were included in the repeated measures analysis. ANCOVA analyses included the variables SNAKE (individual), treatment (TRT), Time (hours since manipulation), and Mass as a covariate. Time was modeled as both a continuous and categorical variable. Models varying the variance-covariance structure and how Time was coded were compared. The model with the lowest AIC value was chosen as the most appropriate model. Cumulative difference in metabolic rate values were tested for deviations from a normal distribution and residuals from the chosen model were tested for

normality and homogeneity. All repeated measures tests were done using R version 2.14.1 (R Foundation for Statistical Computing, 2011) using the nlme library (Pinheiro and Bates, 2000) and assumption testing was done in JMP versions 9.0.2 (SAS Institute Inc.) and Systat 13 version 13.00.05 (Systat Software Inc.).

Morphometric Analyses

Trophic morphology was quantified using geometric morphometrics (GM). Change in head shape from 'pre' to 'post' treatment contains not only actual shape change, but statistical 'noise', in the form of landmark placement error, measurement error, and the inherent individual variability in an organism with a highly kinetic skull (Parker, 1878; Klauber, 1956; Gans, 1961; Franzetta, 1970) . To account for this, we tested for the difference in the magnitude of shape change between control and milked snakes. Shape change in control snakes should contain all the sources of variation except for that involved in venom expenditure. The magnitude of shape change was quantified using Procrustes distance (Bookstein, 1997; Zelditch et al., 2004). Each configuration had a Procrustes distance calculated by taking the square root of the sum of all the squared Procrustes residuals for the difference in the pre and post treatment shape configurations.

Head size was represented by centroid size (CS), which is calculated using GM. The centroid of each shape configuration is the geometric center of gravity for the landmark configurations. Centroid size is determined by calculating the distance between each landmark and the centroid. It is calculated using the equation:

$$CS \text{ (centroid size)} = \sqrt{\sum d_i^2}$$

Change in head shape (CSDiff) was calculated by taking the 'pre' treatment CS and subtracting 'post' treatment CS. If venom glands make up a significant portion of the trophic morphology,

centroid size of milked snakes should significantly differ between pre and post treatment values. We used the absolute value of CSDiff to aide in meeting the assumption of normality

Results

After data collection, we eliminated 1 individual due to a leaky respirometry chamber, and 9 other individuals due to issues with venom expenditure. Most commonly, control snakes were removed from the analysis due to the striking of the tongs or squeeze box during processing. Several milked snakes were also eliminated because they failed to inject any venom during the venom extraction phase. After getting rid of outliers, we used a total of 53 snakes (22 control, 31 milked).

Venom Expenditure and Metabolic Response

Venom was extracted from 31 snakes. Several snakes struck the jars in such a manner that the venom sprayed instead of injecting into the jar. These snakes were allowed to stay in the dataset due to a visible expenditure of venom, but venom mass was not recorded and therefore left out of the following summary results. We obtained an average of 0.1344 grams (SD= 0.068) of venom (wet mass) per individual. These amounts are slightly lower than other reported values for rattlesnake species (McCue, 2006; Mackessy, 2010), but those studies attempted to empty the venom glands, while we simulated a defensive encounter. Snakes typically injected venom on only the first one or two strikes, while the last 2-3 strikes could be considered 'dry bites'. The amount of venom injected into the jars was not correlated to body mass, as a regression of Log_{10} Venom Wet Mass against Log_{10} Body Mass was not significant ($F = 0.2860$, $P = 0.5977$, $R^2 = 0.1024$). Venom was assumed to have a moisture content of 70.9% (McCue, 2006) and protein

content of 5.7kJ/g (Peterson et al., 1999). Venom yields and energetic content is summed in Table 1.

Resting metabolic rate was allometrically related to body mass, described by the equation: CO_2 production = $0.094W^{0.67}$ (Figure 3). The allometric scaling coefficient is lower than reported in previous studies (McCue, 2006; Pintor et al., 2010), but it falls within the range of expected scaling coefficients (Beaupre and Zaidan, 2001; Nagy, 2005). There was no significant difference in average RMR between control and milked snakes (Control = 3.324, SD = 2.003, n = 22; Milked = 3.422, SD = 2.568, n = 31).

Repeated measures ANCOVA model tests revealed the covariance structure of autoregressive with heterogeneous variances was the best fit and the variable of Time was modeled as a factor (Table 2). Models for the repeated measures ANCOVA were as follows:

*CDMR (VCO2[ml/h]) = Body Mass + TRT*Time + Error*

Where: CDMR = cumulative difference in metabolic rate, TRT = treatment, and Time = pre or post manipulation. The model explained significantly more variation than the null model (L-ratio = 65.94129, p <.0001). Only the intercept and the Time variable were significant (Time: $F_{16,866}$ = 2.82505, p = 0.0002), meaning that while CDMR changed over the 96 hour period, it did not differ between treatment (control vs. milked) or in the way both treatments changed over time (TRT*Time: p=0.1730). Both treatments showed elevated metabolic rates for the first 30 hours post-treatment, and then metabolic rates fell below average RMR (Figures 4 & 5).

To make comparisons to the other two studies on costs of venom production in snakes (McCue, 2006; Pintor et al., 2010), we calculated a 72 hour average for each snake and compared it to their average RMR. We calculated a percent change from RMR values (post treatment MR/ RMR) and tested for differences between the treatments. Tests for treatment

differences, using Log_{10} Body Mass as a covariate, found no significant difference between control and milked snakes ($F_{2, 50} = 0.3189$, $p = 0.7285$; TRT: $F_{1, 50} = 0.656$, p = 0.7988). Control snakes actually showed a higher average increase in metabolic rate (2.5%) following manipulation than milked snakes did (1.1%) (LS means of MR ratio: control: 1.025, SE = 0.042; milked = 1.011, SE = 0.035). Examining just the 72 hour post manipulation metabolic rates, we again find no significant difference between control and milked snakes. The ANCOVA was significant for the covariate Log_{10} Body Mass ($F_{1, 50} = 123.7365$, $p < .0001$), but there was no significant difference between treatments ($F_{1, 50} = 0.4943$, $p = 0.4853$). The least square means from the ANCOVA examining the 72 hour post manipulation: control snakes = 2.66 ml CO_2/h (SE = 1.076), while milked snakes = 2.85 ml CO_2/h (SE = 1.064).

Morphological Response to Venom Expenditure

Dorsal and right lateral images had landmarks placed using TPS software (Rohlf, unpublished). Dorsal images had a series of 25 landmarks placed on each photo. Landmarks 1-10 were Type I or III landmarks, while 11-25 were sliding semi-landmarks that estimated the curvature of the snake's head (Table 3 and Figure 6). Lateral images had a total of 34 landmarks placed on each photo. Landmarks 1-9 were Type I or III landmarks, 10-16 were sliding semi-landmarks only the upper jaw, 17-28 were sliding semi-landmarks characterizing the posterior aspect of the head, and 29-34 were sliding semi-landmarks characterizing the anterior aspect of the head (Table 4 and Figure 7).

Treatments did not significantly differ in average SVL or in the relationship between SVL and head size (CS). Therefore, any detected difference in the change of shape or size is due to venom extraction, and not to previously existing differences or a difference in the allometric relationship between head and body size. Our goal was not to quantify the actual shape or size of

the trophic morphology, but rather the magnitude and direction of change due to venom expenditure.

We used an ANCOVA to test for treatment differences in changes to centroid size (CS) and magnitude of shape change. Centroid size difference (CSDiff) was obtained by subtracting the post manipulation CS from the pre manipulation CS. We took the absolute value of the CSDiff and used a square root transformation to meet the assumption of normality. We used log transformed SVL as a covariate, as larger snakes should have greater overall differences in CS.

$$\sqrt{|CSDiff|} = Log_{10}SVL + TRT + Error$$

The magnitude of shape change was quantified using the Procrustes distance (see Methods). We tested for significant treatment differences in Procrustes distance (PD), using an ANCOVA with body size as the covariate. Both variables were log transformed to meet the assumption of normality.

$$Log_{10}PD = Log_{10}SVL + TRT + Error$$

Neither the dorsal nor lateral aspects of trophic morphology showed any significant treatment differences in either magnitude of shape change or change in head size (PD or CSDiff respectively). None of the ANCOVA models were significant, and the covariate was only significance once (Dorsal aspect of PD; LogSVL: $F_{1,50}= 4.768$, $p = 0.034$).

Discussion

Our study showed no significant metabolic or morphological response to venom expenditure by *Crotalus v. viridis*. Other studies have shown significant increases in metabolic rate that are hypothesized to correspond to venom replacement. Increases of 11% (McCue, 2006), 21% (Pintor et al., 2010), and 39% (Nisani et al., 2007) above resting metabolic rate have been reported in four snakes species and a species of scorpion. Contrary to those other studies,

Crotalus v. viridis showed no marked increase in metabolic rate following simulated defensive strikes. Snakes that were allowed to expend venom actually had a lower average change in metabolic rate than control snakes (1.1% and 2.5% increases respectively). Our study suggests that the metabolic costs of venom are most likely not any greater than the typical fluctuations in resting metabolic rate within an individual.

The amount of venom recovered from milked snakes was comparable to those reported in other studies on pit viper species (Mackessy, 2002; McCue, 2006), even though our study was designed to mimic a defensive encounter by a rattlesnake, rather than draining the venom gland. While venom glands were most likely not emptied, we collected enough venom to obtain comparable results as other studies do not truly empty venom glands either (Mackessy, 2010, Pers. Comm.).

Our study may have underestimated the total cost of venom production. Venom replenishment estimates have ranged from 14 to 50 days (Rotenberg et al., 1971; Kochva et al., 1975; Hayes, 2008; Young 2008; Currier et al., 2012). Previous studies ranged in duration, but 72 hours of post envenomation is the most common, and therefore it is what we reported in the results (McCue, 2006; Nisani, 2007). One study did quantify metabolic rates for nine days following the milking process, but found that after the sixth day there were no real detectible differences in metabolic rate from baseline levels (Pintor et al., 2010).

There were multiple reasons we chose the study design. First, the majority of the research suggests that a significant portion of the increase in energy expenditure associated with venom production occurs within the first three days (Rotenberg et al., 1971; Kochva et al., 1975; Oron et al., 1978; Mackessy, 1991; Pinto et al., 2010). Second, we wanted to be able to easily compare

our estimates with those from other studies. Lastly, we wanted to minimize the stress on the snakes by only keeping them in the respirometry chambers for 4-5 days without water.

The determinations of the metabolic and ecological costs of venom production are paramount for our understanding of how envenomation systems evolved. At least one of the leading hypotheses about a snake's ability to control venom expenditure is based upon the assumption that venom is costly (Young, 2008). Previous empirical evidence suggested that cost was metabolic/energetic (McCue, 2006). Pintor et al. (2010) found significant increases in metabolic rate associated with venom production, but when the energetics of venom production were compared to other day to day activities, the amount of energy expended was only a small portion of an annual energy budget. We submit that in at least some species, energetic costs of venom production are lost in the noise of day to day homeostatic maintenance.

Using average values from Table 2 and energetic values from published literature; we obtained expected values of hourly metabolic increases. The average energetic content of venom samples was 223 J, assuming 70.9% water and 5.7 kJ/g (Peterson et al., 1999), and the average RMR was 3.39 ml CO_2 h^{-1}. Using the conversion factor of 27.42 J ml^{-1} CO_2 (Zaidan and Beaupre, 2003), the snake would have to expend an additional 8.13 ml CO_2 to replenish its venom supply. The most recent literature suggests venom production peaks from days 3 to 7 (Currier et al., 2012), so hourly increases in metabolic rate would range from 0.113 to 0.048 ml CO_2 respectively. Expected hourly increases in metabolic rate were 1.5% - 3% of the average hourly RMR value. To detect a significant difference between control and milked snakes would depend on hourly RMR fluctuating by a significantly smaller amount. We conclude that finding a significant difference in metabolic rate due to venom extraction is unlikely given the relatively

small energetic content of venom and time it takes replenish and the normal fluctuations in RMR to maintain homeostasis.

There are still ecological and evolutionary costs that can be associated with venom that may have driven the evolution of venom control. Ecological costs would be associated with time spent producing venom, or time spent without adequate venom stores (Young et al., 2002; Hayes, 2008, Young, 2008). If a snake encounters a predator or prey without enough venom, then there would be a selective advantage in carefully metering out limited venom supplies. There is still some debate as to the extent that snakes are limited by their venom supplies. Field reports of multiple strikes against predators and prey (Cundall and Beaupre, 2001), may suggest snakes are not limited to small quantities of venom. Yet, other reports suggest that snakes only possess enough venom for up to six strikes before glands require replenishment (Young, 2008).

Evolutionary costs would be incurred if snake venom could not adapt to resistance changes in their current prey-base, or changes the species in their prey-base. Snake venom is a complicated mixture of components (Mackessy, 1991), but not complicated enough to never need to change. If a population of snakes cannot adapt to changes in their prey-base, then their venom would become obsolete. Venoms have evolved in a complex environment balancing toxicity, tenderizing ability, and evolution of immunity by mammalian prey items (Barlow et al., 2009; Mackessy, 2010).

Predictions made about shape and size change of trophic morphology due to defensive strikes were not supported. Figures 8 & 9 show the treatment by time means for each group. The treatment means did change between pre and post manipulations, but there was no statistical difference among any of the groups. Biologically, dorsal shape change did change more on the first axis (Relative Warp 1), which explains the most shape variation, while the lateral aspect

changed more along the second axis (Relative Warp 2). The pattern of group change between pre and post manipulation suggests that dorsal shape is affected by envenomation more than lateral shape, but again no statistical significance is present. Overall, there appears to be no morphological consequence of a series of defensive strikes in this species.

Although some estimates suggest venom glands comprise up to 15% of total head volume (McCue, 2006), to our knowledge there has been no study that has actually quantified this relationship. Works commonly cited for increases in venom gland size with increases head size (Forsman and Lindell, 1993; Forsman, 1994, 1996) did not measure venom gland size. It is likely that venom glands may impact trophic morphology, but the highly kinetic skull likely mask the ability to detect it. We do encourage future studies examining trophic morphology, and especially encourage field studies examining venom yields and venom gland composition in regards to seasonal and annual variation (see Willemse et al., 1979 and Williams and White, 1992). Snake trophic morphology provides an excellent area for future study, as we are just now starting to quantify some of the patterns that exist in nature (Smith and Collyer, 2008, Aubret and Shine, 2009).

We suggest the following avenues for future research and experimental design in studies on morphological and metabolic response to envenomation: First, continued work on the metabolic costs associated with venom production is needed. To date almost all of the work has been done in pit vipers or arachnids. The diversity of species studied should be increased. Second, future work should quantify specific time periods associated with venom production. Most of the previous work, including our own, concluded before the completion of the venom production cycle. Expanding metabolic measurements for 14-21 days post milking would provide a much more detailed picture of the metabolic patterns in organisms. Third, increased

sample sizes. Our experiment had the largest sample size to date, and we found no effect, therefore further investigations try to maximize sample size. Fourth, the types of treatments used can be expanded to incorporate molecular techniques (Currier et al., 2012) and compounds that may inhibit venom production, which may provide further insights on the metabolic costs and pathways of venom production (Luna et al., 2009). Lastly, field studies aimed at the ecological patterns and costs associated with venom and venom production provide an avenue that is difficult due to sample size and tracking issues, but one that desperately needs more empirical data.

In conclusion, we provide the first data that suggest that metabolic costs of venom production may be negligible. We also report that there is no significant response in trophic morphology to the envenomation process. Control of venom expenditure has abundant research opportunities and important implications for understanding the evolution of venom in snakes.

Acknowledgements

Our project would not have been possible without the aid of my coauthors; Jason Ortega, Dr. Steven Beaupre, and Joe Agugliaro. The design and execution of the project was a combination of all four of us contributing details and skills that the others did not possess, and the end product is much improved because of it. I also want to thank Drs. Mike Douglas, Michael Collyer, and Mark Davis for their input and comments during varying stages of this project. Lab-mates Craig Lind and Brenna Levine also provided may useful insights along the way. I thank my dissertation committee (Dr. Gary Huxel, Dr. Giovanni Petris, and Dr. William Etges) for their help and comments along the way. This work was approved by the University of Arkansas IACUC committee (Protocol #'s 12047 & 08042) and snakes were collected under scientific collection

permits from North Dakota Game and Fish (#GNF02388267 & GNF02599962) and the South Dakota Department of Game, Fish, and Parks (#37 & 51).

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FIGURES

FIGURE 1: Graph of pilot study data on the metabolic rate of *Crotalus viridis viridis*. Graph shows that snakes showed a small degree of metabolic depression, or chamber acclimation. CO_2 production exhibited a declining pattern the longer the snake remained in the chamber. For this reason, the first 24 hours were thrown out when we estimated RMR.

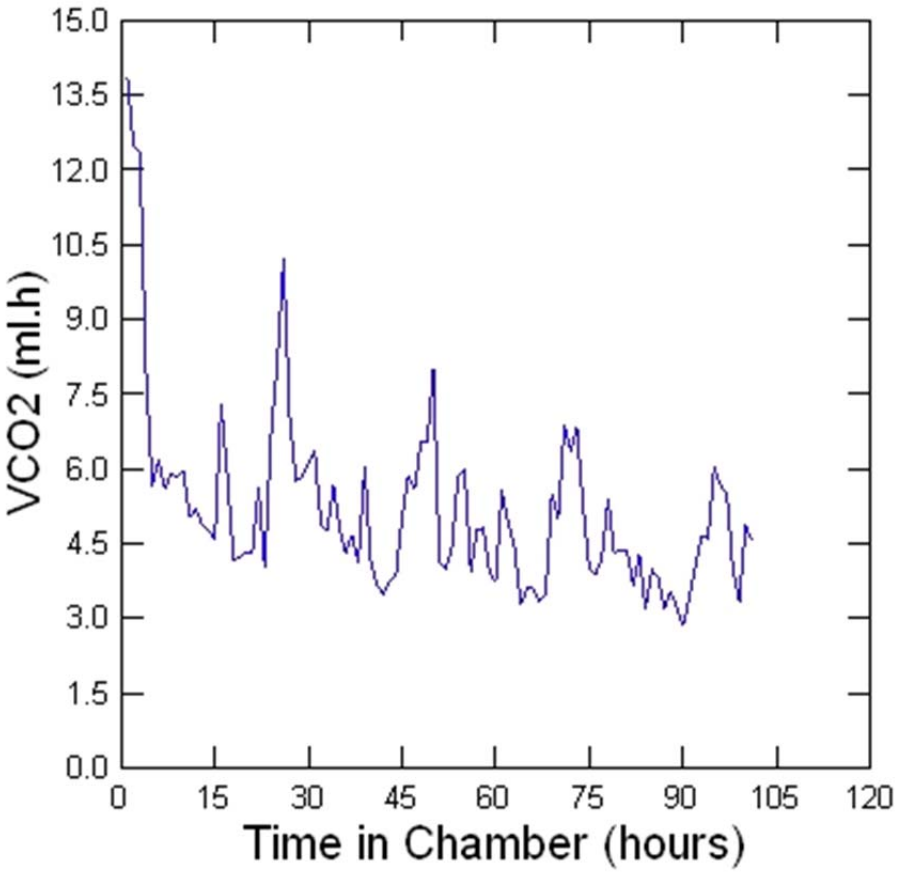


FIGURE 2: Graphs showing the predicted response in the cumulative difference in metabolic rate (CDMR) and the difference in metabolic rates (DMR) after milking. If there is a high cost associated with venom production, we should see primarily positive values when looking at the difference of post milk MR and RMR. Along those same lines, CDMR should show a steady increase until it plateaus as the snake returns to RMR levels.

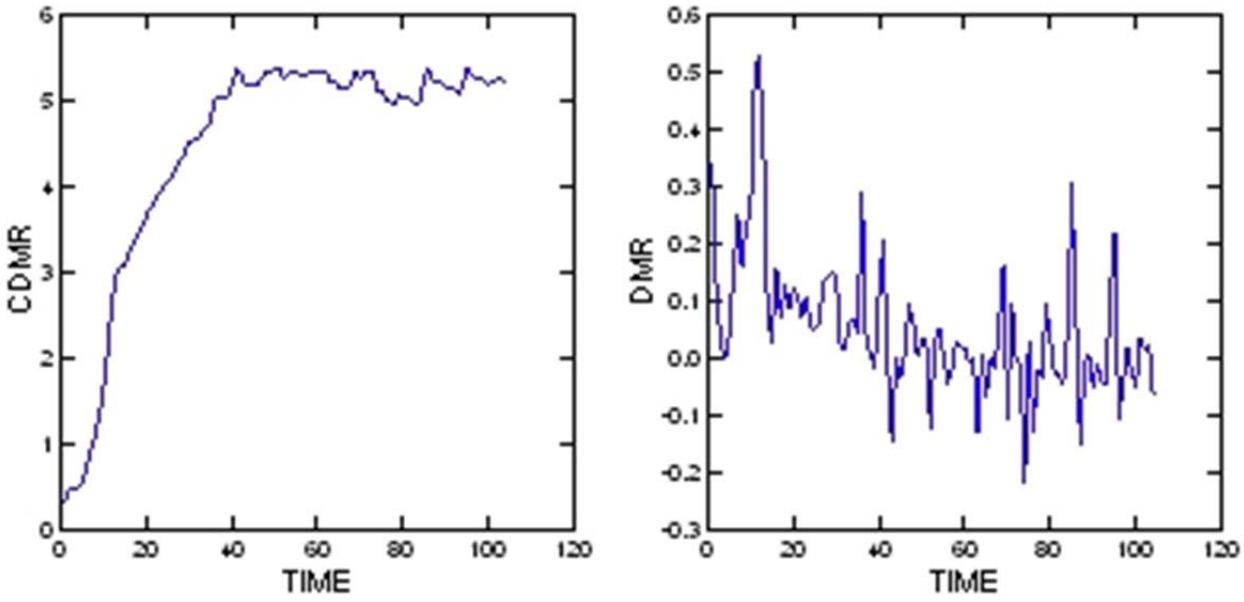


FIGURE 3: Average RMR (ml CO2/h) values for all snakes plotted against snake body mass (g). These data were used in determining the allometric relationship between metabolic rate and body mass for *Crotalus v. viridis*.

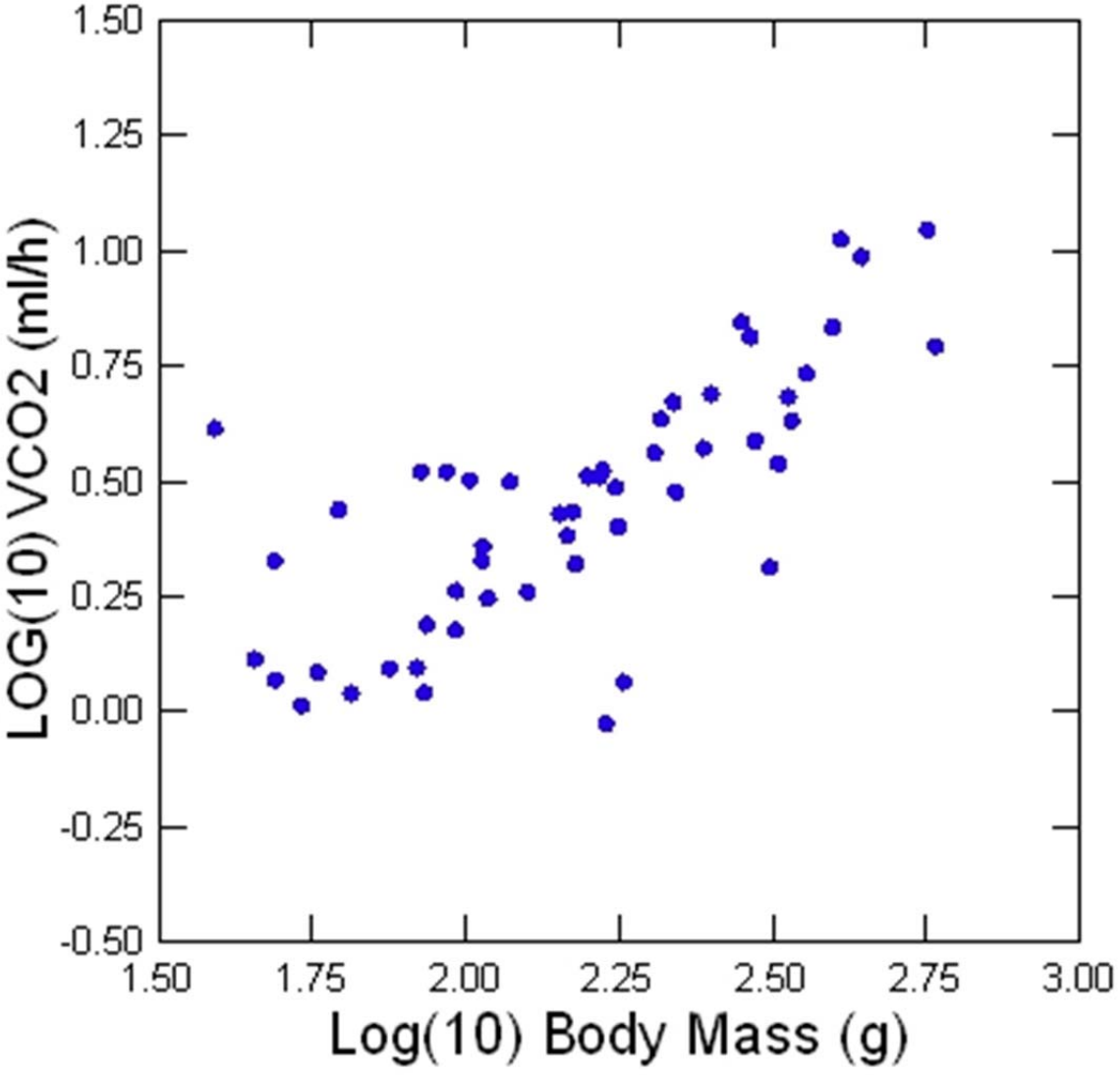


FIGURE 4: Graph of CDMR value over the 96 hours of post manipulation for both control and milked snakes. Lines represent treatment means at each of the 17 time points.

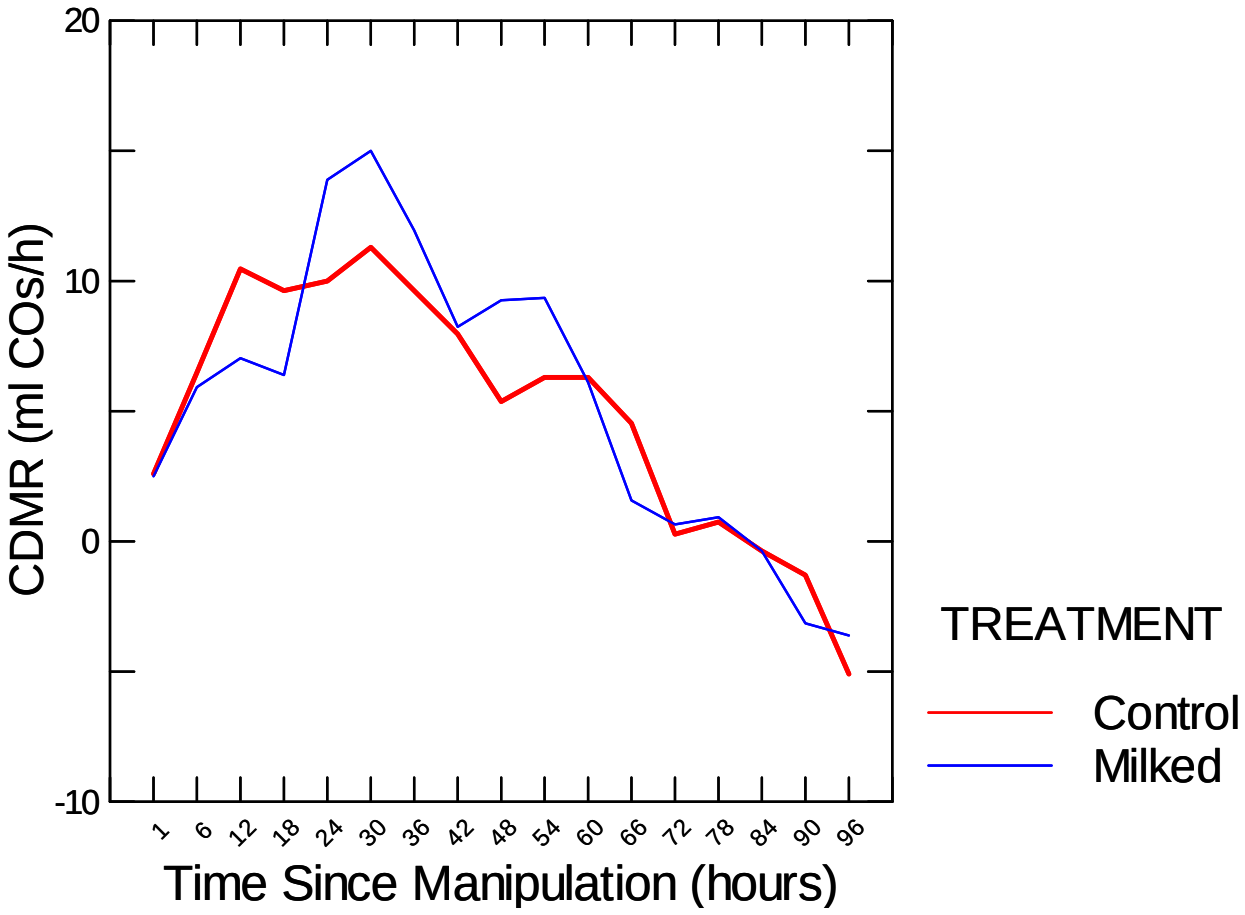


FIGURE 5: Mean values for the cumulative difference in metabolic rate (expressed as VCO2/h) for each treatment over the 96 hours of the experiment. Values are the same as in Figure 4, but with error bars of +/-1 SE added.

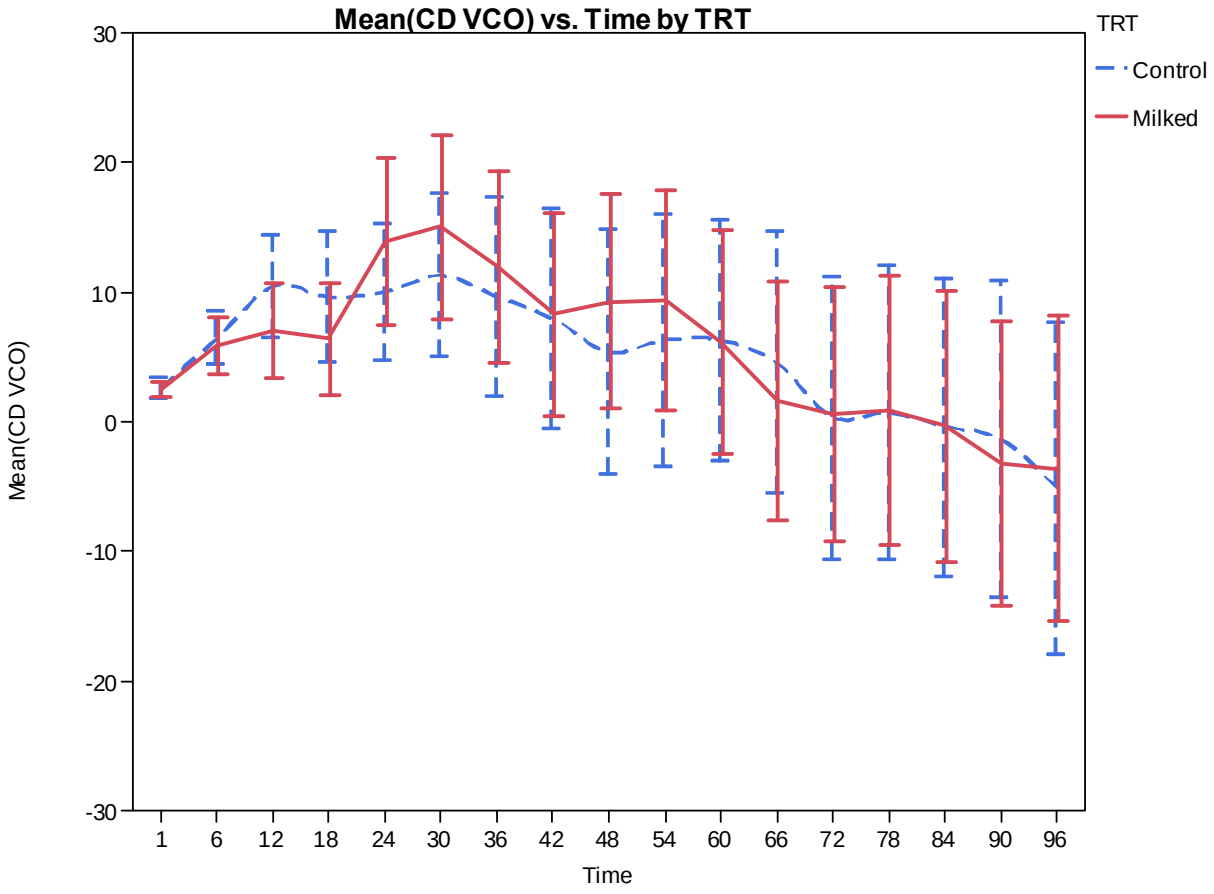


FIGURE 6: Visual representation of the landmarks placed on the dorsal aspect of snake trophic morphology. Landmarks are numbered in order of placement and correspond to TABLE 3.



FIGURE 7: Visual representation of landmarks placed on the lateral aspect of snake trophic morphology. Numbers correspond to order of placement and TABLE 4.

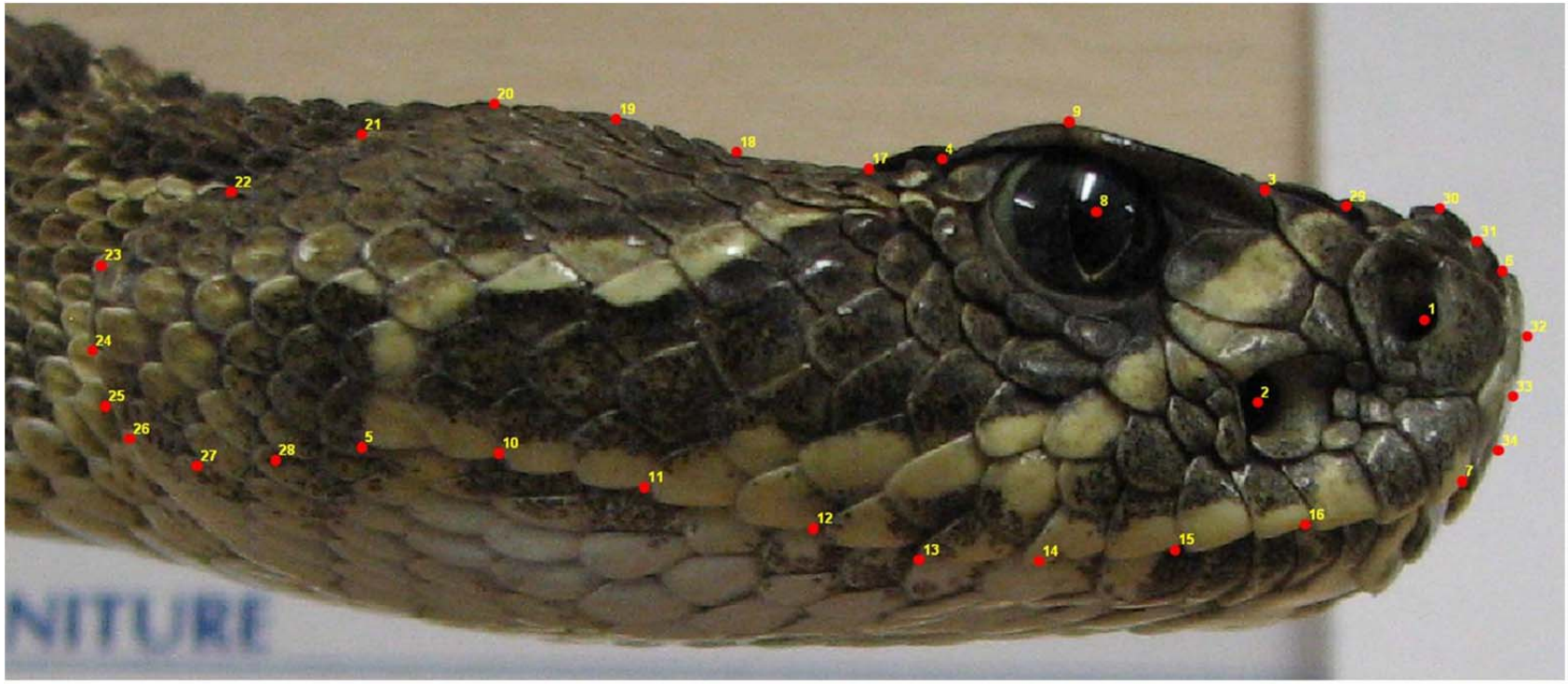


FIGURE 8: Relative warp plot for the dorsal aspect of snake trophic morphology. Plots are of the first two relative warp scores (LS Means) for each treatment and time combination. Control snakes pre-manipulation (Triangle), Control snakes, post manipulation (rectangle), milked snakes, pre manipulation (star), and milked snakes, post manipulation (circle). Graph shows how shape changes and that RW1 is associated with a narrowing of the head.

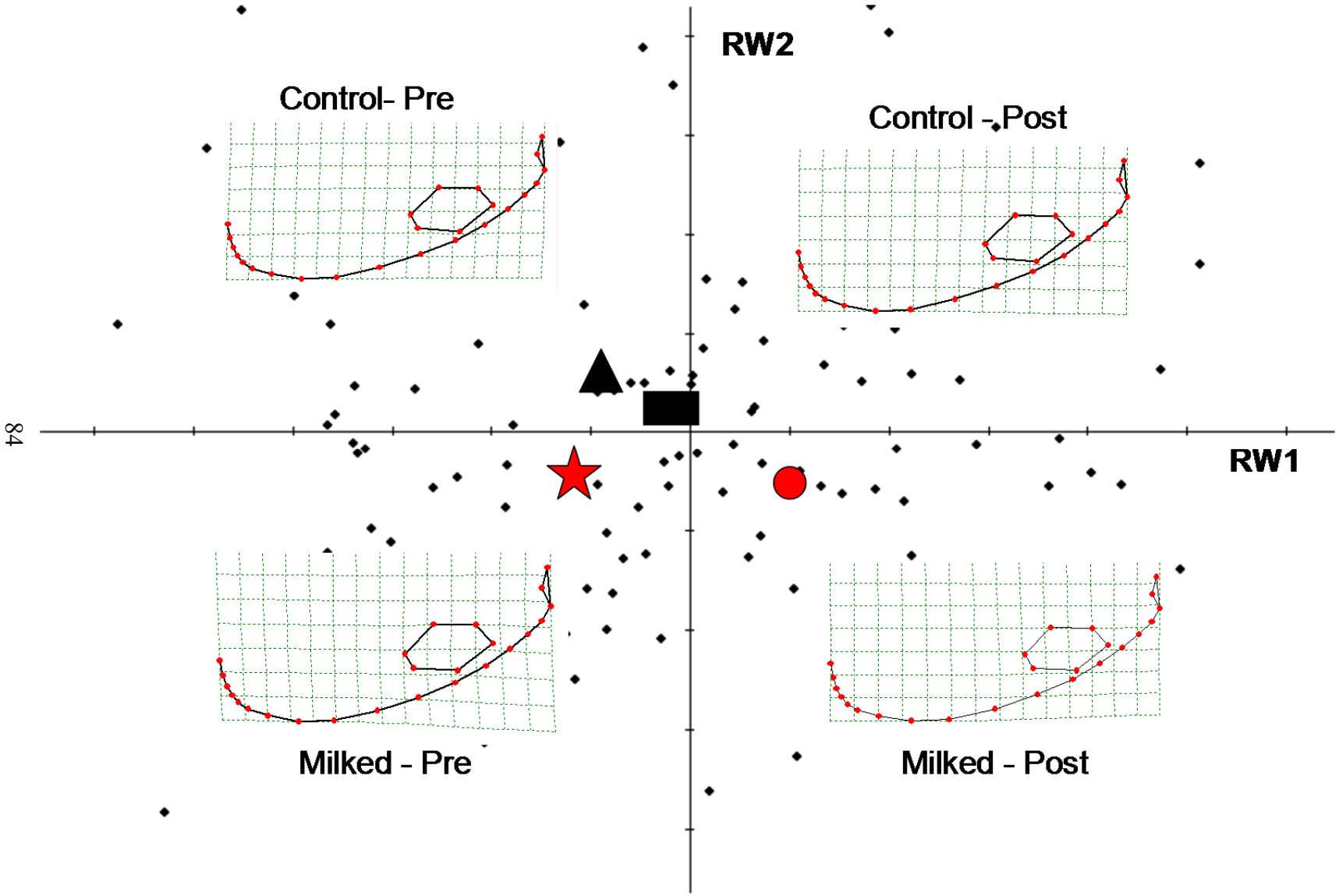
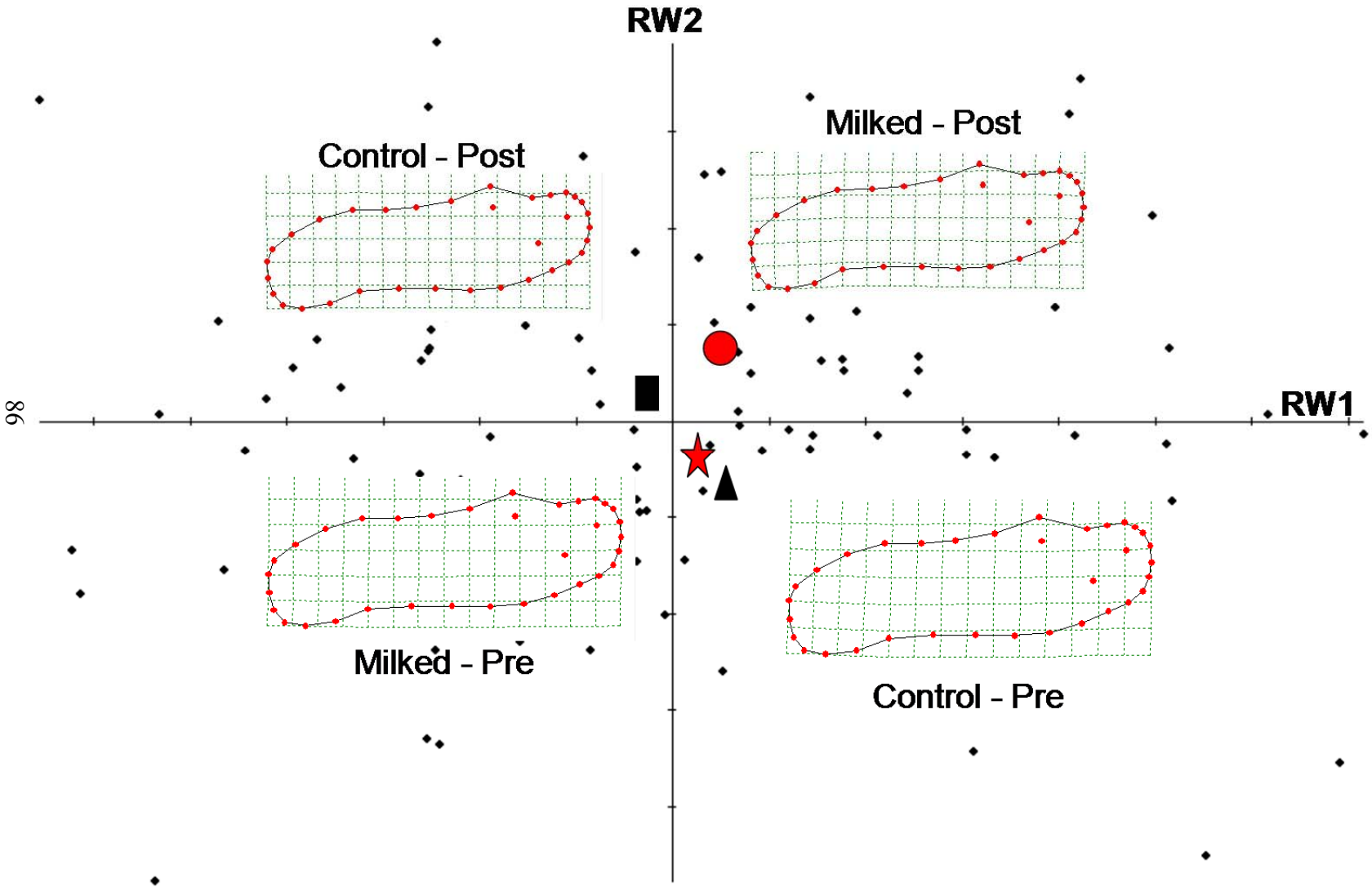

RW2
RW1
Control- Pre
Control - Post
Milked - Pre
Milked - Post
84

FIGURE 9: Plot of the first two relative warp scores for each TRT*Time group for the lateral aspect of snake trophic morphology. Deformation grids for each TRT*Time grouping are placed next to the markers for their LS Means. There was no significant difference in trophic morphology in the lateral aspect. Unlike the dorsal aspect of trophic morphology, the most visible difference in pre and post milking morphology was associated with the Y-axis (RW2). The second relative warp axis is associated with head depth.


RW2
RW1
98
Control - Post
Milked - Post
Milked - Pre
Control - Pre

TABLES

TABLE 1: Table summarizing the venom yields, venom wet mass, venom dry mass (assuming 70.9% water), and energy content (assuming 5.7 kJ/g) for all snakes milked in this study whose venom could be collected.

Snake	Mass (g)	Venom Injected (g)	Wet Mass (mg)	Dry Mass (mg)	Venom Protein (kJ)
273-2	39.19	0.0527	52.7	15.3357	0.087413
299-11	45.57	0.0981	98.1	28.5471	0.162718
299-4	49.05	0.1209	120.9	35.1819	0.200537
23	55.14	0.0636	63.6	18.5076	0.105493
291-6	56.9	0.1308	130.8	38.0628	0.216958
296-1	62.4	0.0874	87.4	25.4334	0.14497
60	65.42	0.1283	128.3	37.3353	0.212811
14	75.48	0.1446	144.6	42.0786	0.239848
4	83.53	0.1289	128.9	37.5099	0.213806
10	86.83	0.0966	96.6	28.1106	0.16023
63	93.46	0.107	107	31.137	0.177481
287-2	96.66	0.1352	135.2	39.3432	0.224256
44	108.64	0.0605	60.5	17.6055	0.100351
55	109.31	0.0921	92.1	26.8011	0.152766
289-9	147.28	0.2268	226.8	65.9988	0.376193
276-7	150.48	0.1815	181.5	52.8165	0.301054
298-5	151.82	0.192	192	55.872	0.31847
292-2	158.77	0.0819	81.9	23.8329	0.135848
276-18	165.98	0.2714	271.4	78.9774	0.450171
277	174.56	0.2281	228.1	66.3771	0.378349
276-16	179.8	0.2187	218.7	63.6417	0.362758
286	209.73	0.1454	145.4	42.3114	0.241175
268	233.21	0.074	74	21.534	0.122744
289	288.66	0.017	17	4.947	0.028198
278*	328.49	0.1937	193.7	56.3667	0.32129
278	332.84	0.2617	261.7	76.1547	0.434082
298	387.95	0.0266	26.6	7.7406	0.044121
282	401.89	0.1355	135.5	39.4305	0.224754
262	560.95	0.1961	196.1	57.0651	0.325271

TABLE 2: Tested covariance structures for the repeated measures ANCOVA for the metabolic rate dataset and their associated AIC values. Δ AIC values were used to choose the best fitting covariance structure.

Covariance Structure	Time Variable Structure	AIC	Δ AIC
Compound Symmetry	Continuous	8325.346	2068.796
Autoregressive	Continuous	6636.695	380.145
Autoregressive w/ Heterogeneous Variance	Continuous	6456.077	199.527
Continuous Autoregressive	Continuous	6633.695	377.145
Compound Symmetry	Factor/Categorical	8191.482	1934.932
Autoregressive	Factor/Categorical	6514.778	258.228
Autoregressive w/ Heterogeneous Variance	Factor/Categorical	6256.55	0
Continuous Autoregressive	Factor/Categorical	6514.778	258.228

TABLE 3: Detailed information about the landmarks used for geometric morphometric analysis of dorsal head shape.

Landmark	Type	Description
1	I	Posterior, medial point of the rostral scale
2	I	Left lateral point of the rostral scale
3	I	Right lateral point of the rostral scale
4	II	Inflection point of the head and neck (skeletal)
5	I	Caudolateral corner of the supraocular scale (where supraocular meets postocular [lateral])
6	I	Caudomedial point of supraocular and caudal border post-oculars
7	I	Craniolateral point of the supraocular (where supraocular meets the last canthal, laterally
8	I	Craniomedial point of articulation between the last canthal and the supraocular
9	II	Maximal lateral curvature of the supraocular
10	II	Maximal medial curvature (point) of the supraocular
11	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
12	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
13	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
14	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
15	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
16	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
17	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
18	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
19	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
20	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
21	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
22	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
23	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
24	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head
25	Sliding	Located between points 3 and 4, estimate the lateral curvature of the upper jaw and head

TABLE 4: Detailed information about the landmarks placed on the lateral aspect of trophic morphology for quantifying shape using geometric morphometrics.

Landmark	Type	Description
1	III	Middle of the nostril opening
2	III	Middle of the opening of the pit organ
3	I	Anterior point of the supraocular scale
4	I	Posterior point of the supraocular scale
5	I	Rictus of the jaw - where upper and lower jaw converge
6	I	Superior point of the rostral scale
7	I	Anterior/Inferior point of the 1st supralabial scale
8	III	Middle of the eye
9	II	Maximum curvature of the supraocular scale
10	Sliding	Series of points between landmark #5 and #7, tracing the upper jaw
11	Sliding	Series of points between landmark #5 and #7, tracing the upper jaw
12	Sliding	Series of points between landmark #5 and #7, tracing the upper jaw
13	Sliding	Series of points between landmark #5 and #7, tracing the upper jaw
14	Sliding	Series of points between landmark #5 and #7, tracing the upper jaw
15	Sliding	Series of points between landmark #5 and #7, tracing the upper jaw
16	Sliding	Series of points between landmark #5 and #7, tracing the upper jaw
17	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
18	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
19	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
20	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
21	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
22	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
23	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
24	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
25	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
26	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
27	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
28	Sliding	Series of points between landmark #4 and #5, tracing the posterior aspect of the head
29	Sliding	Series of points between #3 and #6, tracing the area anterior to the supraocular
30	Sliding	Series of points between #3 and #6, tracing the area anterior to the supraocular
31	Sliding	Series of points between #3 and #6, tracing the area anterior to the supraocular
32	Sliding	Series of points between #6 and #7, tracing the front of the head
33	Sliding	Series of points between #6 and #7, tracing the front of the head
34	Sliding	Series of points between #6 and #7, tracing the front of the head

CHAPTER 3: MORPHOLOICAL RESPONSE OF A GAPE-LIMITED PREDATOR TO EXTENDED PERIODS OF STARVATION

Abstract

The size and type of prey items a gape-limited predator can ingest is constrained by their trophic morphology. The ability of an organism to successfully capture and ingest prey items has direct consequences on their survival and fitness, therefore trophic morphology plays a vital role in the evolution of gape-limited species. Understanding the role of mass-energy allocations and developmental responses to food deprivation can provide inferences into the survival, reproductive fitness, and phenotypic plasticity of a population. Here we studied the response of a gape-limited predator to extended periods of starvation, and quantified changes in their trophic morphology. The responses of juvenile and adult Prairie rattlesnakes to periods of starvation lasting 100-200 days were very distinct. Adult snakes exhibited very little change in their trophic morphology or its allometric relationship with body size. Juvenile snakes showed a more pronounced change in their trophic morphology. Starved snakes had a significantly different allometric relationship between the size of their trophic morphology (head) and overall body size, exhibiting a decrease in head size while overall body size remained unchanged. The results suggest that extended periods of food deprivation result in smaller head sizes at any given body size. Starvation induced differences in head size could result in prey size limitations and fitness costs. Comparisons of adult and juvenile responses documented a significant difference in trophic morphology between the two groups, suggesting trophic morphology undergoes an ontogenetic shift as a snake grows.

Introduction

Very few, if any, natural populations are free from the impacts of resource limitation. The limited resource could be that of space, time (active season), food, or any combination. Populations have evolved in response to spatial and temporal variation in resource environments. Not surprisingly, many species/populations have evolved multiple strategies for dealing with such variation in resources such as life-history trade-offs (Wilbur et al., 1974; Stearns, 1976; Forsman and Lindell, 1991; Skúlason and Smith, 1995; Zera and Harshman, 2001), alterations of growth rates or activity patterns (Metcalfe and Monaghan, 2001), and changes in the mobilization of endogenous resources (McCue, 2010).

One of the extreme examples of resource limitation is starvation. Starvation refers to the biological condition in which a post-absorptive animal is able and willing to ingest prey items, but is unable to do so due to extrinsic limitations (McCue, 2010). Starvation is commonly considered to be a critical factor in mortality and determinant of growth in many species (Shan et al., 2009). The likelihood that a population will persist through periods of starvation not only depends on the food resource structure within their habitat, but also the species' ability to cope with food limitations (Navarro and Gutierrez, 1995; Metcalfe and Monaghan, 2001; Zera and Harshman, 2001; Mangel and Munch, 2005; McCue, 2010).

Starvation is commonly considered to be a significant factor in mortality and development (Wang et al., 2006; Shan et al., 2009) Periods of food deprivation/starvation can alter normal trajectories of development in organisms (Metcalfe and Monaghan, 2001). Limitation of energy during key points in development will impact all future life-history decisions, altering the physiology and morphology of individuals and the evolutionary trajectory of populations (Metcalfe and Monaghan, 2001). Understanding how organisms respond to

periods of starvation during various stages of ontogeny provides insights into the plasticity and evolution of developmental pathways and mechanisms (Sadeh et al., 2011).

Previous research has identified species groups that are well suited for surviving periods of prolonged starvation (Navarro and Gutierrez, 1995; Wang et al., 2006). Among those groups with the ability to endure long periods of starvation, snakes must be considered one of the most impressive. Snakes are among one of only a handful of taxa that can successfully tolerate periods of starvation lasting over a year (Klauber, 1956; Wang et al., 2006; McCue, 2007a 2007b; 2010, 2012).

Until recently, very little work on the mechanisms that snakes employ to survive extended periods of starvation had been conducted (McCue, 2007a). Reptiles in general exhibit a unique evolutionary history and should provide significant insights into the evolution and mechanisms of starvation tolerance (for review see: McCue, 2010 and McCue 2012). First, unlike many other reptile species, snakes are obligate carnivores, and are often at the top or near the top of their respective food chains (Beaupre and Montgomery, 2007; Beaupre and Douglas, 2009). Secondly, snakes are all gape-limited predators (Arnold, 1993). The size and shape of trophic morphology dictates what prey items a snake can successfully forage upon (Forsman, 1996a, b; Shine, 1991; 2002; Bonnet et al., 2000). Lastly, snakes often possess long generation times (Klauber, 1956; Ernst and Ernst, 2003). Longer-lived species face additional life-history decisions based on allocation between growth and reproduction that short-lived, semelparous species do not. Studying a long-lived group of organisms like snake, while posing some challenges, provides insights that are more applicable to higher vertebrates like mammals and even humans.

To date, the majority of snake starvation research has focused on starvation physiology. The metabolic mechanisms and pathways studied have elucidated several possible strategies for dealing with starvation (for reviews see McCue, 2010; 2012). Snakes have displayed multiple mechanisms for mobilizing endogenous resources such as lipids, proteins, and carbohydrates to meet their energetic demands during starvation (Hung et al., 1997; McCue, 2010). Snakes can also adaptively reduce the energetic requirements during periods of food limitation by suppressing their metabolic rate (McCue, 2007a). Snakes undergo dramatic changes in their relative body composition during starvation by sacrificing lipid stores to maintain a store of structural proteins (McCue, 2007a, b).

Physiological pathways are often temporarily altered during starvation (McCue, 2010). Morphological development may be impeded or altered by starvation and those alterations are often not reversible (Shan et al., 2009). While physiological pathways provide the mechanisms underlying morphological development, it is the morphology itself, the phenotype, on which selection will act. Investigations into the life-history evolution of morphology, phenotypic plasticity, and adaptive morphology all need to account for periods of starvation and how they may alter normal developmental trajectories.

The body of work on the effects of starvation on morphology and development is far less established. Studies have investigated morphological impacts of food limitation tend to focus on vertebrate groups that undergo metamorphosis (Shan et al., 2009). Morphological changes due to the onset starvation (Shan et al., 2009), duration of starvation (Sheen and Whiteman, 1998), and diet (Mittelbach et al., 1992; Mittelbach et al., 1999) have been demonstrated in both fish and amphibian species.

The current study examines the morphological impacts of extended periods of starvation on the trophic morphology and the allometric relationship between body size and trophic morphology of *Crotalus viridis viridis*. Previous research on snake trophic morphology has focused on how diet affects trophic morphology (Cammilleri and Shine, 1990; Queral-Regil and King, 1998; Aubret et al., 2004; Schuett et al., 2005), but a few have studied resource level (Forsman, 1996a; Bonnet et al., 2001; Krause et al., 2003). To date, we know of no study that has examined the effects of long or short term starvation on the trophic morphology of snakes. Our goal is to empirically determine if periods of starvation affect the allometric relationship between body size and trophic morphology and if snake trophic morphology has a detectable response to periods of starvation.

Methods and Materials

Study Species

Prairie rattlesnakes (*Crotalus v. viridis*) used in this study were captured from sites in western North and South Dakota in the summers of 2007 and 2008 (Smith, 2006) as part of a larger study on trophic morphology. All juvenile snakes used in this study were born in the laboratory, but the adults were wild-caught. All the juveniles used in this study were born in August or September in 2007. Juveniles were fed 25% of their body mass once a month until late November. Juveniles were then artificially hibernated until mid-April. Upon emergence from hibernation, snakes were offered three meals approximately two weeks apart prior to beginning the study. Adults were captured in 2007 and 2008. They were not hibernated, but kept at an active season temperature and light:dark photoperiod for their entire stay in the lab. Adults were offered a meal of thawed pre-killed rodents once a month, with meal size ranging from 15%-25% of their body mass. All females that had previously given birth to a litter in the lab were given extra-large meals and all

snakes (juveniles and adults) were deemed to be in good physiological condition prior to entering the study.

Snakes were maintained in plastic containers of varying sizes (14-31 liters), given water *ad libitum*. All containers were housed under a 12L:12D photoperiod at 24° C (+/- 1° C). Cages were cleaned as needed prior to the study, but during the course of the study cages were cleaned on the same day, regardless of treatment, in order to standardize disturbance patterns.

There was no overlap in body size or mass between the juveniles and adults at the onset of the study. The juvenile study included 46 individuals, randomly selected from a group of ~65 regularly feeding males and females. Although the snakes that entered the study were selected at random and randomly assigned a treatment, it should be noted that only juvenile snakes that were in good physiological condition and eating on a regular basis were sampled. Juvenile snakes ranged from 18 to 54.7 grams (mean = 36.64, SD = 7.85) in mass and 27.08 to 38.96 cm (mean = 34.0, SD = 2.63) in snout to vent length (SVL). The adult study contained 33 individuals (both sexes) that ranged in size from 162.56 to 628.72 grams in mass (mean = 312.0, SD = 113.62) and 59 to 97.9 cm in SVL (mean = 78.86, SD = 9.72).

Experimental Design

Snakes were randomly assigned to one of three treatments. Treatment one (Fed) consisted of snakes that were fed a meal that was 25% +/- 2% of their body mass every 25 days for 200 days. Treatment two (Starved) was the starved treatment; these snakes received only water *ad libitum* and no food for 200 days. Treatment three (Reversed) snakes were starved for the first 100 days, and then 'reversed'; receiving a meal that was 25% +/- 2% of their body mass every 25 days for the next 100 days. Sample sizes for the treatments were: Treatment 1 (16 juveniles, 11 adults), Treatment 2 (15, 11), and Treatment 3 (15, 11). One of the adults in the starved treatment

(Treatment 2) died on day 198 and was removed for all data analysis to keep sample sizes balanced among time points.

Snake morphology was measured on days 0, 100, and 200. Snakes were weighed to the nearest 0.01 grams and SVL was measured to the nearest 0.1 cm. Snout to vent length (SVL) in adults was measured by placing the snakes in a squeeze box (Quinn and Jones, 1974), but juveniles were photographed. Digital images of the ventral surface of juveniles also contained a ruler for scale. Digital images were measured three times each, and the average SVL was used. The three separate measurements rarely differed by over 1 cm (average SD over repeated measurements was 0.42 cm), which is around 1%-3% measurement error and falls within levels of error associated with squeeze box methods (Beaupre, Pers. Comm.).

Trophic morphology was quantified through the use of geometric morphometrics (GM). Digital images of the dorsal and right, lateral aspects of the snake's trophic morphology (head and neck) were taken at each time interval (Days 0, 100, 200). Digital images were taken of the dorsal and right lateral aspects of the head using a Cannon ® PowerShot S3 IS 6.0 mega pixel camera. All digital images were taken at specially built camera table setups (one for the dorsal and one for the lateral), that control camera and snake positioning and provided a number of rulers for scale bars (for more detailed description, see Smith, 2006).

GM Data Acquisition

Geometric morphometrics (GM) examines the relationship in landmarks or outlines of the specimens. Landmarks need to be: 1.) derived from anatomical reference points, 2.) homologous among the individuals in the sample, 3.) discernible, and 4.) able to be replicated over time (Bookstein, 1997). Landmarks, once placed on the digital images, are given X and Y Cartesian coordinates, and a landmark configuration is the set of all landmarks for a specific individual (or

group mean) as defined by their Cartesian coordinates. Landmark configurations contain all the variation due to shape, size, position, and orientation. Generalized Procrustes analysis (GPA) accounts for all non-shape variation (size, position, and orientation) (Rohlf and Slice, 1990).

Once centered and rotated, landmark configurations undergo thin-plate spline (TPS) analysis which describes the uniform shear and the bending energy of each landmark configuration. Thin-plate splines produce Principle Warps, which are eigenvectors of the bending energy matrix which describe all shape variation. Shape variables are calculated by combining the partial warps and the uniform shear (Bookstein, 1997). Shape variables are Euclidean, and therefore suitable for parametric statistics methods.

Head size was represented by centroid size (CS), which is calculated using GM. The centroid is the geometric center of gravity for the landmark configurations. Centroid size is calculated by:

$$CS\ (centroid\ size) = \sqrt{\sum d_i^2}$$

Centroid size is determined for *i* landmarks where d_i is estimated from subtracting each *X* and *Y* from their mean values. Centroid size was calculated for each snake, trophic aspect, and time period. Dorsal and lateral CS were used as estimates of trophic morphology size, and tested separately for their allometric relationship to SVL.

Statistical Analysis

Juveniles and adults were analyzed separately. We had different predictions about the response to starvation depending on size-class, therefore it is appropriate to analyze these datasets separately (so that one trend does not mask the other) and qualitatively compare the trends/patterns afterward.

Body mass, size (SVL) and head size were analyzed using repeated measures ANOVA/ANCOVA (Weerahandi, 2004). Data were Log_{10} transformed to achieve normality. Dorsal centroid size and lateral centroid size (DCS and LCS respectively) were compared among treatments using ANCOVA with SVL as a covariate. All non-shape variables were tested for differences among treatments and time periods (TRT and Day) and their interaction (Time*Day). For each repeated measures analysis, several variance-covariance (VCV) structures were tested and compared. The VCV structure (Zar, 1999; Weerahandi, 2004) was selected using information theoretic methods and model AIC values. If models were not greater than $\Delta 2$ AIC apart, the models were considered equally good and the VCV structure that most logically fit our design was used to report parameter values and significance levels.

The allometric relationships between head size (DCS and LCS) and body size (SVL) were examined using regression and ANCOVA methods. The Fed treatment acts as the control and therefore comparisons of Starved and Reversed treatment the Fed treatment provide information on deviation from normal development. We examined Days 100 and 200 separately, modeling head size with SVL as a covariate with TRT as a fixed effect and the interaction (SVL*TRT). We tested regression models for slope and intercept heterogeneity.

Likewise, we calculated absolute growth rate (GR) for each treatment as the change in SVL from one time period to another (0-100 & 100-200). Growth rates difference among treatments were compared by ANOVA methods. Analysis of growth rate data provides insight into the presence or absence of compensatory growth in the reversed treatment. Negative values of growth were assumed to be due to measurement error and factors associated with snake position and body condition, but not due to true ‘shrinking” (Luiselli, 2005).

Trophic morphology shape was analyzed using MANCOVA for each time period (Zar, 1999; Petris, Pers. Comm.). To test for significant differences in overall shape among treatments, shape variables (relative warp scores [RW]) were tested by using multivariate analysis of covariance. The appropriate centroid size was used as a covariate, while TRT and its interaction with the covariate were used as fixed effects. At each time period, the RW scores were subjected to a Principle Component Analysis (PCA) on the covariance matrix. Since RW scores were originally obtained from the dataset containing all time periods, a PCA will rotate each time period's data to best explain the variation present in the RW scores.

Dorsal and lateral head shape were not our primary concern, as the more biologically intriguing question is if the change in head shape differs among treatments. Change in shape between treatments can occur in one of two ways. First, the magnitude of shape change can differ between treatments. The magnitude of shape change was quantified using Procrustes distance (Bookstein, 1997; Zelditch et al., 2004). Each configuration had a Procrustes distance calculated by taking the square root of the sum of all the squared Procrustes residuals for that specimen. The Procrustes distance is the amount of deviation of a particular configuration from the mean (Figure 1).

Second, the disparity of head shape within a treatment can change. Disparity is a measure of the amount of variation in head shape a particular group has (Viguier, 2002; Zelditch et al., 2003). Disparity is calculated using Procrustes residuals, which are the squared sum of all the landmarks away from the mean of that landmark (Bookstein, 1997; Zelditch et al., 2003; Zelditch et al., 2004). Observed values of treatment disparity were calculated at each time interval, and then data were shuffled between rows. New disparity values are calculated for each randomization, 9999 iterations were run noting each time a value was greater than or equal to the

observed value (Manly, 1991). Disparity allowed us to investigate whether shape differences are due to one group having a more variable shape than another and also to investigate if there are any differences in variation that do not result in a difference in mean shape (see Figure 2).

All analyzes were performed using R version 2.14.1 (R Foundation for Statistical Computing, 2011) using the nlme library (Pinheiro and Bates, 2000). Additional testing, validating, and graphics were done using JMP versions 9.0.2 (SAS Institute Inc.), Excel 2003 (Microsoft Corporation) with the PopTools addin (Version 3.2, www.poptools.org), and Systat 13 version 13.00.05 (Systat Software Inc.). For all tests, a α of 0.05 was used to determine statistical significance. When multiple comparisons were made, a Bonferroni correction of α/n was used to determine statistical significance.

Results

After removal of the one fatality, treatment sample sizes were 11 Fed, 10 Starved, and 11 Reversed. There were 46 juvenile snakes used in this study, with sample sizes of 16, 15, and 15 respectively. Analysis of non-shape variables with repeated measures used the selected covariance structure (see Table 1 & 2) (Weerahandi, 2004).

Dorsal and right lateral landmarks were placed using TPS software (Rohlf, unpublished). Dorsal images had a series of 25 landmarks placed on each photo. Landmarks 1-10 were Type I or III, while11-25 were sliding semi-landmarks that estimated the curvature of the snake's head (Chp. 2: Figure 6 and Table 3). Lateral images had a total of 34 landmarks placed on each photo. Landmarks 1-9 were Type I or III landmarks, 10-16 were sliding semi-landmarks only the upper jaw, 17-28 were sliding semi-landmarks characterizing the posterior aspect of the head, and 29-34 were sliding semi-landmarks depicting the anterior aspect of the head (Chp. 2: Figure 7 and Table 4).

There were no significant differences among treatments for adult snakes in SVL, Mass, DCS, or LCS. Overall TRT*Day means are available in Table 3. SVL showed no significant difference among TRT ($F_{2,87}$=0.8, p = 0.9227), but was significant for Day ($F_{2,87}$=5.96, p= 0.0037). Adult body mass showed significantly different patterns of change among treatments. The selected model had significant values for Day ($F_{2,87}$ = 49.529, p <0.0001) and the interaction term (TRT*Day) ($F_{4,87}$ = 76.953, p <0.0001). Starved and Reversed snakes decreased in mass over the first 100 days. Starved snakes continued to lose mass until Day 200; in fact weight loss was much more dramatic from Day 100 to 200 than it was from Day 0 to 100. Reversed snakes rapidly began to add mass once feeding resumed at day 101 (Table 3, Figure 3). Fed snakes continually put on mass over the 200 days.

Head size showed distinctively different patterns between the dorsal and lateral aspect. Dorsal centroid size (DCS) changed significantly over time (Day) ($F_{2,86}$ = 7.64, p = 0.0009) and the covariate of SVL was significant ($F_{1,86}$ = 117.75, p <0.0001). The model for lateral centroid size (LCS) had only one significant parameter, the covariate SVL ($F_{1,86}$ = 158.75, p <0.0001).

Unlike adult snakes, juvenile snakes exhibited several significant treatment differences. Change in SVL was significantly affected by TRT ($F_{2,129}$ = 4.14, p = 0.018), Day ($F_{2,129}$ = 25.46, p <0.0001), and their interaction ($F_{4,129}$ = 4.13, p = 0.0035). As expected, Fed snakes increased in SVL throughout the duration of the study. Surprisingly, the most dramatic increase in SVL came from the Reversed snakes during the first 100 days when they were being starved (Figure 4, Table 4) By Day 200, there was a significant difference in SVL among treatments. The Starved snakes were significantly shorter than the Fed and Reversed (Table 4, Tukey HSD in Table 5) at Day 200, but not at any other time period. The initial period of growth in all treatments over the

first 100 days was surprising, but most likely due to the ample lipid and protein stores the snakes possessed since entering the study in good physiological condition (McCue, 2007a).

Mass also significantly differed for all modeled parameters (TRT: $F_{2,129} = 41.268$, $p < 0.0001$, Day :$F_{2,129} = 191.926$, $p < 0.0001$, and TRT*Day: $F_{4,129} = 124.429$, $p < 0.0001$). Fed snakes had an increase in mass throughout the study, starved snakes showed a fairly steady decrease in mass over the 200 days, and reversed snakes decreased the first 100 days and then increased the last 100 (Figure 5).

Dorsal centroid size (DCS) was significantly different among TRT ($F_{2,128} = 4.66$, $p = .0111$), Day ($F_{2,128} = 68.54$, $p < 0.0001$), and their interaction (TRT*Day; $F_{4,128} = 16.86$, $p < 0.0001$). The covariate of SVL was also significant ($F_{1,128} = 108.89$, $p < 0.0001$). All three treatments showed slight decreases in DCS from Day 0 to 100. From Days 100 to 200, after adjustment for SVL, Fed and Reversed snakes showed significant increases in DCS while starved snakes did not (Table 6). Lateral centroid size (LCS) did not show any significant differences among treatments or the interaction between Day and TRT. LCS did significantly vary over time (Day: $F_{2,128} = 19.38$, $p < 0.0001$) and with body size (SVL: $F_{1,128} = 65.15$, $p < 0.0001$).

We tested for the presence of compensatory growth (see Discussion) by comparing changes in SVL and Mass over the course of the study of Fed and Reversed snakes. We used permutation methods (Manly, 1991) to test for significant differences. Observed F-ratios were compared to ratios obtained from permutations, the data was shuffled for 999 iterations. Permuted values greater than or equal to our observed value were tabulated and *P*rand (p-value obtained through randomization/permutation methods) values determined significance.

Adults had no significant comparisons when testing for accelerated growth rates in Reverse treatment snakes. Reversed snakes did put on mass and body length during the fed portion of the study, but there was no evidence that the rates significantly higher than baseline. Juvenile snake comparisons yielded three significant contrasts with significantly higher F-ratio values than expected by chance (*Prand* = 0.009, 0.001, and 0.002 respectively). Reversed snakes exhibited rates of SVL and Mass increase from Day 100 to 200 that were 2-3 times higher than those in Fed snakes from Day 0 to 100 (GRS1 & GRM1: see Table 7). There was no difference in Reversed treatment rates when compared to rates of Fed snakes over the same time period.

More detailed examination of possible differences in the allometric relationship between head and body size yielded only a few significant results. Adult and juvenile allometric relationships were examined at each time period separately. Repeated measures suggested that the relationship between SVL and DCS might be significantly different in juveniles for at least one of the time periods. All age classes and time periods were tested to ensure no differences were missed. Slope heterogeneity tests suggested that there were significant differences in the relationship between DCS and SVL in juvenile snakes at Day 200 (TRT: $F_{2,40} = 10.864$, $p = 0.0017$; SVL*TRT: $F_{2,40} = 3.219$, $p = 0.05054$) and between LCS and SVL for adults at Day 100 (SVL*TRT: $F_{2,26} = 4.538$, $p = 0.0204$) (Figures 6 & 7).

We did a PCA on the first 17 relative warp scores (RW) before doing a MANCOVA on each age class and time period combination to test for shape differences with head size (centroid size) as a covariate. We used the first 17 RW instead of all available RW scores to eliminate the issues with running out of degrees of freedom. No set published method is available for determining the cutoff, so we used the criteria that a RW had to explain at least 0.5% of the variation (Collyer, Pers. Comm.). RW scores are projected from shape space into tangent space

via a PCA already, so that RW1 explains the largest proportion of shape variation. We used 17 RW in both the dorsal and lateral analysis, accounting for 95.6% and 96.28% of shape variation respectively.

Dorsal head shape showed no significant differences at any time period for both adults and juveniles. No shape differences existed at the onset of the study and it appears starvation (whether for 100 or 200 days) did not significantly alter head shape from control snakes. Lateral head shape was not significantly different among treatments at any time period. Neither juveniles nor adults showed any divergence in head shape during the course of the study.

Magnitude of shape change was quantified using Procrustes distance at two time intervals; change from Day 0 to 100 (Prodist1), and change from Day 100 to 200 (Prodist2). Repeated measures ANCOVA methods were used to test for differences among treatments. Adults showed no significant differences in magnitude of shape change among treatments or time periods (Table 8). Dorsal and lateral trophic aspects differed slightly in the pattern of Procrustes distance, but neither aspect had any significant trends (Figures 8 and 9).

Juvenile snakes showed no significant differences among treatments for the dorsal aspect, but there was a significant treatment affect for the lateral aspect (Treatment: $F_{2,85}$=4.9314, p = 0.0094). Starved snakes had a smaller magnitude of shape change from Day 0 to 100 when compared to Fed, although this difference was not significant once multiple comparisons was accounted for (Table 9). Figures 10 & 11 show the patterns of change over time for both the dorsal and lateral aspects of morphology in juvenile snakes.

Disparity in head shape throughout the study yielded only two significant comparisons. Only contrasts to control treatments (Fed snakes) were made, as comparisons between Reversed and Starved have little biological meaning. Juvenile snakes had a significant difference between

Fed and Reversed snakes at Day 100 for the lateral aspect (Obs. F-ratio [F/R] = 1.4615, *Prand* = 0.0471). Fed snakes had a significantly higher amount of variation in head shape than you would expect by chance, the ratio value of 1.4615 suggests Fed snakes had almost 50% more variation in shape than Reversed snakes. Adult snakes had a significant difference in the disparity between Fed and Starved snakes at the onset of the study (Obs. F-ration = 1.82, *Prand* = 0.0168), meaning the Fed treatment was more variable than the Starved treatment before the study began.

Discussion

Subjecting snakes or most any vertebrate to extended periods of starvation has many predictable outcomes. Our study documented predictable changes in SVL and body mass. Fed snakes showed an overall increase in both variables, while starved snakes generally decreased in mass and had little to no increase in body size (SVL). Starved juvenile snakes were significantly smaller than the other two treatments by day 200. There were also marked differences between adults and juveniles in their responses to starvation through SVL and mass. Adult snakes had very few significant treatment differences during 200 days of starvation.

Despite the fact that vastly different landmarks were placed without any fore-knowledge regarding how head shape and size might change over time, it remains difficult to explain why one aspect of trophic morphology (LCS) changes so divergently from the other (DCS). Unfortunately, there is no real way to statistically test for differences in the response of DCS and LCS, as they are derived from different data sets and their normal relationship with body size may significantly differ. Qualitatively though, each variable displayed a unique pattern of change over the course of the study. Perhaps different portions of the head have different developmental windows/periods and our study did not encompass both. One aspect of morphology may be more influenced by soft tissues, while the other aspect's shape is due to primarily skeletal components.

Research on environmental influences on trophic morphology has found differing responses to sets of linear measurements (Forsman, 1996a; Scudder-Davis and Burghardt, 1996; Bonnet et al., 2001). There are many possible explanations for the divergence in LCS and DCS, and provide ample avenues of future research.

We found no evidence to suggest that juvenile or adult snakes will increase growth rate in SVL or mass upon restoration of resource levels after a period of starvation. Change in mass from Days 100 to 200 in Reversed adult snakes exceeded that of Fed adult snakes, but the comparison was right at the 0.05 level ($p = 0.0558$) and was most likely a product of smaller adult sample sizes. Juvenile snakes in the reversed treatment showed a significantly higher rate of growth in SVL and Mass from Day 100 to 200 than Fed snakes during the first 100 days of the experiment. Snakes have shown differences in growth potential based on age/size (Dmi'el, 1967; Scudder-Davis and Burghardt, 1996; Beaupre et al., 1998), so differences in SVL between the time periods and treatments compared could confound any possible inferences about growth rate. Reversed snakes were not significantly different from Fed snakes at Day 200, so comparisons with growth rates of Fed snakes from that time period is most appropriate. Given the data, it is unlikely that any true compensatory growth occurred during the study.

Many groups of organisms display periods of compensatory growth (CG) after periods of food limitation (Metcalfe and Monaghan, 2001; Olsson and Shine, 2002; Ali et al., 2003; Mangel and Munch, 2005). Initially thought to be universally beneficial, many hidden costs of CG have more recently been uncovered (Metcalfe and Monaghan, 2001). There is often an initial increase in fitness and/or survival following CG, but many documented cases show trade-offs later in life with fecundity, longevity, reproductive fitness, and overall survival (Metcalfe and Monaghan, 2001; Olsson and Shine, 2002; Mangel and Munch, 2005; Stoks et al., 2006; Sadeh

et al., 2011). Trade-offs between growth, storage, and reproduction are common among snakes and other organisms (Stearns, 1976; Reznick, 1985; Forsman and Lindell, 1991). Snake body size influences locomotion, endurance, feeding capacity, fecundity, mating success, energetic demands, and ability to withstand food shortages (Forsman, 1993). The balance of costs and benefits associated with increased growth rate and mass-energy trade-offs depend on relative abundance of prey items, fluctuations in resources, size-based predation and other causes of mortality, and environmental variation.

Overall, juvenile snakes that undergo extended periods of starvation should exhibit CG once resources levels increase if: 1) survival increases with body size, 2) reproductive fecundity is correlated to body size, 3) increased body size increases prey base, or 4) there are reliable environmental cues to future resource levels and/or periods of starvation are infrequent. Conversely, CG would not be expected and/or beneficial if: 1). Survival did not increase with body size and/or predation risk increased with body size, 2) the maintenance energetic demands of increased size conveyed a cost at current resource levels, or 3) there was a high degree of environmental variation in resource level and/or little to no reliable cues.

The lack of definitive proof for compensatory growth in our study could be due to several factors. First, snakes in the Reversed treatment were only starved for 100 days, this period may not have been sufficient enough to promote an increase in growth rate once feeding resumed. Second, these populations may have evolved under conditions that do not favor CG. Future work should examine longer periods of starvation, population and size specific survival rates, and environmental factors influence the presence/absence of compensatory growth.

Specific tests examining the allometric relationship between DCS and LCS with SVL provided only two points that demonstrated statistical differences among treatments. In both

cases Starved snakes had significantly shallower slopes than Fed snakes. In juvenile snakes at Day 200, DCS vs. SVL regression lines showed a significant difference in the slopes of the allometric relationship, with Starved snakes having shallower slopes than the other two treatments, but a significantly higher intercept. In adult snakes at Day 100, regression lines showed Fed snakes had a higher slope than the other treatments, but there were no differences in intercept values. Given the smaller adult sample sizes and the inherent large effect of any outliers, an ephemeral difference in the allometric relationship in adults may be a spurious outcome. Juvenile sample sizes were larger and snakes were initially less variable so the significant difference at Day 200 most likely represents an alteration of mass-energy allocation due to starvation.

While this study was able to document changes in allometric relationship over time and treatment, there is not enough clear evidence to describe a predictable pattern due to starvation. The experimental design may have used too large of time periods, being unable to capture changes that occurred at smaller steps between measurements. Future work should use smaller time steps and attempt to elucidate the exact duration of food deprivation that triggers a change in the allometric relationship. We can conclude that snakes did alter allometric relationships over time, so allocation decisions between head size and body size are made.

Gape-limited predators, snakes in particular, provide a model organism for the study of allometry. Both body size and head size have significant impacts on survival and overall fitness (Arnold, 1983; Forsman, 1994). Juvenile snakes decreased their head size relative to their SVL during an extended period of starvation. Stress, like extended periods of resource limitation, can have dramatic effects on trophic morphology and symmetry (Badyaev, 1998; Badyaev and Foresman, 2000; Badyaev et al., 2005; Young and Badyaev, 2007). Development during periods

of increased stress resulted in trade-offs between overall size and canine symmetry in grizzly bears (Badyaev, 1998), and morphological variation in shrew mandibles (Badyaev and Foresman, 2000). Extended periods of starvation may be stressful enough to alter allocation decisions in snakes. If the allometric relationship documented at day 200 in our study persists, snakes exposed to extended periods of starvation early in their development will be more constrained in the type and size of prey items they can ingest. Possible increased prey limitations would have dramatic effects on the overall fitness of those individuals. Future research should examine if the change in the allometric relationship is reversible and impacts on gape size during adulthood.

Shape analyzes provided only a few significant differences, and no consistent patterns. Overall, it appears starvation did not affect the shape of trophic morphology, the magnitude of shape change, or the amount of shape variation present in the treatments. Examining the deformation grids and relative warp plots (Figures 12-17) provides a clearer picture on how shape changed throughout the study.

It appears that shape does not show a large degree of variation across any comparison. The most interesting observation is that juveniles and adults are consistently separated on the relative warp plots (Figures 14 & 17). The large degree of separation between adults and juveniles suggests some sort of ontogenetic shift in trophic morphology. Using the figures to make some generalizations about trophic morphology, it appears adult snakes have a distinct change in snout morphology in the dorsal aspect, along with the broadest portion of the head moving slightly posterior towards the quadrate bone. On the lateral aspect, there was less of a distinct change, but adults were characterized by more robust snout and ocular areas, as well as slight changes in the posterior aspect.

There has been limited work on ontogenetic shifts in trophic morphology in snakes (but see Vincent et al., 2004), and even less using landmark based geometric morphometrics. It is clear that more work needs to be done in this area before we can fully understand how resource level, prey base, ecology, and body size all merge together to influence the all-important trophic morphology in snakes. Changes in trophic morphology of gape-limited predators have important ecological and evolutionary influences (Arnold, 1983; Shine, 1991; Forsman, 1993; 1994; 1996a; Schuett et al., 2005). A better understanding of how trophic morphology changes over an individual's lifetime could provide a great deal of insight into population ecology (Schuett et al., 2005). Further investigations into the magnitude of shape change, its ontogenetic patterns, and relationship to resource availability would provide insights into the possible mass-energy trade-offs, resilience, and development of trophic morphology

Beyond responses of SVL and Mass, to our knowledge no one else has specifically looked at the effects of starvation on trophic morphology and its relationship with body size. Traditionally most morphological studies on the effects of starvation have been limited to species with multiple life stages, i.e. those species that undergo metamorphosis (see Introduction). Other work on the relationship between head size and body size has focused on high and low resource populations/years, not specifically on extended periods of starvation (Forsman and Lindell, 1991, 1997; Forsman, 1993, 1996a).

Since trophic morphology in gape-limited predators is rarely associated with any task other than prey acquisition and ingestion (Shine, 1991), any theory on allocation decisions associated with trophic morphology and its relationship to body size should be based on survival and feeding performance (Diller and Wallace, 2002). The costs of continued growth at the expense of physiological condition should be offset by an increase in prey base or survival

(Forsman, 1993; Forsman and Lindell, 1997; Diller and Wallace, 2002; Vincent et al., 2006; Vincent et al., 2009). Unlike determinate growers, reptiles have no true asymptote of growth, but rather growth slows as size increases (indeterminate growth) (Sebens, 1987). The significance of indeterminate growth is that allocation decisions between growth and reproduction are continuous (Forsman and Lindell, 1991). Body size of has important influences on survival (Forsman, 1993; 1996a; Forsman and Lindell, 1997), reproduction (Dmi'el, 1967; Shine, 1991; Lourdais et al., 2002), and net energy gain (Forsman and Lindell, 1991; Forsman, 1996b). Any significant alterations in trophic morphology and/or its relationship to body size should have both ecological and evolutionary implications for gape-limited predators, especially those with indeterminate growth.

Previous research does suggest a predicted divergence in the response to starvation between mature and juvenile organisms. Snakes vary in their growth rates (Beaupre et al., 1998) and growth efficiency over time (Scudder-Davis and Burghardt, 1996); and survival in snakes is often associated with body sizes (Forsman, 1993). Newborn and juvenile snakes are most likely susceptible to a larger number of predators than adults (Diller and Wallace, 2002; Smith, Pers. Obs.) The increased chance of predation in juvenile snakes suggests that rapid growth would be beneficial (Forsman, 1994; Webb et al., 2003). Increased size also increases the diversity of prey items that an individual can consume (Forsman, 1993; Diller and Wallace, 2002). Qualitatively our study supports the theory that overall body size will influence how they will respond to that period. Adults showed very few significant treatment differences in any measured variable; while juveniles showed not only greater magnitude of change in almost all the variables, but also had more significant treatment differences and a clear response to starvation.

Several factors of our design could have influenced the results. First, the juvenile snakes were selected from a subset of snakes that were consistently feeding. The selection of snakes violates the assumption of a random sample of the population. While it is necessary to use snakes that are healthy and feeding in most studies, it could be argued that the responses shown here are an artifact of the snakes selected and not to starvation. Second, these populations were specifically chosen to investigate influences on trophic morphology because of previous research. Prior studies suggest that these populations display significant temporal and spatial variation in trophic morphology (Smith, 2006; Smith and Collyer, 2008). Third, laboratory conditions may have precluded behaviors that may have mediated some of the effects of starvation (thermal regime, foraging behavior, etc.). Lastly, a majority of the adult snakes used in this study were not captured for the sole intent of this project, but because they were pregnant at the time of capture. Most of the adults used in the study (~60%) had given birth to litters in the lab 2-3 years prior to conducting the adult portion of this study. While there was ample time for females to recuperate from giving birth and all snakes were in good physiological condition prior to the start of the study; there remains that chance that female snakes that have already successfully reproduced would respond differently than males or females that had yet to reproduce.

In conclusion, extended periods of starvation can significantly alter the relationship between trophic morphology and body size in a gape-limited predator. Starved snakes exhibited smaller increases in dorsal and lateral head size (metrics for trophic morphology size) than in overall body size (SVL). If this pattern is irreversible, snakes exposed to early periods of resource deprivation should have increased restrictions on the size and type of prey items they can ingest. These restrictions could have life-long fitness impacts and result in decreased

reproduction, smaller adult body size, delayed maturation, or decreased fitness. Differences in adult and juvenile response to starvation may reflect ecological differences in the costs and benefits associated with increased body size and mass-energy allocation decisions

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Acknowledgements

I thank Dr. Steve Beaupre for his insights and input into this project as well as the logistic support he offered. I thank my dissertation committee: Drs. William Etges, Gary Huxel, and Giovanni Petris for their input and direction through the course of my degree. This project would not have been possible without the input from fellow lab members: Jason Ortega, Joe Agugliaro, James Van Dyke, Marshal McCue, and Lara Douglas. Suggestions on experimental design, setup, and data analysis from Drs. Mark Davis and Michael Collyer were instrumental in the completion of this project. This project was conducted with the approval of the University of Arkansas IACUC committee (Protocol # 08042) and the primary author was supported by a Walton Distinguished Academic Fellowship and Graduate Teaching Assistantship from the University of Arkansas. All snakes were obtained under the proper permits from both the North Dakota Game and Fish (#GNF02388267 & GNF02599962) and the South Dakota Department of Game, Fish, and Parks (#37 & 51).

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FIGURES

FIGURE 1: A visual representation of Procrustes distance and the equation used to calculate it for each individual. Procrustes distance was used to quantify the magnitude of shape change.

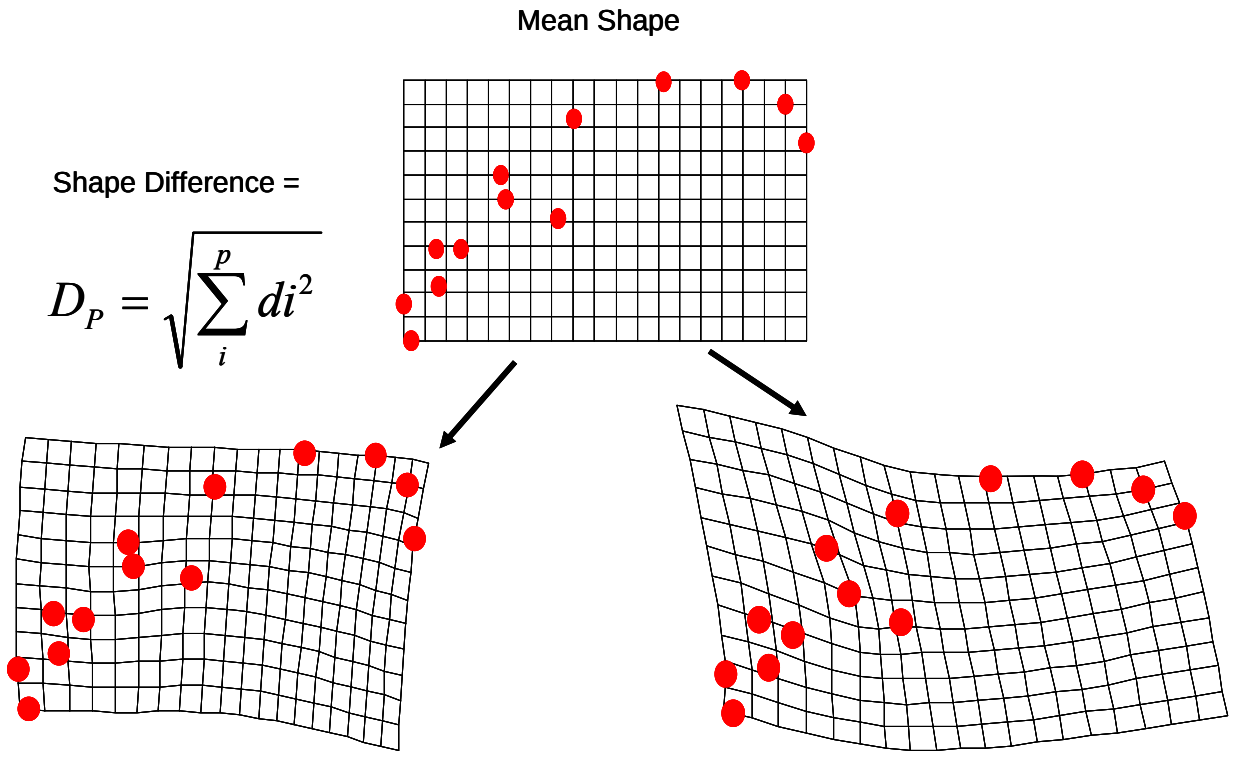


FIGURE 2: Graphical representation of the within group disparity in head shape. In this example, Fed snakes had a smaller amount of shape variation on Day 0. Disparity measures provide more insight into patterns of morphology and possible morphological changes that may not be statistically significant, but still provide some biologically relevant information about shape.

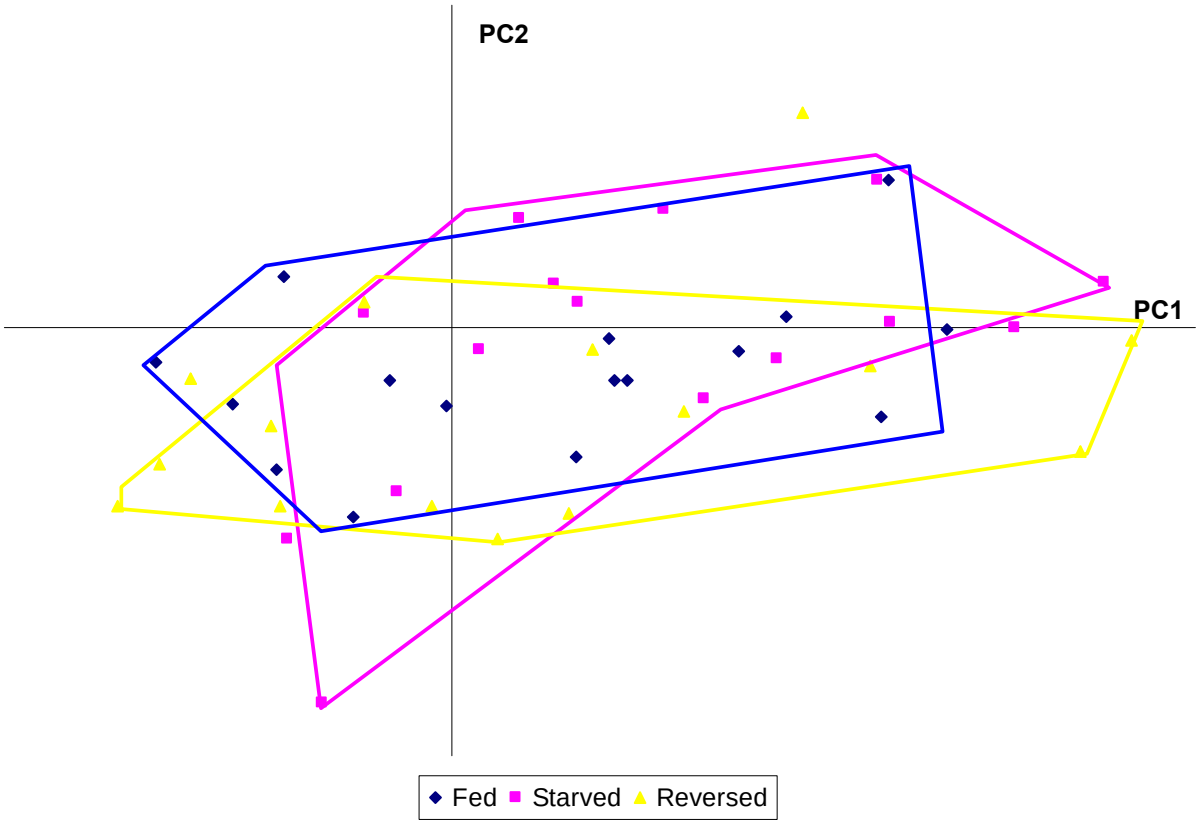


FIGURE 3: Average values of body mass (LogMass) for adult snakes over the course of the study. Treatments were never significantly different, but mean SVL did change over time and the pattern of changes was significantly difference among treatments. Mean value is plotted at each time point and error bars represent 95% confidence intervals.

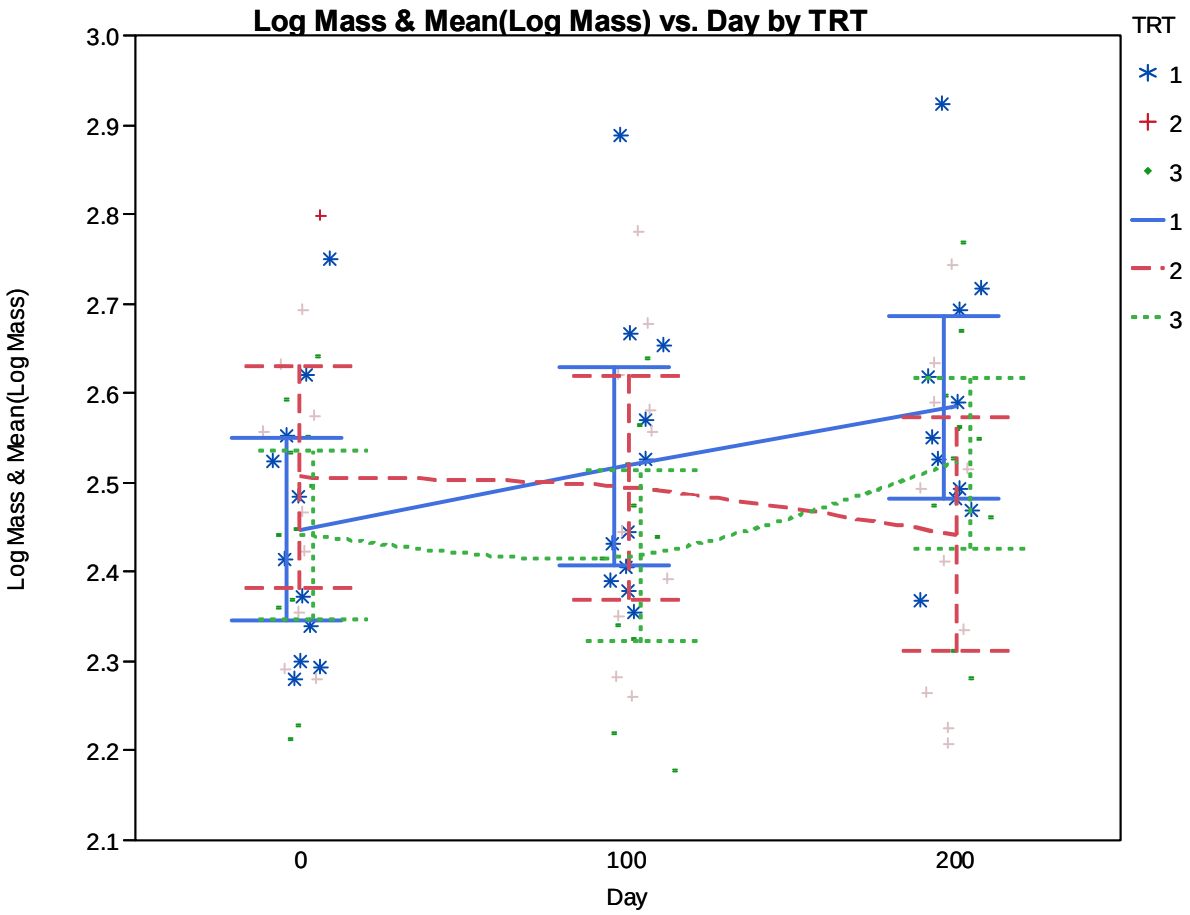


FIGURE 4: Graph of mean treatment SVL for juvenile snakes over the course of the study. Fed (1) and Reversed (3) treatments were significantly larger (in SVL) than starved (3) snakes by Day 200. Error bars represent 95% C.I..

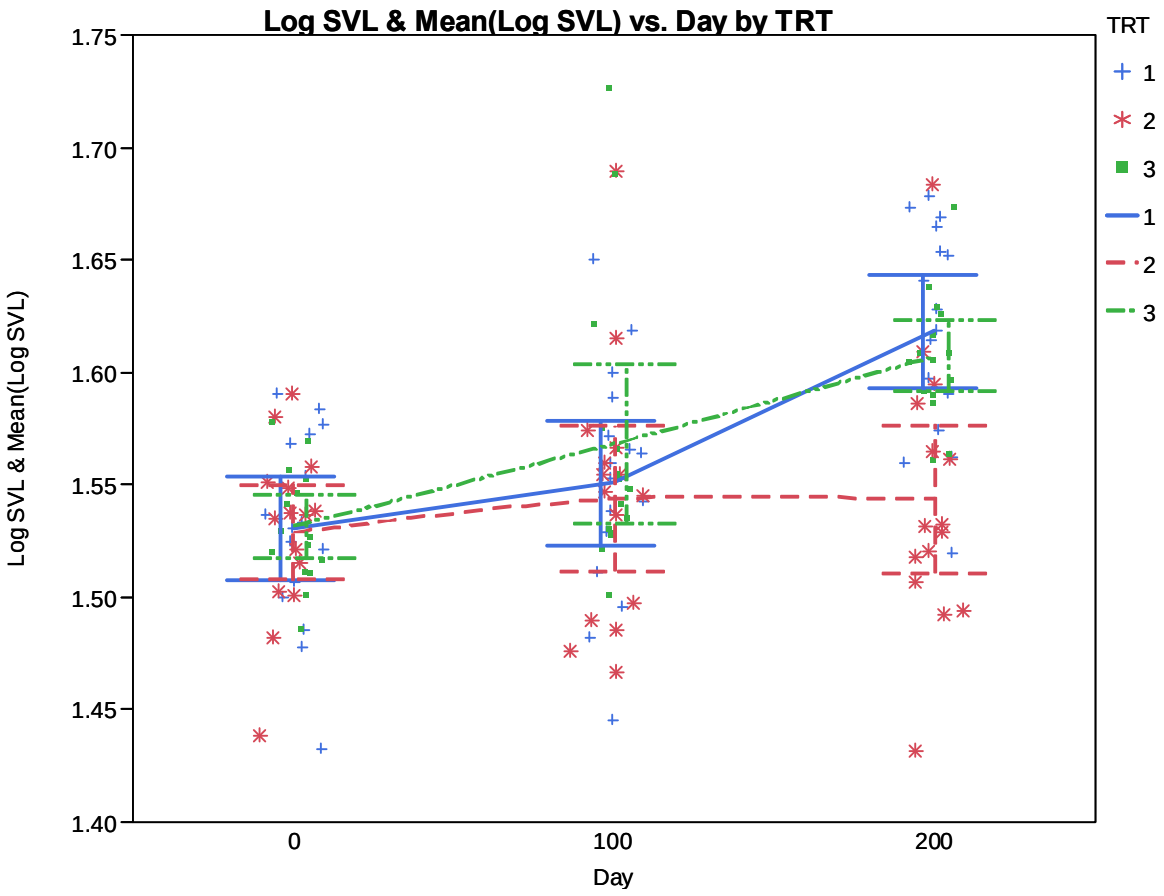


FIGURE 5: Plot of treatment average values for body mass (LogMass) over the course of the study. Treatment means are graphed along the line with 95% C.I. presented by error bars. At Day 200, starved snakes (2) weighed significantly less than Fed (1) and Reversed (3) treatment snakes.

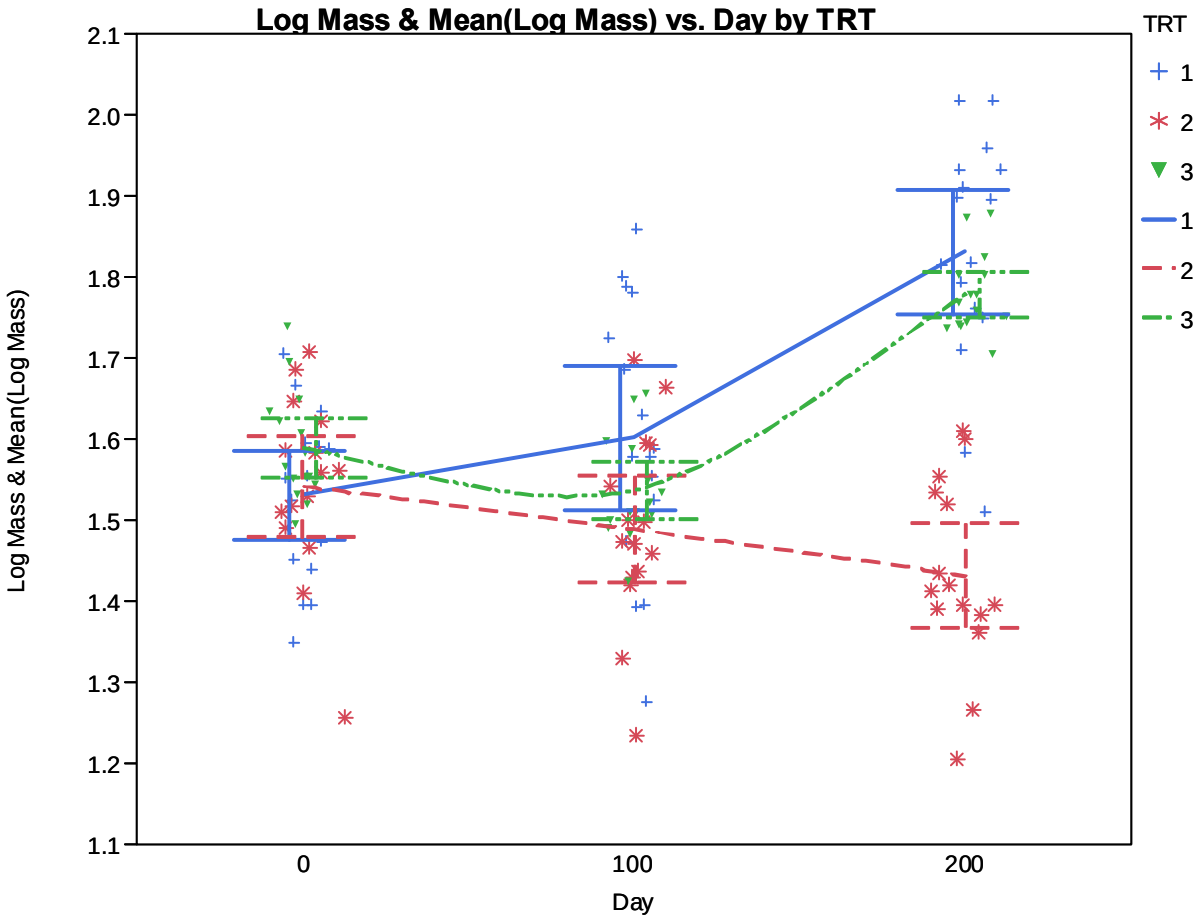


FIGURE 6: Plot of the separate regression lines depicting the allometric relationship between body size (LogSVL) and dorsal centroid size (LogDCS) in juvenile snakes at Day 200 of the study. Starved snakes had a significantly smaller value for the slope of the regression line, indicating a significant alteration to the allometric relationship in starved snakes.

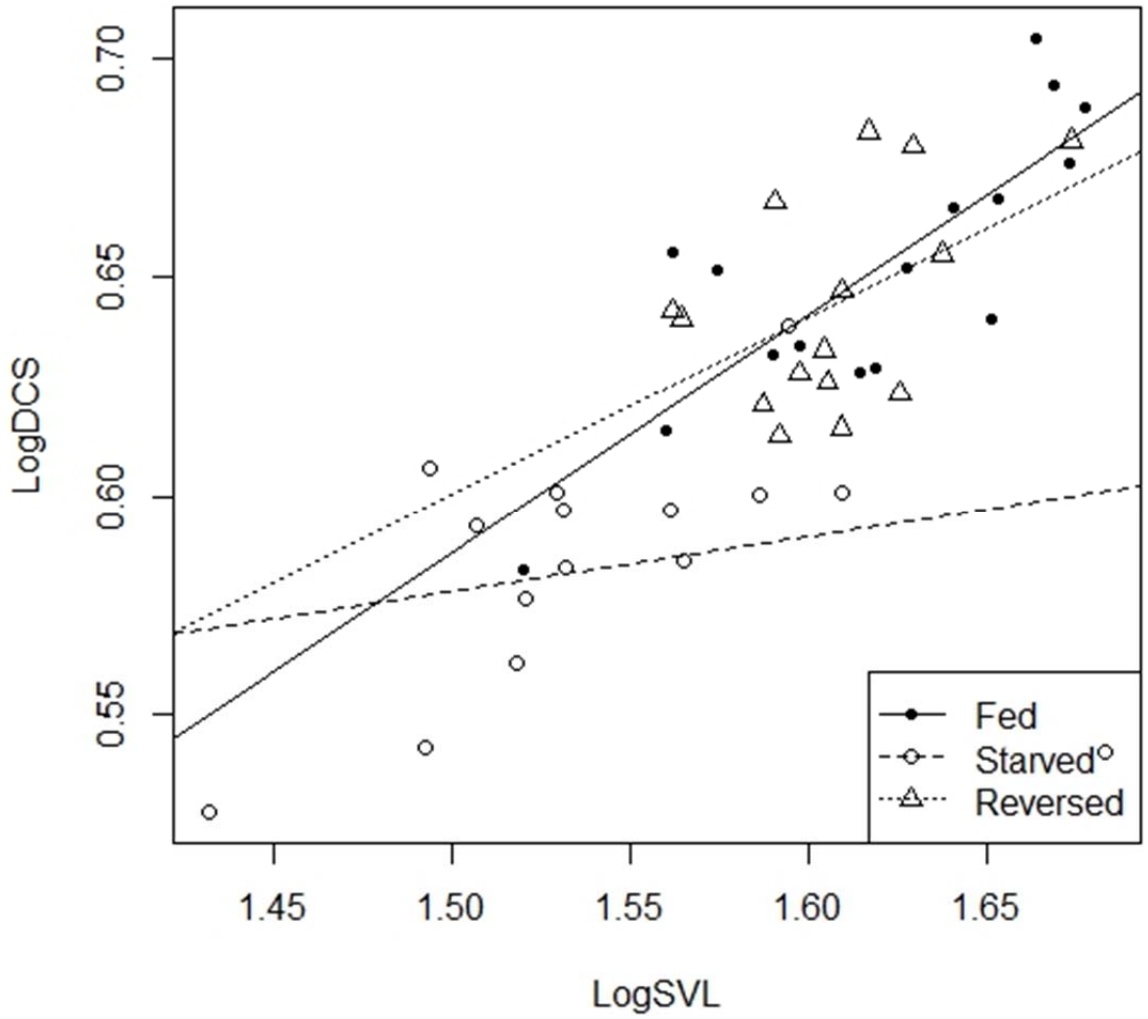


FIGURE 7: Plot of the allometric relationship between body size (LogSVL) and lateral centroid size (LogLCS) for adult snakes at Day 100. Fed snakes had a significantly higher value for the slope of their regression line, but this significant difference was only present at this one time interval.

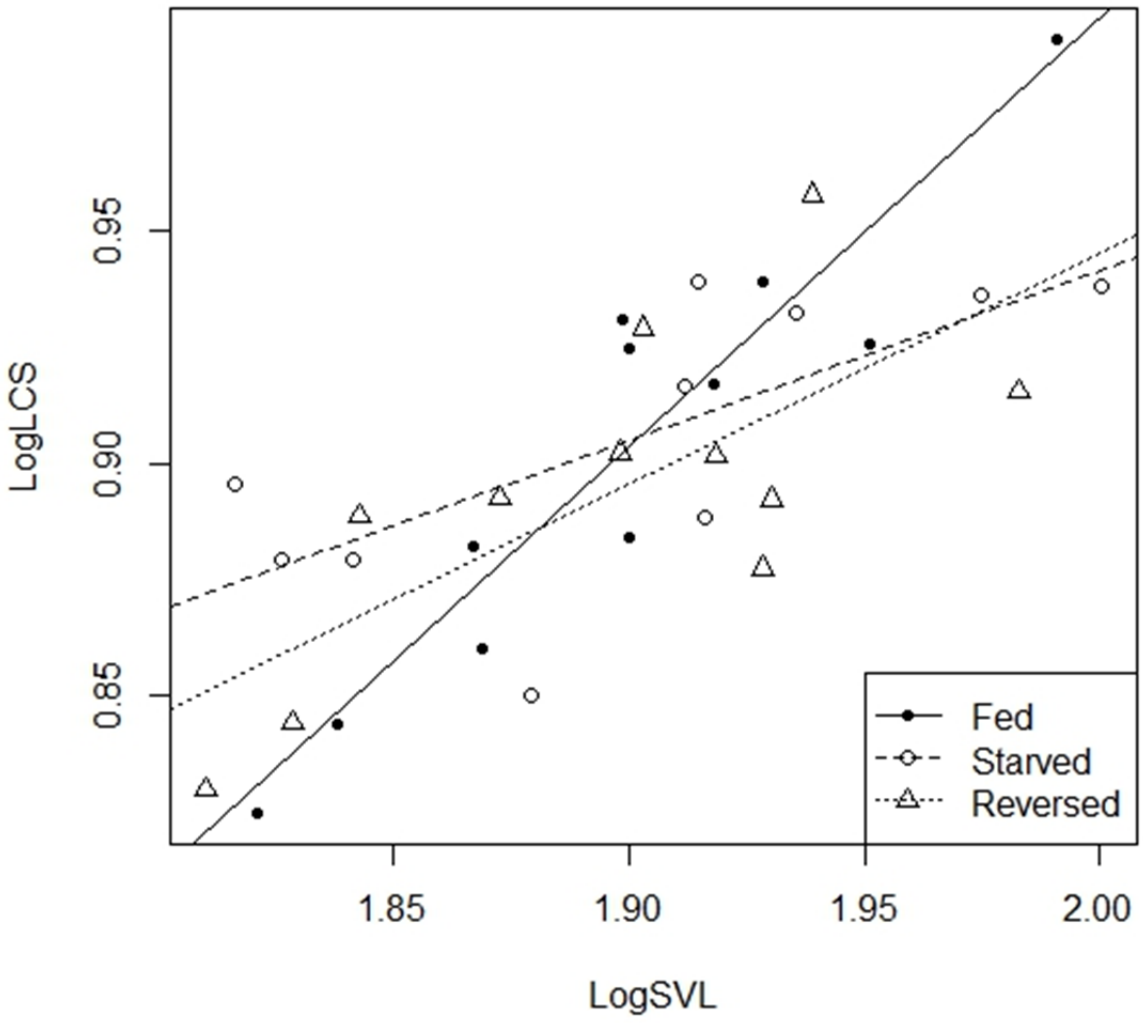


FIGURE 8: Graph of Procrustes Distance over time for the dorsal aspect of trophic morphology for adult snakes. The magnitude of shape change was quantified using Procrustes Distance for configurations from Day 0 to 100 (Day 0) and from Day 100 to 200 (Day 1). Error bars represent 95% C.I..

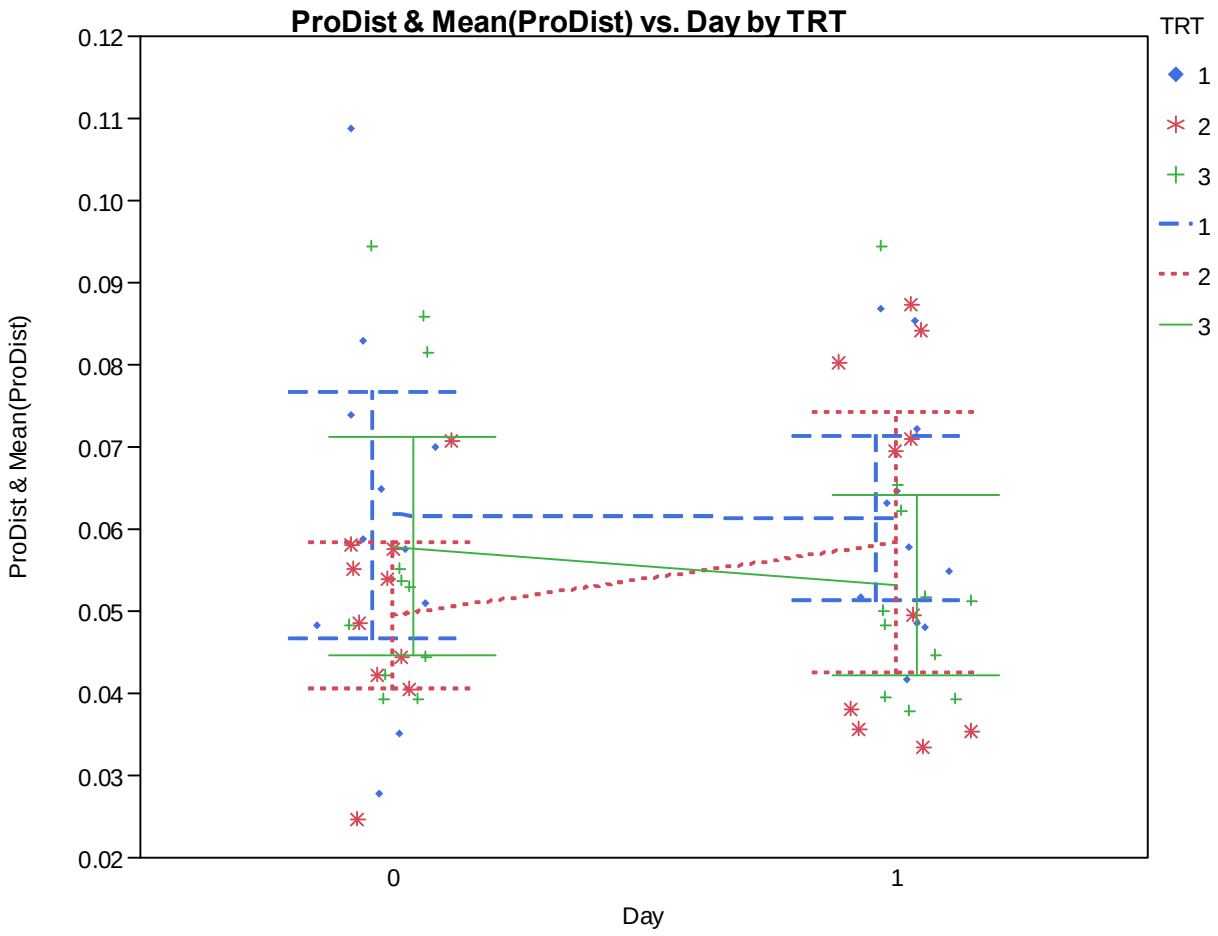


FIGURE 9: Plot of the magnitude of shape change in the lateral aspect of trophic morphology in adult snakes. Shape change was quantified using Procrustes Distance. No significant differences among treatments or time periods were detected. Treatment numbers correspond to 1-Fed, 2- Starved, and 3-Reversed; while the day listed on the X-axis correspond to the first day of the comparison (0 to 100 or 100 to 200).

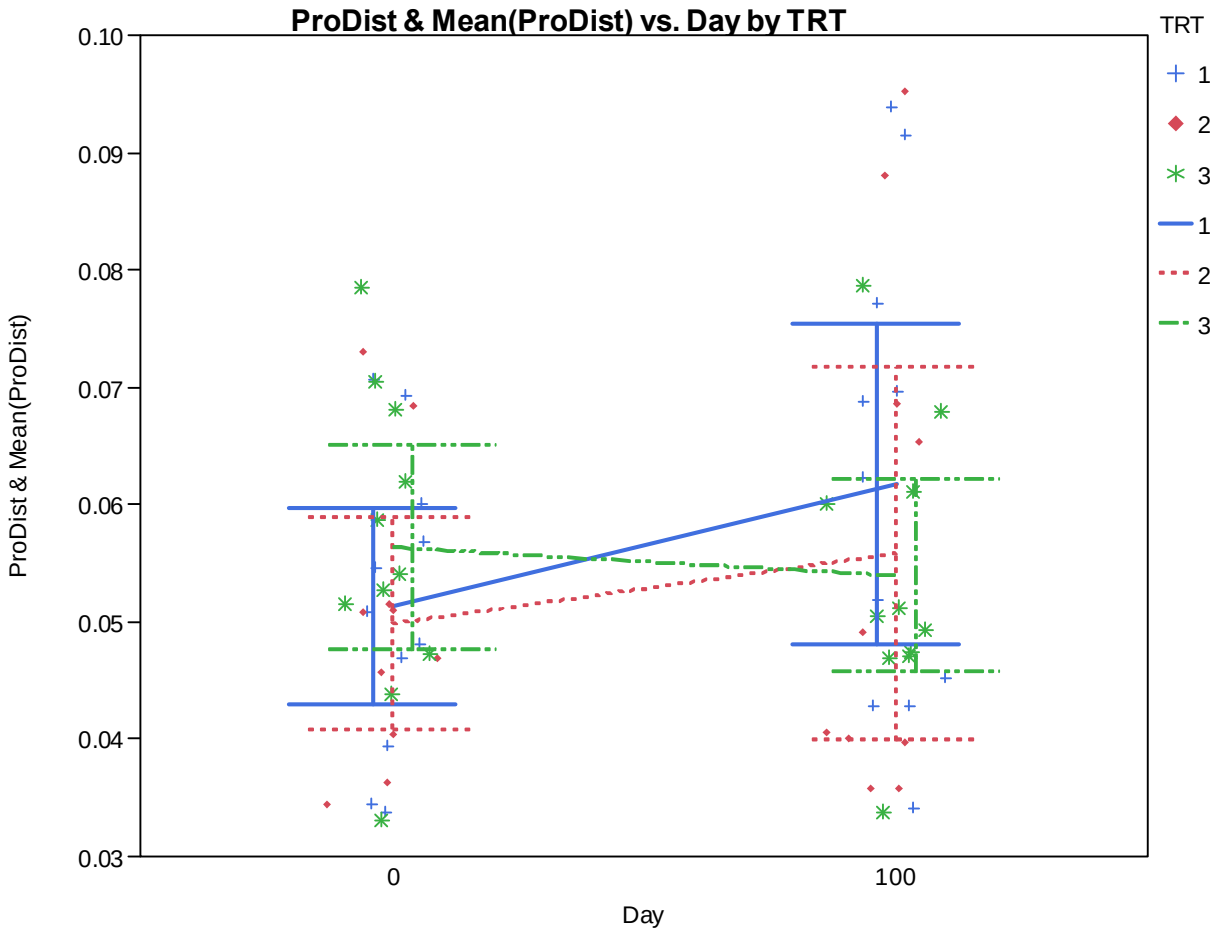


FIGURE 10: Graph of Procrustes Distance for the dorsal aspect of trophic morphology in juvenile snakes. Magnitude of shape change (ProDist) is plotted for each comparison, Day 0 to 100 (0) and Day 100 to 200 (1). Error bars represent 95% C.I. No significant differences among treatments were detected. Treatments are: Fed-1, Starved -2, and Reversed – 3.

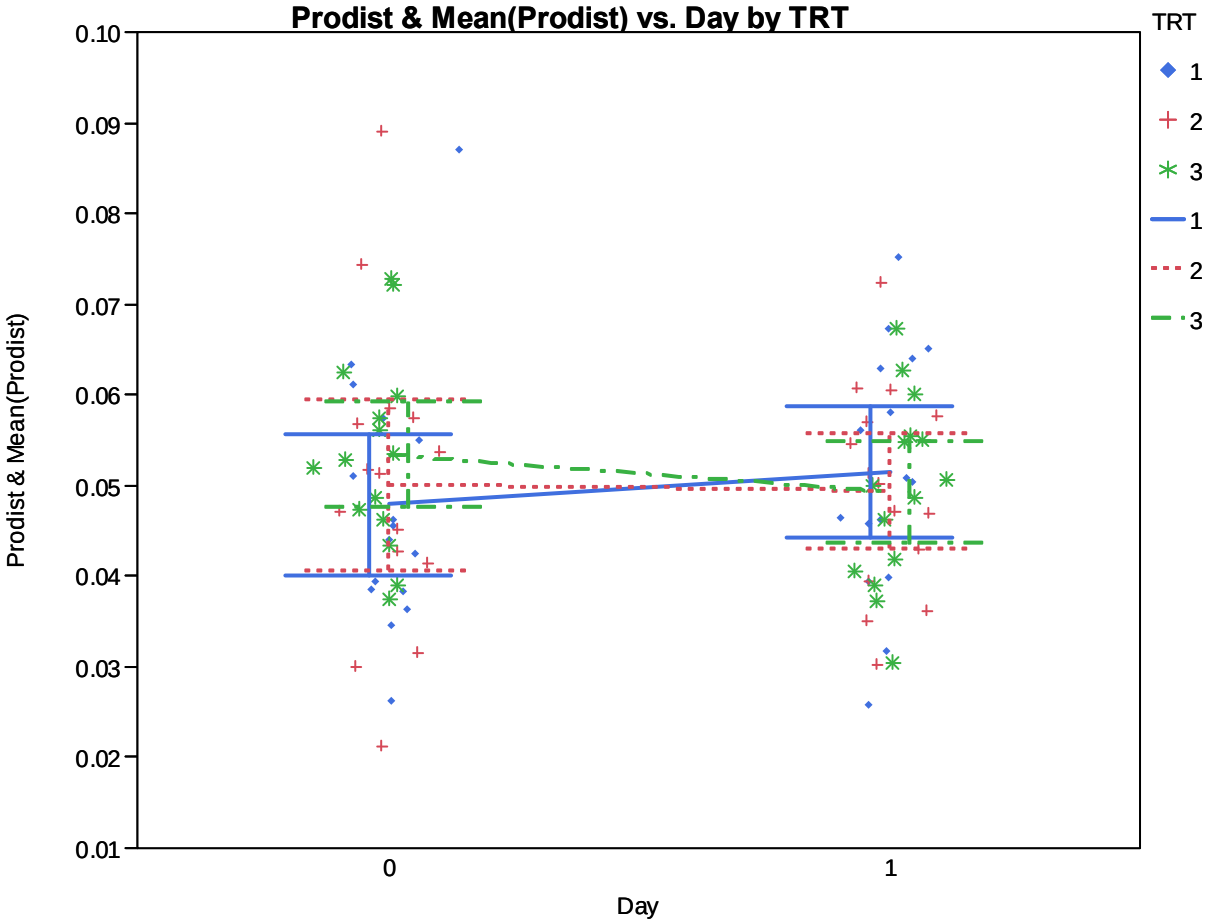


FIGURE 11: Graph of the magnitude of shape changes (ProDist) for the lateral aspect of trophic morphology in juvenile snakes. Treatments did not significantly differ in the magnitude of shape change for either comparison (Day 0 too 100 [Day 0] or Day 100 to 200 [Day 100]). Error bars represent 95% C.I. and treatments are: Fed-1, Starved - 2, and Reversed – 3.

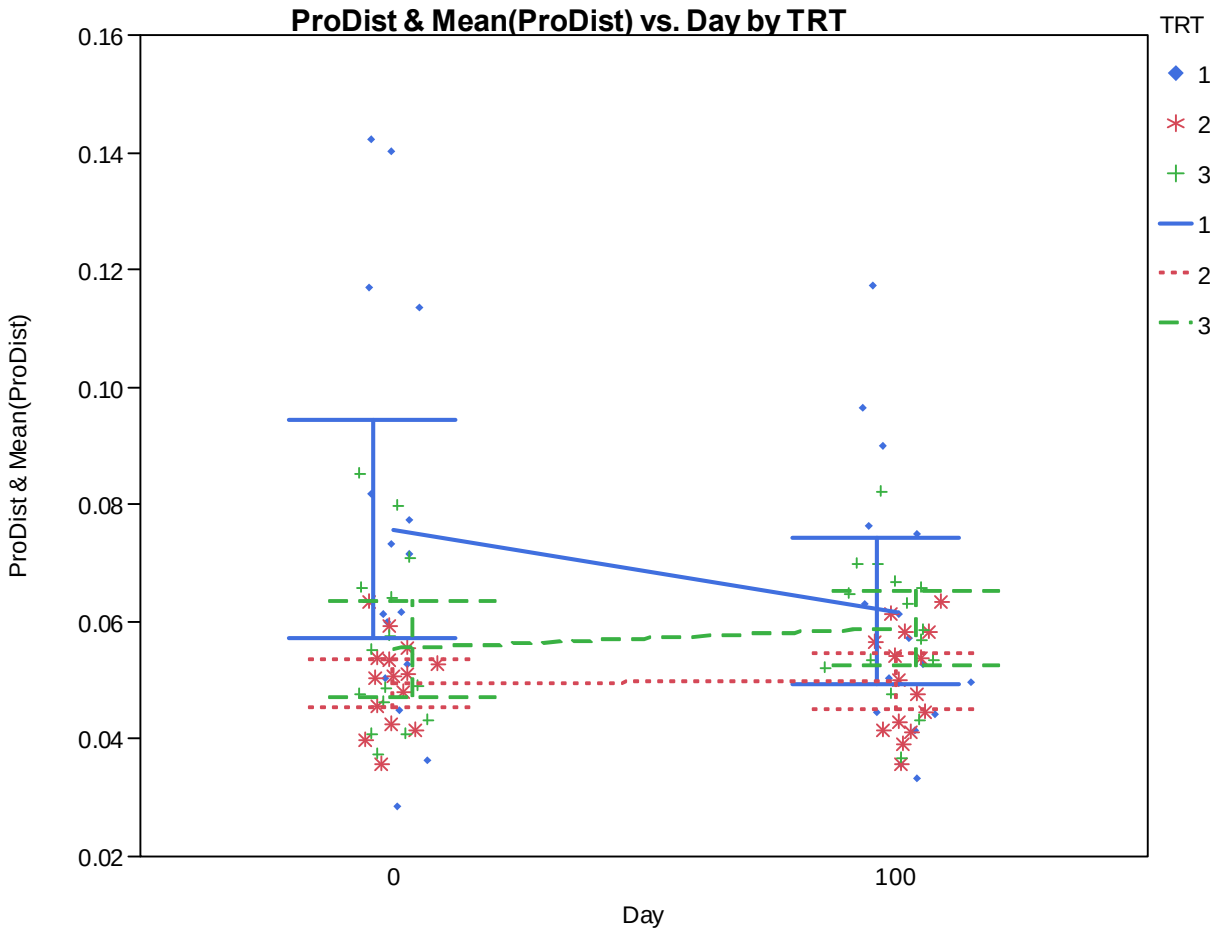


FIGURE 12: Deformation grids illustrating the slight shape change in each adult treatment throughout the course of the study. Fed treatments show a slight widening of the head, while Starved snakes show a slight narrowing; but none of these changes were statistically significant.

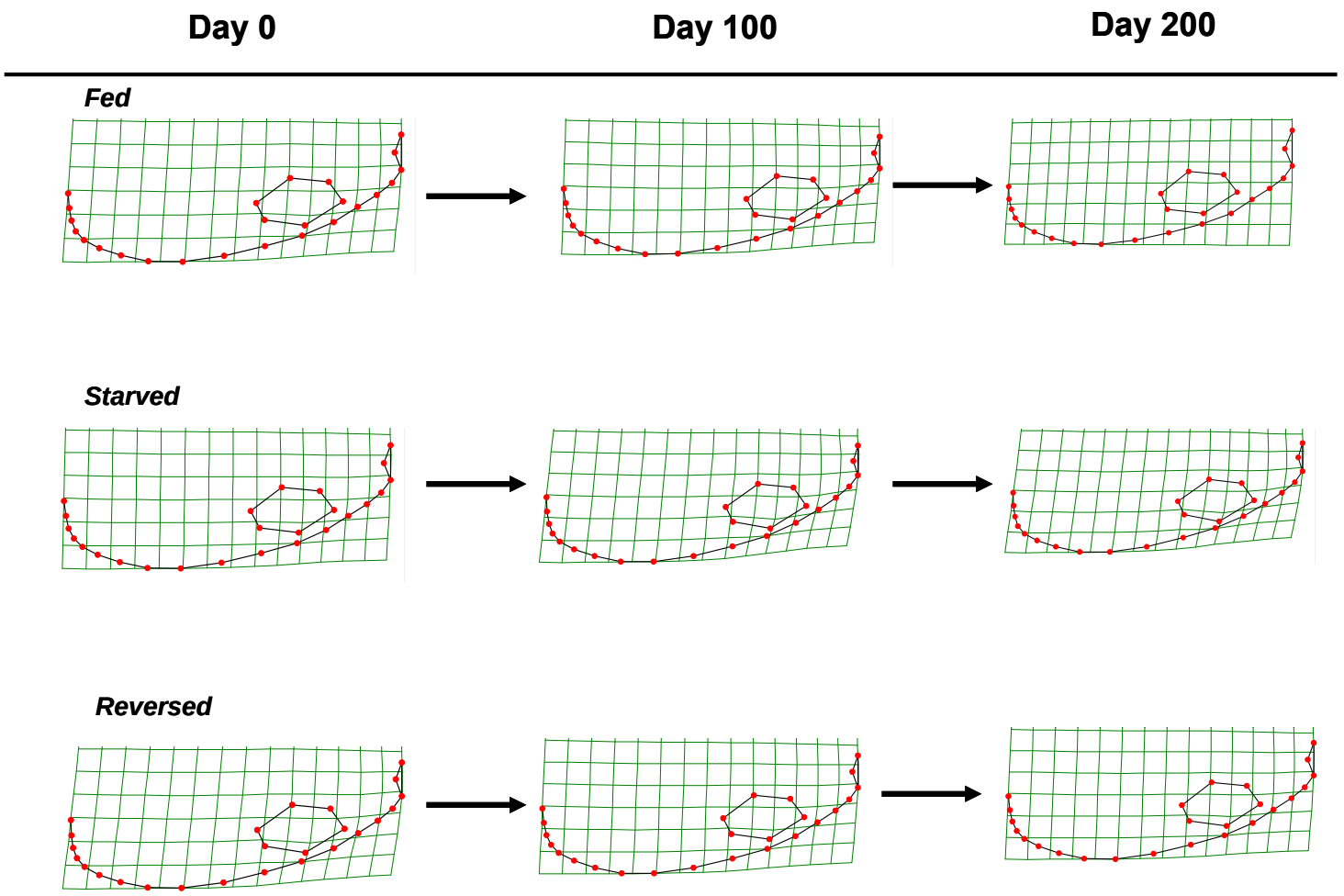


FIGURE 13: Deformation grids showing the change in the dorsal aspect of juvenile trophic morphology over the course of the study. There were no statistical shape differences among treatments or detectible patterns of shape change.

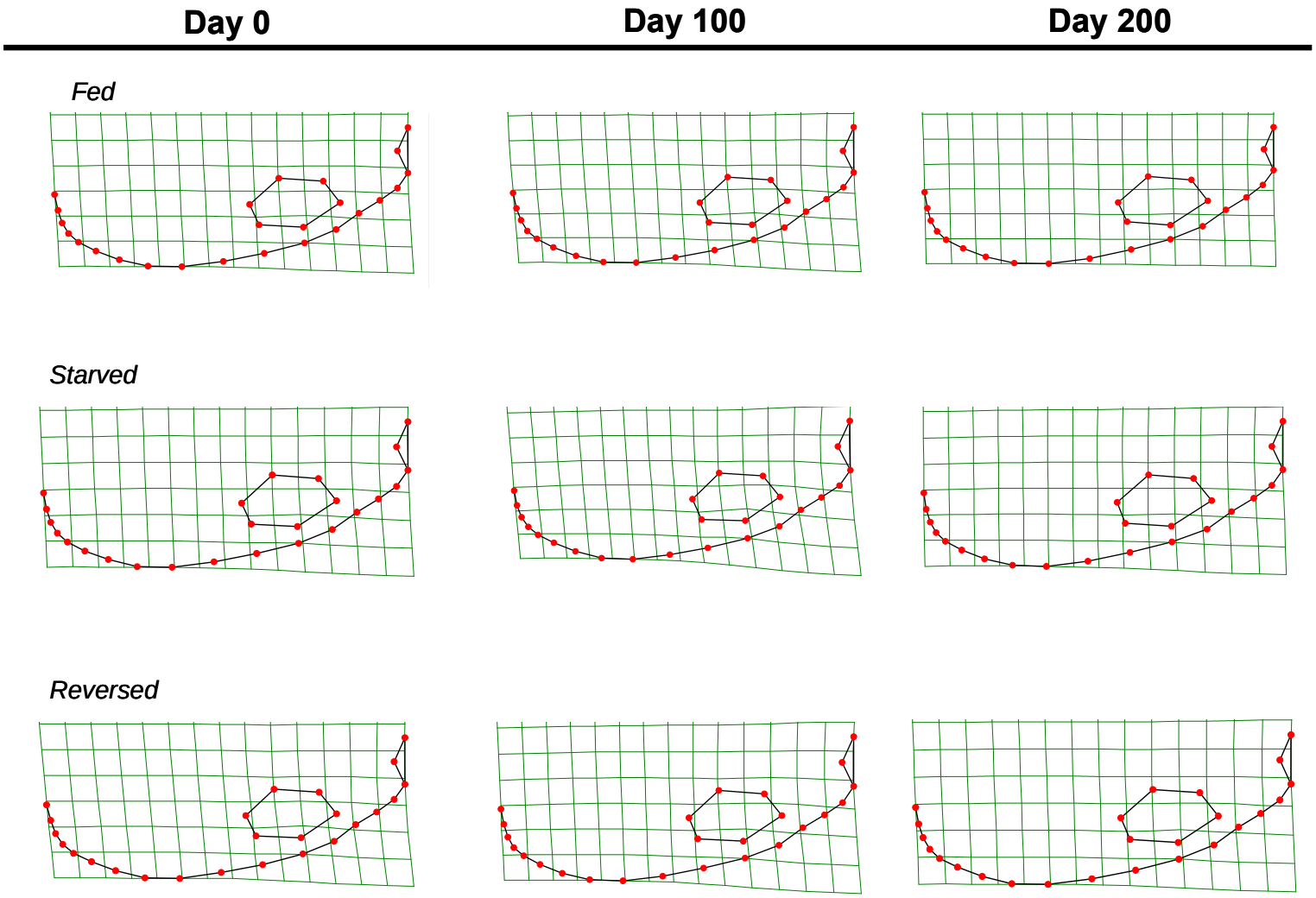


FIGURE 14: Plot of the relative warp scores for the entire dataset, adults and juveniles. Deformation grids show the extreme morphology representative of the ends of each of the RW axes. RW1 was best classified as changes to the anterior aspect of the head and snout, while RW2 was associated with changes in the posterior aspect of the head. On the plot adults are marked with ‘o’ and juveniles with ‘x’. It is clear there is an ontogenetic shift in trophic morphology in this species.

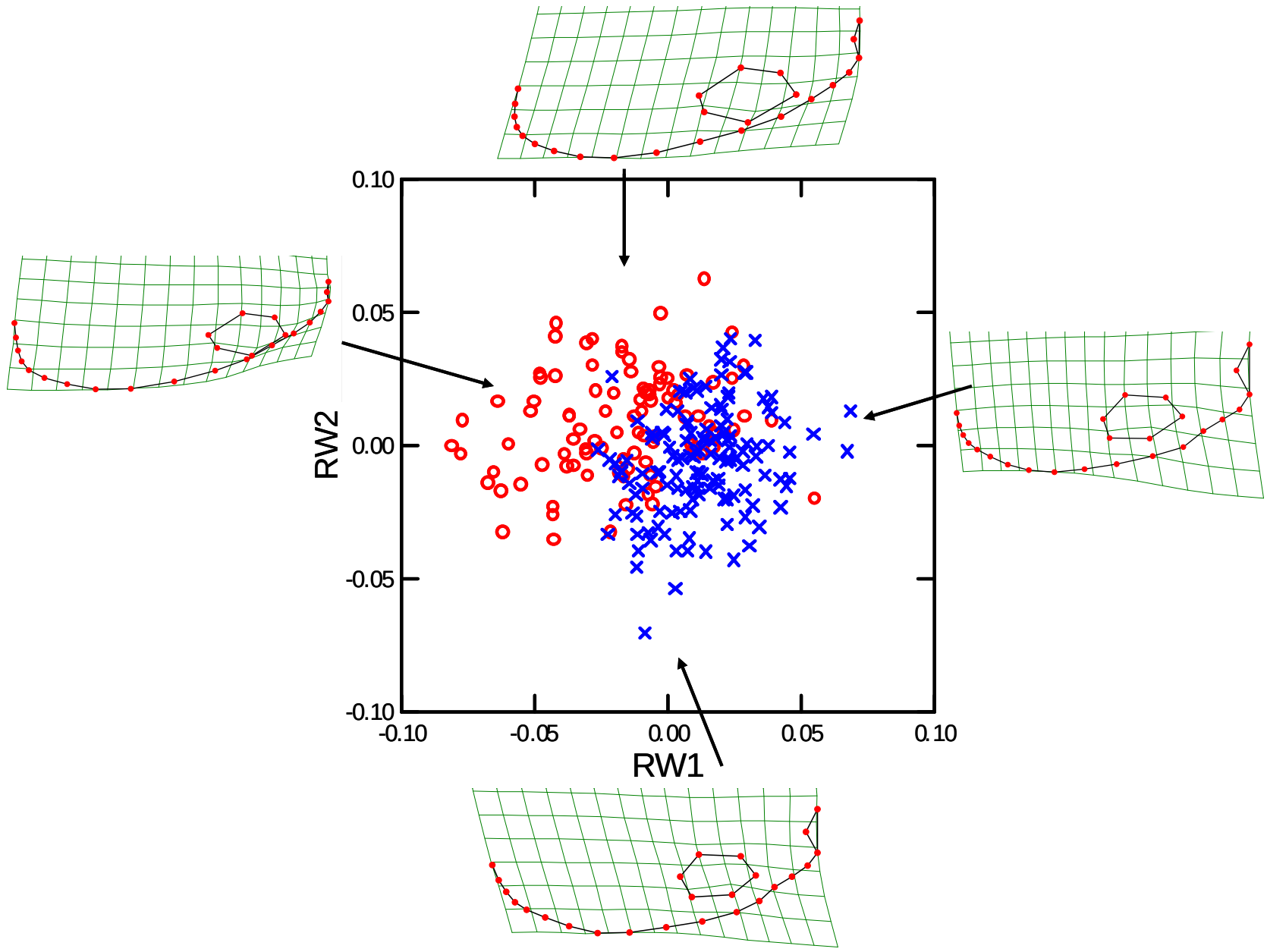

0.10
0.05
0.00
-0.05
-0.10
RW2
-0.10
-0.05
0.00
0.05
0.10
RW1

FIGURE 15: Deformation grids showing the slight change in lateral trophic morphology in adult snakes throughout the experiment. No statistically significant differences were present and very little noticeable shape change occurred.

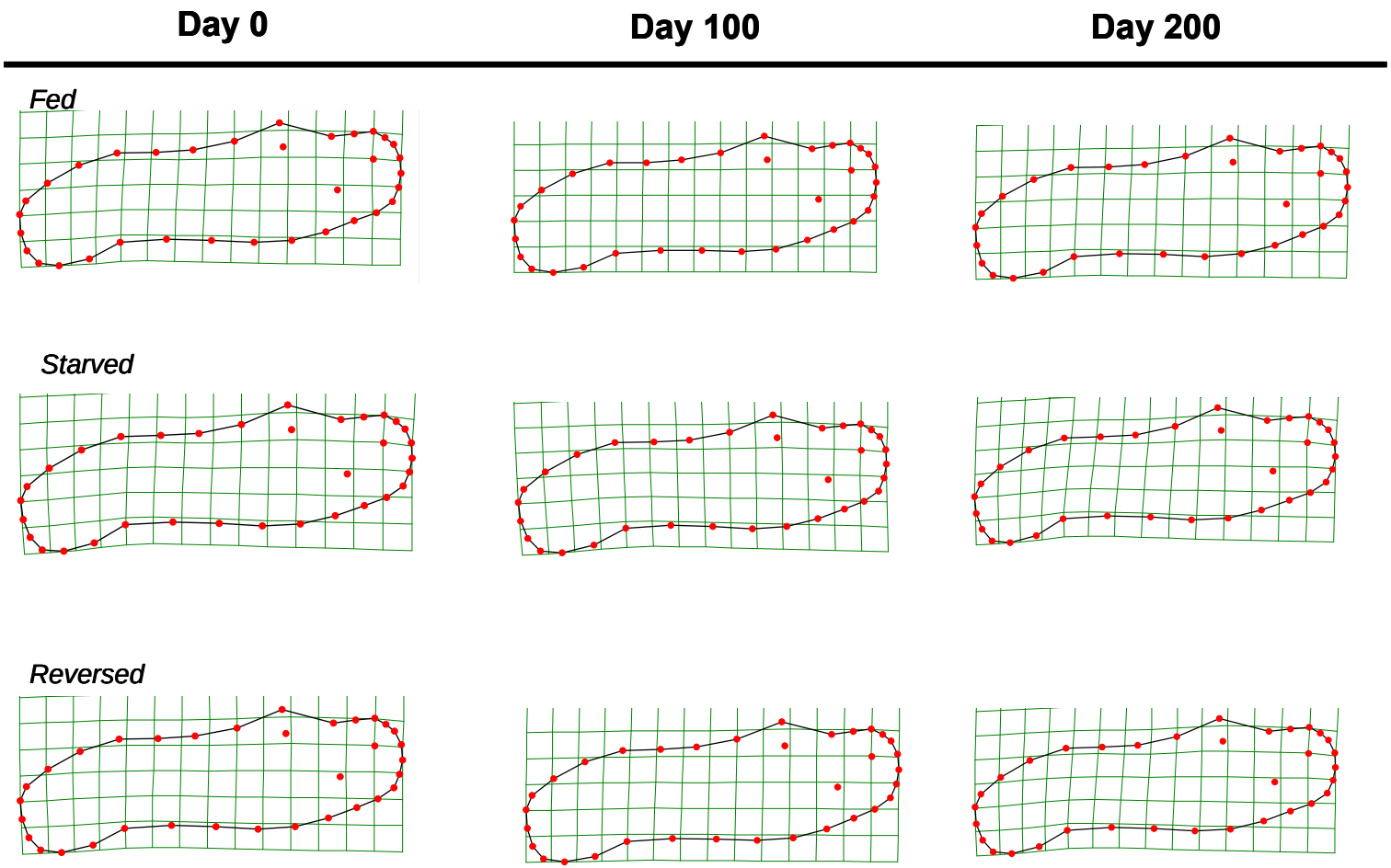


FIGURE 16: Deformation grids showing lateral shape change in juvenile snakes over the course of 200 days. Lateral morphology showed very little shape change within treatments and no significant differences among treatments.

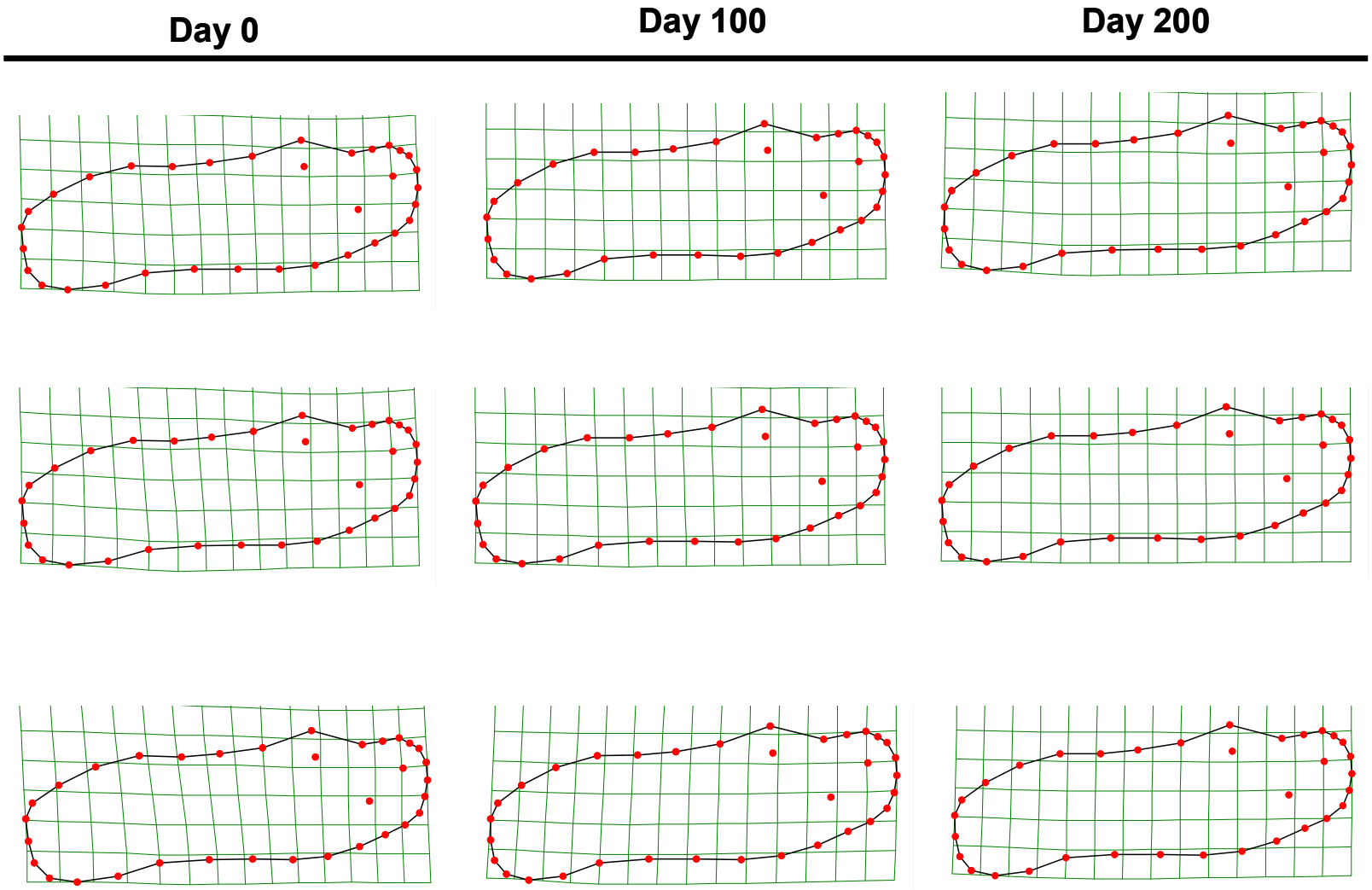
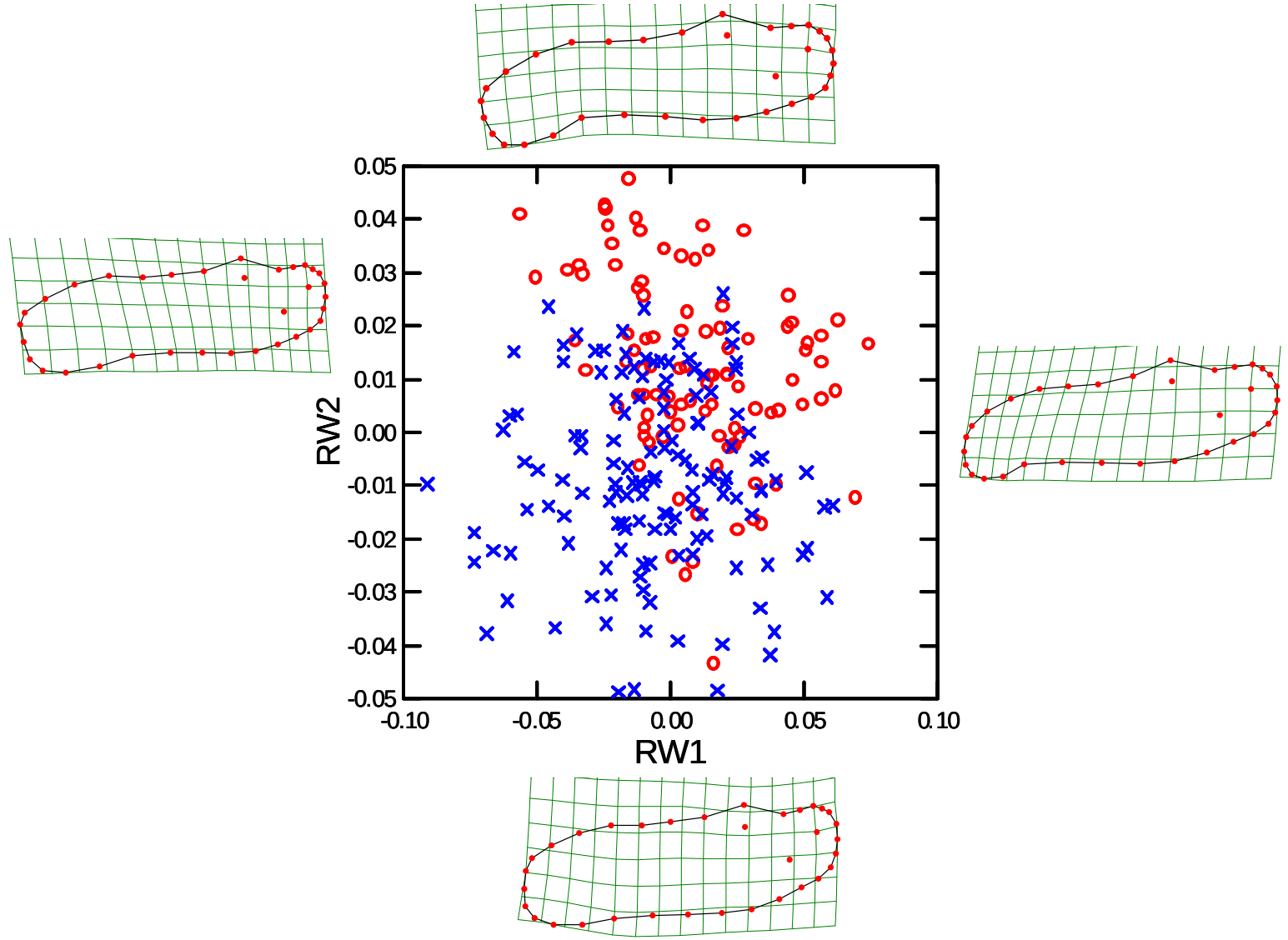


FIGURE 17: Relative warp plot showing the entire lateral data set, adults and juveniles. Adults (o) and juveniles (x) show a large degree of separation along the RW2 axis, suggesting an ontogenetic shape change in this species. Deformation grids show how shape changes along each axes.


0.05
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.04
-0.05
RW2
-0.10
-0.05
0.00
0.05
0.10
RW1

TABLES

TABLE 1: Model selection results for the covariance structures for the repeated measures ANOVA/ANCOVA methods for the adult snakes. Only the top 4 covariance structures are listed and the best fit model was chosen using ΔAIC and is shown in bold and italics.

	AIC value for specified variable			
Covariance Structure	**Log SVL**	**Log Mass**	**Log DCS**	**Log LCS**
Compound Symmetry	***-321.0316***	-233.2889	***-362.124***	-351.4335
Unstructured	-318.9178	-237.4779	-355.9689	-344.6462
Auto Regressive	-307.2087	***-243.7073***	-356.0719	***-351.6633***
Auto Regressive w/ heterogeneous variance	-304.7441	-241.1623	-352.3152	-347.9462

TABLE 2: Model selection results for the covariance structures used for repeated measures ANOVA/ANCOVA methods for juvenile snakes. Only the top 4 covariance structures are listed and the best fit covariance structure was selected using ΔAIC and is shown in bold and italics.

	AIC value for specified variable			
Covariance Structure	**Log SVL**	**Log Mass**	**Log DCS**	**Log LCS**
Compound Symmetry	-378.1023	-297.3418	-589.3392	-395.2611
Unstructured	-380.959	-310.7033	-590.6097	-417.0825
Auto Regressive	-377.2049	-300.0739	-593.7746	-395.9184
Auto Regressive w/ heterogeneous variance	***-382.314***	***-309.2339***	***-594.2611***	***-419.6888***

TABLE 3: Treat*Day group mean values for body size variables repeated measured on adult snakes. Snout to vent length (SVL), body mass, dorsal centroid size (DCS), and lateral centroid size (LCS) were measured every 100 days during the starvation experiment. Values are group means followed by 1 SE (Mean – SE).

Day	TRT	SVL (cm)	Mass (g)	DCS	LCS
0	Fed	77.28 - 2.99	297.58 - 34.87	7.51 - 0.2495	7.80 - 0.278
100	Fed	79.59 - 3.01	354.79 - 39.76	7.82 - 0.2299	7.99 - 0.218
200	Fed	81.84 - 2.78	407.97 - 41.59	7.83 - 0.2297	7.87 - 0.226
0	Starved	80.84 - 3.13	349.21 - 36.57	7.32 - 0.2513	7.73 - 0.292
100	Starved	80.67 - 3.16	338.9 - 41.7	7.55 - 0.2411	7.86 - 0.228
200	Starved	81.92 - 2.91	319.52 - 43.62	7.85 - 0.2409	8.2 - 0.237
0	Reversed	77.40 - 2.99	279.23 - 34.87	7.32 - 0.2397	8.01 - 0.279
100	Reversed	78.83 - 3.01	265.01 - 39.78	7.80 - 0.23	7.93 - 0.217
200	Reversed	79.62 - 2.77	337.16 - 41.59	7.86 - 0.2296	7.93 - 0.225

TABLE 4: Treatment means for non-shape variables measured on juvenile snakes. Group means are given along with standard error (Mean (SE)).

Day	TRT	SVL (cm)	Mass (g)	DCS	LCS
0	Fed	34.11 (0.71)	34.89 (1.95)	3.94 (0.05)	5.26 (0.101)
100	Fed	35.8 (1.29)	42.66 (2.72)	3.92 (0.065)	5.39 (0.104)
200	Fed	41.77 (1.05)	71.09 (3.45)	4.39 (0.07)	6.84 (0.206)
0	Starved	33.93 (0.73)	35.86 (2.01)	3.79 (0.052)	5.36 (0.112)
100	Starved	35.28 (1.33)	31.95 (2.81)	3.78 (0.067)	5.36 (0.108)
200	Starved	35.28 (1.08)	27.91 (3.57)	3.96 (0.072)	5.86 (0.211)
0	Reversed	34.07 (0.73)	39.3 (2.01)	3.91 (0.05)	5.32 (0.111)
100	Reversed	37.41 (1.33)	34.77 (2.83)	3.86 (0.066)	5.4 (0.107)
200	Reversed	40.56 (1.08)	60.43 (3.56)	4.35 (0.072)	6.52 (0.213)

TABLE 5: Results from the Tukey HSD pairwise comparison, testing for significant differences in SVL (LogSVL) among treatments. Fed (1) and Reversed (3) snakes were not significantly different, but Starved (2) snakes were significantly shorter than the other two treatments. Comparisons were considered significant after if p-values were less than 0.017.

Level		Least Sq Mean
1	A	41.769375
3	A	40.554000
2	B	35.283333

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	6.486042	1.504499	2.83396	10.13813	0.0003*
3	2	5.270667	1.528573	1.56015	8.98119	0.0036*
1	3	1.215375	1.504499	-2.43671	4.86746	0.7003

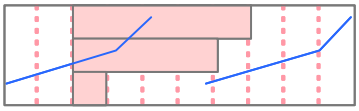


TABLE 6: Results from the Tukey HSD pairwise comparison of dorsal centroid size at Day 200 for juvenile snakes. Results show that Starved (2) snakes were significantly smaller than Fed (1) and Reversed (3) treatments. Analysis was conducted in JMP and an α of 0.017 was used to determine significance.

Level		Least Sq Mean
1	A	0.64268814
3	A	0.63879661
2	B	0.59786502

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	0.0448231	0.0108706	0.018413	0.0712332	0.0005*
3	2	0.0409316	0.0105084	0.015402	0.0664617	0.0010*
1	3	0.0038915	0.0090516	-0.018099	0.0258823	0.9034

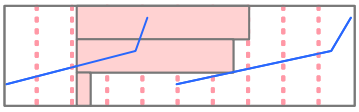


TABLE 7: Permutation comparisons of the observed F-ratio values for compensatory growth in juvenile snakes. Only comparisons between Fed and Reversed treatments were conducted. GRM and GRS refer to changes in Mass and SVL (respectively) for each time comparison. Values were calculated by comparing change in mass and SVL from Day 0 to 100 (1) and Day 100 to 200 (2). GTS and GTM represent the total change in SVL and Mass from Day 0 to 200. SVL2 and SVL3 are the body size values for each snake at Days 100 and 200 respectively.

Contrast	Obs. F-ratio	# Tests >= Obs	Valid Iterations	*Prand*
GRS1	*1.974771*	*8*	*999*	*0.009*
GRM1	*3.093896*	*0*	*999*	*0.001*
GRS2	0.74663	967	999	0.968
GRM2	0.902726	871	999	0.872
GTS	0.847164	808	999	0.809
GTM	0.583794	999	999	1
SVL2	*1.13276*	*1*	*999*	*0.002*
SVL3	0.970942	781	999	0.782

Table 8: Group means and standard errors for Procrustes distances. Magnitude of shape change was measured from Days 0 to 100 and Days 100 to 200 by using Procrustes distance. Snakes are grouped by age class, treatment (1-Fed, 2-Starved, 3-Reversed), and trophic morphology aspect.

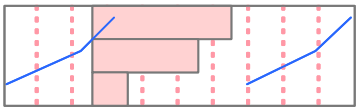
Aspect	Age Class	TRT	Time Period	Mean	SE
Dorsal	Adults	1	0-100	0.0617	0.0068
Dorsal	Adults	2	0-100	0.0496	0.0040
Dorsal	Adults	3	0-100	0.0579	0.0060
Dorsal	Juveniles	1	0-100	0.0479	0.0036
Dorsal	Juveniles	2	0-100	0.0501	0.0044
Dorsal	Juveniles	3	0-100	0.0534	0.0027
Dorsal	Adults	1	100-200	0.0614	0.0045
Dorsal	Adults	2	100-200	0.0519	0.0053
Dorsal	Adults	3	100-200	0.0590	0.0064
Dorsal	Juveniles	1	100-200	0.0516	0.0034
Dorsal	Juveniles	2	100-200	0.0495	0.0030
Dorsal	Juveniles	3	100-200	0.0493	0.0026
Lateral	Adults	1	0-100	0.0514	0.0038
Lateral	Adults	2	0-100	0.0499	0.0040
Lateral	Adults	3	0-100	0.0564	0.0039
Lateral	Juveniles	1	0-100	0.0758	0.0087
Lateral	Juveniles	2	0-100	0.0496	0.0019
Lateral	Juveniles	3	0-100	0.0555	0.0038
Lateral	Adults	1	100-200	0.0618	0.0061
Lateral	Adults	2	100-200	0.0558	0.0070
Lateral	Adults	3	100-200	0.0540	0.0037
Lateral	Juveniles	1	100-200	0.0618	0.0059
Lateral	Juveniles	2	100-200	0.0499	0.0023
Lateral	Juveniles	3	100-200	0.0589	0.0030

TABLE 9: Results from the LS Means Tukey HSD pairwise comparison of lateral Procrustes Distance in juvenile snakes. Starved (2) snakes showed a smaller degree of shape change (ProDist) from Day 0 to Day 100 than Fed snakes (1), although this only approached significance (significance α= 0.017).

Level		Least Sq Mean
1	A	-1.162720
3	A B	-1.271846
2	B	-1.307033

Levels not connected by same letter are significantly different.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
1	2	0.1443130	0.0503925	0.021885	0.2667415	0.0175*
1	3	0.1091263	0.0504443	-0.013428	0.2316804	0.0895
3	2	0.0351868	0.0514774	-0.089877	0.1602508	0.7743



CHAPTER 4: MORPHOLOGICAL RESPONSE OF *Crotalus viridis viridis* TO DIET MANIPULATION: IS TROPHIC MORPHOLOGY OF SNAKES INDUCIBLE BY VARIATION IN PREY SIZE AND AMOUNT?

Abstract:

Gape-limited predators are restricted in the type and size of prey items they can ingest by the shape and size of their trophic morphology. Often gape-limited predators are ectotherms and occupy niches that endotherms cannot, making them ecologically important species in many ecosystems. Evolutionary theory predicts that gape-limited predators should possess plasticity in their trophic morphology to allow them to respond to environmental cues about their prey base. In this study, we examined the effects of three possible influences over trophic morphology in the pit-viper *Crotalus viridis viridis*. Diet manipulations were carried out for 480 days that varied the amount and size of prey items. Snakes from three populations were used, selected such that two populations were only separated by a short distance (~15 km), while the third was more distantly removed (~180 km). The experimental design accounted for the possible influence of prey size, resource level, and geographic differences in morphological response. By Day 480, snakes from the two prey size treatments exhibited significantly different head shapes. Snakes reared on whole rodents had broader heads, while snakes force-fed homogenized prey had narrower heads. Shape differences were only present in two of the three populations. The third population did not show any significant alterations of head shape due to prey differences, but did possess relatively larger heads throughout the study. Our results show that trophic morphology of rattlesnakes is plastic, at least in some litters, and can be induced by prey items.

Introduction:

The ability of organisms to successfully acquire adequate of amounts of energy is the main determinant of growth, survival, and reproductive fitness of an individual. Therefore, an organism's ability to obtain resources and the structures associated with resource acquisition should directly influence their fitness (Forsman, 1994). Selection acts on an organism's ability to respond to change in its environment and the variation in those responses (Stearns, 1989; Skúlason and Smith, 1995; Pigliucci, 2001; Casselman and Schulte-Hostedde, 2004).

There are many examples of predator-prey interactions that result in a plastic response and morphological change in the prey species (Tollrian and Harvell, 1999, Agrawal, 2001; Whitman and Agrawal, 2009). Phenotypic plasticity in many traits have been ascribed to the presence of predation: plants alter their chemistry (Baldwin, 1988), aquatic invertebrates develop spines, keels, and helmets (Harvell, 1986; Dodson, 1989), amphibian larva alter their morphology and coloration (Relyea, 2001; Gomez-Mestre and Diaz-Paniagua, 2010) and invertebrate and vertebrate immune systems can be induced by pathogens (Harvell, 1990). Less work has been done on phenotypic plasticity in both species in predator-prey interactions (Agrawal, 2001), and the response of predators to changes in their prey (Padilla, 2001).

A predator's plastic response to changes in its prey or its prey base has been referred to as an inducible offense (Padilla, 2001; Kopp, 2003). Many taxa of predators exhibit plastic responses in morphology such as: stolons of bryozoans (Harvell and Padilla, 2000), jaw morphology of fish (Wimberger, 1991; Svanback and Eklov, 2002; Andersson, 2003, Kahilainen and Ostbye, 2006), body size and jaw structure of larval salamanders (Johnson et al., 2003), jaws of insects (Greene, 1989), and the chelae of crustaceans (Smith and Palmer, 1994). The plastic

responses of predator enhance their feeding ability and are generally expressed during the lifetime of a single individual (Padilla, 2001).

The trophic morphology of predators varies significantly among species (Klauber, 1938) and is correlated to prey characteristics (Skúlason and Smith, 1995; Forsman and Shine, 1997; Magnhagen and Heibo, 2001; Svanback and Eklov, 2002; Johnson et al, 2003; Andersson et al., 2004; Kishida et al., 2006; Marcil et al., 2006). Trophic morphology also shows a large degree of intraspecific sexual dimorphism (Shine and Crews, 1988; Camilleri and Shine, 1990; Vincent et al., 2004; Bulté et al., 2008), prey availability (Bonnet et al., 2001; Magnhagen and Heibo, 2001), population (Forsman, 1991; Shine, 1991; Aubret and Shine, 2009), body size (Shine et al., 1998), community structure (Padilla, 2001), and habitat (Svanback and Eklov, 2002).

Gape-limited predators are constrained in the prey they can consume by their trophic morphology. Trophic morphology encompasses any structure associated with the acquisition and ingestion of prey items. The size and shape of trophic morphology determines the maximum ingestible prey size and prey shape (Forsman, 1994). Differences in the size of prey that can be consumed are primarily considered to be determined by body size (Pough and Groves, 1983; Shine, 1991; Shine et al., 1998; Aubret and Shine, 2009), but the shape of trophic morphology and its allometric relationship with body size can also influence maximum prey size (Forsman, 1994). Individuals with larger relative trophic morphology can ingest larger prey items compared to similarly sized snakes with small relative trophic morphology (Forsman and Lindell, 1993).

The phenotype of an organism, in this case specifically its trophic morphology, is a combination of genetic and environmental influences and their interactions over the lifetime of the organism (Schuett et al., 2005). Phenotypic plasticity is the expression of alternative phenotypes due to environmental influences (Stearns, 1989), and is prevalent in the trophic

morphology of many taxa (Kishida et al., 2006). Plasticity in morphology is influenced by temperature (Blouin and Brown, 2000; DeWitt and Scheiner 2004; Georga and Koumoundouros, 2010), diet (Magnhagen and Heibo, 2001; Krause et al., 2003), and resource level (Queral-Regil and King, 1998; Bonnet et al., 2001).

Snakes are model organisms for investigations of phenotypic plasticity and prey fluctuations for two important reasons. First, snakes are gape limited predators (Arnold, 1993), and trophic structures (e.g. the jaws) are not used in any other activities such as mating or combat; and snakes cannot tear or chew their prey and therefore must ingest their prey whole (Shine, 1991). Thus, head shape and size dictate the type and size of prey that a snake can ingest (Forsman, 1996a; Shine, 2002). Second, of all the reptile species whose prey base is limited by trophic structure, snakes are more commonly a dominant predator and key indicator of environmental conditions (Beaupre and Douglas, 2009).

While some species and/or populations have shown moderate levels of morphological change due increases in prey size (Queral-Regil and King, 1998; Aubret et al., 2004), others have been unresponsive (Bonnet et al., 2001). Plastic responses to environmental cues have shown to vary between sexes (Camilleri and Shine, 1990; Scudder-Davis and Burghardt, 1996; Krause et al., 2003), ontogenetic stage (Vincent et al., 2004), population (Aubret et al., 2004), and species (Phillips and Shine, 2004). To date no consistent pattern or predictable trend has been elucidated in snakes (Table 1 for history). If patterns of adaptive plasticity can be determined, researchers will be able to better gauge the ability of a snake species to adjust to prey base fluctuations and changes in community structure.

The focus of the current study is test if trophic morphology can be induced by varying resource levels and prey sizes. Specifically, do snakes fed significantly larger and/or more prey

items have significantly larger or differently shaped trophic morphology. Trophic morphology was quantified as two variables; shape and size (relative size). Changes in the relative size and the shape of trophic morphology provide a dual perspective on possible responses to environmental factors. Landmark-based geometric morphometrics (GM) were used to quantify the shape of trophic morphology (Rohlf, 1999). Variation in landmark data includes variation associated with shape, size, position, and orientation. Geometric morphometrics allows for all non-shape variation to be removed and shape can be analyzed as the size-free entity. These methods provide the opportunity to better examine shape variation and its sources (Collyer et al., 2005). These methods provide visual advantages over traditional approaches based on linear distance measures (e.g. Caldecutt and Adams 1998, Adams and Rohlf 2000, Rüber and Adams 2001). The use of GM methods also provides an estimate of size. A centroid is determined for each of the landmark configurations, and is the geometric center of gravity for each specimen. Centroid size is calculated as the square root of the sum of all the squared distances of each landmark from the centroid (Rohlf and Slice, 1990).

While previous research investigating trophic morphology has examined effects of food size and resource level, to date no experiments have manipulated both in order to eliminate any possible interaction between prey size and resource level. Much of the previous research that manipulated diets in the laboratory used small sample size and/or individual litters (Forsman, 1996a, b; Bonnet et al., 2001; Schuett et al., 2005).

Given the lack of a consistent pattern of trophic plasticity in snakes (Table 1) and the areas of possible improvement, the current investigation focuses on the influence of litter and environmental factors on the trophic morphology of snakes. Specifically, our goal is to quantify the relative impacts of litter/population of origin, resource level, and prey size on the

development of trophic morphology and its allometric relationship to body size in a group of juvenile snakes. The populations used in this study have previously been documented to display significant temporal and spatial variation in trophic morphology (Smith, 2006; Smith and Collyer, 2008), which provided evidence for both genetic and environmental influences on snake morphology.

We focus on whether different populations respond differently to feeding treatments, whether larger prey size has a significant positive influence on trophic morphology (do bigger prey items result in larger head sizes and/or difference shapes), and whether the amount of resources available to a snake directly or indirectly influences trophic morphology. We measured the change in trophic morphology and its allometric relationship to body size over the course of 480 days to provide insights into how snakes respond to diet manipulations and whether those responses varied among populations.

Methods:

Seventy-two juvenile Prairie rattlesnakes (*Crotalus v. viridis*) were used in this study. All snakes were born in the lab in the August and September of 2008. Gravid females were captured from previous research sites (Smith, 2006; Smith and Collyer, 2008) in North and South Dakota in the summer of 2008 (June-July). All females were housed in the lab under identical conditions until parturition. Snakes were from one of 3 populations (Gill, Kuhn, or Miller) and 8 litters. Snakes from the Gill population came from 3 litters (n=12, 9, 9); 4 litters from Kuhn (n = 7, 5, 7, 4), and a single litter from Miller (n = 20).

After the first shed, all snakes were weighed, measured, sexed and housed in their own container. Snakes were housed in 14-liter plastic containers lined with newspaper. Each enclosure contained a hide-box and water dish. All individuals were housed in the same room,

under a 12L:12D photoperiod (7am-7pm) and a room temperature of 24°C (+/- 1° C). Snakes were given water *ad libitum* and enclosures were cleaned as needed.

Snakes were fed thawed pinky and fuzzy mice every two weeks until mid-November. In November, snakes were artificially hibernated until mid-April. Upon emergence from artificial hibernation, snakes were offered a thawed rodent every two weeks for 6 months. All snakes considered for this study came from litters with at least 4 individuals, had fed at least three times before hibernation, survived hibernation, and successfully ingested at least one 'fuzzy ' (a mouse that is 10-13 days old and 4.5-7.99 grams in mass, Rodentpro.com) post-hibernation. Since this was a feeding study, only litters that were consistent feeders were considered. All snakes had consumed at least two meals consisting of 'fuzzy' mice prior to entering the study.

Candidate litters were evaluated in November of 2009. Eight litters ranging in size from 4 to 20 individuals were selected. Snakes were randomly assigned to one of 4 treatments within their litters. The only stipulation put on treatment assignment was that litters in multiples of four needed to have equal representation. Treatments combined resource level and prey size/type. Resource level was either high or low. The high resource level consisted of a meal every 14 days, while the low resource level consisted of a meal every 25 days. Prey size/type represented the extreme case of dietary divergence. Half of the treatments were fed thawed rodents, starting with 'fuzzy' mice and increasing in size as the snakes grew, such that at the conclusion of the experiment weanling and small rats (11.43 – 15.24 cm in length and 45-84.99 grams in mass) were being used. The other half of the treatments were fed a homogenized rodent puree through a rubber catheter tube (Allegiance Urological Catheter, Cardinal Health, McGaw Park, IL; size 22 Fr = 7.3 mm in diameter). Rodents were homogenized by grinding whole, frozen rodents through a meat grinder (output was re-ground 2 more times), then placed in a food processor

with a few milliliters of water and blended until a uniform consistency was present. The rodents used from the homogenate were the same sizes (from the same batches) that were used in the other treatments. Specifically, if snakes were being fed whole thawed large mice, frozen large mice were used to make the homogenate. This standardized the nutritional composition of the two diets and should have eliminated (or at least minimized) any energetic values between the diets. Although water was added to the ground rodent, it was only done so to obtain a consistency that could be fed through a catheter tube without any blockage. The amount of water added never exceeded 30 ml (1 ounce) per 200 grams of homogenate. The added water is minimal on a per mouse basis: for example 'fuzzy' mice weighing 6 grams each would require 33.33 mice to make up the 200 grams of homogenate. With less than 30ml of water added to every 200grams, it amounts to each mouse drinking less than 1ml of water prior to being consumed.

Combinations of these two factors resulted in four treatments. Treatment 1 (n= 17) consisted of snakes fed thawed whole rodents every 14 days, treatment 2 (n=19) was fed rodent homogenate every 14 days, treatment 3 (n=18) was fed thawed whole rodents every 25 days, and treatment 4 (n=18) was fed rodent homogenate every 25 days. All meals sizes were standardized for each treatment. Meals were 25% (+/- 2%) of the average body mass for the entire treatment. Thawed rodents were fed ahead of time to determine meal size; rodent homogenate was made by grinding frozen rodents with the combined mass that equaled 35% of the total treatment snake masses. Each batch of homogenate was tested prior to use to determine how much 1 cc of homogenate weighed and each snake had the appropriate volume (in cubic centimeters) injected through a syringe into the catheter. Homogenate varied very little over the course of the experiment and normally 1 cc of homogenate equaled 0.9-1.1 grams in mass. The preloaded

syringe of homogenate was weighed before and after force feeding each snake to obtain the exact mass (to the nearest 0.1g) of homogenate administered.

The force-feeding process consisted of the catheter tube, a 60 ml syringe with catheter tip (BD, Franklin Lakes, NJ), the homogenized rodents, and some animal lard for lubricating the catheter tube. Snakes were restrained in a plastic tube and the lubricated catheter tube was gently forced into the mouth and down the esophagus. The catheter was gently pushed down approximately 1/3 of the body length, to just before the stomach. Once in place the homogenate was slowly injected from the syringe into the snake (the catheter tube was preloaded with homogenate to eliminate any air bubbles). Once the appropriate amount of homogenate was injected, the catheter was slowly removed and reweighed with the syringe. No individuals regurgitated a meal (force-fed or otherwise) during the course of the study.

Measurements

Our feeding study was designed to last 480 days. Full sets of morphological measurements were made every 120 days and just prior to the onset of the experiment. Snakes were weighed prior to every meal (either 14 or 25 days). Mass was recorded and used to compute meal size. Mass of prey ingested was recorded for every meal. All snakes were weighed, measured for snout to vent length (SVL) and tail-length (TL) every 120 days (and Day 0). Mass was recorded to the nearest 0.1 grams and SVL and TL were recorded to the nearest 0.1 cm. Snout to vent length and TL were measured through the use of a squeeze box (Quinn and Jones, 1974).

Trophic morphology was quantified through the use of geometric morphometrics (GM). Digital images of the dorsal and right, lateral aspects of the snake's trophic morphology (head and neck) were taken at each time interval (Days 0, 120, 240, 360, & 480). Digital images were

taken using a Cannon ® PowerShot S3 IS 6.0 mega pixel camera. All digital images were taken at specially built camera table setups (one for the dorsal and one for the lateral), that control camera and snake positioning and provided a number of rulers for scale.

GM Data Acquisition

Geometric morphometrics (GM) examines the relationship in landmarks or outlines of the specimens. Landmarks need to be: 1.) derived from anatomical reference points, 2.) homologous among the individuals in the sample, 3.) discernible, and 4.) able to be replicated over time. Type I landmarks are points of discrete juxtaposition of tissues or other definitive anatomical structures, Type II are maximum points on curvatures, Type III are extreme points, like endpoints, centroids, or points farthest away from a reference (Bookstein, 1997). Shape is ultimately quantified using relative warp scores. Relative warp scores are obtained through a principal component analysis on partial warp and uniform scores obtained through a thin plate spline on the aligned coordinates (for more detail see: Bookstein, 1997 or Zelditch et al., 2004).

Dorsal and right lateral images had landmarks placed using TPS software (Rohlf, unpublished). Dorsal images had a series of 25 landmarks placed on each photo. Landmarks 1-10 were Type I or III landmarks, while11-25 were sliding semi-landmarks that estimated the curvature of the snake's head (Chp. 2: Figure 6, Table 3). Lateral images had a total of 34 landmarks placed on each photo. Landmarks 1-9 were Type I or III landmarks, 10-16 were sliding semi-landmarks only the upper jaw, 17-28 were sliding semi-landmarks characterizing the posterior aspect of the head, and 29-34 were sliding semi-landmarks characterized the anterior aspect of the head (Chp. 2: Figure 7, Table 4). Shape is quantified by relative warps scores. Relative warps scores are obtained

Head size was represented by centroid size (CS), which is calculated using GM. The centroid is the geometric center of gravity for the landmark configurations. Centroid size is the square root of the sum of all the squared distances of each landmark to the configuration's centroid (see Bookstein, 1997). Centroid size was calculated for each snake, trophic aspect, and time period. Dorsal and lateral CS (DCS & LCS respectively) were used as estimates of trophic morphology size, and tested separately for their allometric relationship to SVL.

Statistical Analysis

Snake mass, SVL, DCS, and LCS were analyzed for treatment (TRT) and population (POP) differences over time using repeated measure ANOVA/ANCOVA methods. Repeated measures (RM) models examined the significance of TRT, POP, and time (Day), and the interaction of Day with TRT or POP. All repeated measure models were tested for the best fitting covariance structure. Models were evaluated using AIC for the following covariance structures: compound symmetry, unstructured/symmetrical, auto-regressive, auto-regressive with heterogeneous variance, Gaussian, and linear. All reported results are from models using the appropriate covariance structure.

Analysis of DCS and LCS used SVL as a covariate. ANOVA/ANCOVA methods at each time period were also used to confirm the results of the repeated measures. Investigation of the allometric relationship between DCS and LCS also included model tests to determine if the interaction of treatments and SVL or population and SVL was significant. Significant interaction terms indicated significant differences in the slope of the allometric relationship between head and body size. If the interaction term was not significant, but ANCOVA methods suggested TRT or POP was significant, the intercept of the regression line was significantly different, but not the allometric slope.

Amount of prey consumed and change in SVL were evaluated at the end of the experiment (Day 480). Differences among treatments and populations were tested using ANOVA/ANCOVA analyses. Change in SVL was calculated by subtracting the snake's SVL at Day 0 from its final SVL at Day 480. Mass of prey consumed was taken as the sum of the mass of all meals successfully ingested and retained. While some snakes refused some meals of thawed rodents, no force-fed snakes regurgitated any meals over the course of the study.

Trophic morphology shape was analyzed using MANCOVA for each time period (Zar, 1999). To test for significant differences in overall shape among treatments and populations, shape variables were tested by using multivariate analysis of covariance with centroid size (CS) as the covariate. Previous model selection investigations on this species suggest that CS is the best covariate to use, as opposed to SVL (Smith, 2006). Treatment, population, and their interaction with the covariate were used as fixed effects, while Litter was used a random effect.

At each time period, the RW scores were subjected to a Principle Component Analysis on the covariance matrix. Since RW scores were originally obtained from the dataset containing all time periods, a PCA will rotate each time period's data to best explain the variation present in the RW scores. PCA on shape data is basically a data transformation method, where models are evaluated using the PC scores, but shapes are visualized from the original data (RW scores).

Change in shape between time periods for each individual was also measured. Geometric morphometrics allow researchers to quantify any change in shape, whether from a consensus configuration or between two individuals. The magnitude of shape change was quantified using Procrustes distance (Bookstein, 1997; Zelditch et al., 2004). Each configuration had a Procrustes distance calculated by taking the square root of the sum of all the squared Procrustes residuals for that specimen. The Procrustes distance is the amount of deviation of a particular

configuration from the mean. Procrustes distance values for each individual over the 4 times steps (Day 0 to 120, Day 120-240, etc.) were calculated and tested for POP and TRT differences using methods similar to those employed for SVL, DCS, and LCS.

Tests to determine if TRT had an effect on the amount of variation in head shape were conducted by calculating TRT disparity. Disparity is a measure of the amount of variation in head shape a particular group has (Viguier, 2002; Zelditch et al., 2003). Disparity is calculated using Procrustes residuals, which are the squared sum of all the landmarks away from the mean of that landmark (Bookstein, 1997; Zelditch et al., 2003; Zelditch et al., 2004). Observed values of treatment disparity were calculated at each time interval, and then data were shuffled between rows. New disparity values were calculated for each randomization, 9999 iterations were run noting each time a value was greater than or equal to the observed value.

All analyzes were performed using R version 2.14.1 (R Foundation for Statistical Computing, 2011) using the nlme library (Pinheiro and Bates, 2000). Additional testing, validating, and graphics were done using JMP versions 9.0.2 (SAS Institute Inc.), Excel 2003 (Microsoft Corporation) with the PopTools addin (Version 3.2, www.poptools.org), and Systat 13 version 13.00.05 (Systat Software Inc.).

Results

Snakes in TRTs 1 and 3 significantly varied in the number of meals they voluntarily ingested. Snakes were not force-fed whole rodents to avoid possible confounding influences on trophic morphology. Some individual refused up to 60% of their meals. To reduce the consequences of poor feeding behavior on our analyses, we eliminated three snakes from TRT 1 (287-10, 290-5, and 297-2) and four snakes from TRT 3 (287-4, 291-6, 292-4, and 297-1). Snakes were eliminated if the amount of prey they consumed fell outside the 95% confidence interval for their

treatment. Sample sizes after the elimination of non-feeders were: TRT 1 = 14, TRT 2 = 19, TRT 3 = 14, and TRT 4 = 18, for a total sample size of 65 individuals from 3 populations and 8 litters.

Force-fed treatments could not 'refuse' meals, so they were unaffected, but the refusal of meals also resulted in significant differences among all four treatments in the amount of prey consumed (ANOVA – $F_{3,41} = 165.3287$, $P < .0001$). To get around this large difference in resource level, treatments were reorganized based on prey size alone (whole rodents – R, force fed – T, sample sizes were 28 & 37 respectively), while prey consumed was changed to a continuous variable to be investigated with the fixed effects of TRT and POP. There was still a significant difference in the amount (grams) of prey consumed between R (avg. = 369.53 g, SE = 33.56)& T (avg. = 497.59g, SE = 29.2) treatments (ANOVA – $F_{1,63} = 8.2874$, $P = 0.0054$), but since it was incorporated as a continuous covariate, those differences can be accounted for.

Snout to vent length (log transformed) showed no significant differences between prey size treatments during the course of the study. Repeated measures procedures indicated that the unstructured covariance structure was most appropriate (AIC comparisons), and the ANOVA returned only the variable of Day as significant ($F_{3,315}=760.09$, $P<.0001$). Whereas each treatment showed an increase in SVL over time, neither the treatment means nor the pattern of change over time was significant (Food type: $F_{1,315}=1.11$, $P = 0.2918$; Food*Day: $F_{4,315}= 1.57$, $P = 0.1823$; Figure 1).

Body size (SVL) comparisons among populations suggested there was a significant difference in the change in SVL over time. Repeated measures (unstructured covariance) was significant for Day ($F_{4,310}=788.6$, $P <.0001$) and the interaction of Day and POP (Day*POP: $F_{8,310}= 2.67$, $P =0.0075$), but not for POP ($F_{2,310} = 2.8$, $P = 0.0622$). Examination of each time period individually suggested that snakes from the Kuhn population were significantly small than

Miller snakes at Day 0, but all other comparisons were not significant. The significance of the interaction term in the model stems from the one change from Day 0 to Day 120 (Figure 2).

Repeated measures ANOVA on mass (LogMass) revealed the same pattern as SVL. Only the variable Day was significant for the model testing for prey size differences (Day: $F_{4,315}$ = 371.016, P <0.0001). Figure 3 shows that while mass consistently increased during the course of the study, food type treatments were never significantly different. Comparisons of populations yielded significant Day and POP terms (Day: $F_{4,310}$=394.92, P <0.0001, POP: $F_{2,310}$= 13.92, P <0.0001). Once again comparisons at each time period separately suggested the significance stems from the Kuhn snakes being significantly smaller than the other populations at Day 0 (Kuhn: avg. = 22.55g, SE=1.13; Gill: avg. = 32.38, SE=1.01; Miller: avg. = 32.3, SE=1.13). Figure 4 shows the log transformed data over time, illustrating that Day 0 had the largest separation during the course of the study.

Dorsal and lateral head size (DCS & LCS) were significantly different for both prey type (Food) and population (POP). Repeated measures ANCOVA for DCS with SVL as a covariate varied significantly over time (Day: $F_{4,314}$=38.21, P <0.0001) and treatments (Food: $F_{1,314}$ = 4.28, P 0.0393). The model suggested that force-fed snakes had smaller head sizes at the onset (Figure 5), but once Population was accounted for, treatment differences were no longer significant. The same could not be said for LCS, which showed significant differences among Days ($F_{4,309}$ = 17.48, P <0.0001) and Food ($F_{1,309}$ = 8.74, P = 0.0034). When POP was accounted for, Food was still significant ($F_{1,312}$ = 7.57, P = 0.0063), but when each time period was examined, the significant difference was only present at Day 0.

Populations were significantly different for both DCS and LCS for the entire duration of the study. DCS was significantly different over time (Day: $F_{4,309}$ = 32.23, P <0.0001), among

populations (POP: $F_{2,309} = 15.86$, $P < 0.0001$), and differed in how it changes over time (POP*Day: $F_{8,309} = 2.00$, $P - 0.0455$). Regression analysis of the allometric relationship for each time period revealed that while the slope of the line relating DCS to SVL was not significantly different, Miller snakes consistently had higher intercept values (Figure 6). LCS demonstrated a similar pattern, with the exception of a significant POP*Day interaction term. (Day: $F_{4,309} = 18.39$, $P < 0.0001$, POP: $F_{1,309} = 12.56$, $P < 0.0001$). Allometric regression analysis revealed the same pattern as DCS, with no significant difference in the slope of the regression lines, but a significantly higher intercept value for the Miller population (Figure 7).

Comparisons of the magnitude of shape change over each successive time period yielded no significant comparisons. Shape change was quantified using Procrustes distance for both the dorsal and lateral aspects of morphology. Dorsal shape change did not differ among populations or prey sizes at any point in the study (Figures 8 and 9). Change in lateral shape showed the same pattern, with no significant differences among treatments or populations during the course of the study (Figures 10 and 11).

Variation in head shape, as measured through disparity, yielded no consistent patterns. Since no patterns were apparent, and the difference in prey consumed between food treatments varied over time, Days 0 and 480 were the only days examined in detail for differences in disparity. At Day 0, the Gill population was less variable than the other two populations (G/M = 0.669, *Prand* = 0.0029, G/K = 0.694, *Prand* = 0.0066) in lateral shape variation, but no population differences were present for dorsal shape. Prey type treatments differed at Day 0 in the dorsal aspect (R/T = 0.674, *Prand* = 0.0126), with whole-rodent fed snakes having about 32% less variation in head shape. There were no differences in shape variation in the lateral aspect between food treatments. Disparity at Day 480 lacked any significant differences among

populations or treatments. Neither the dorsal nor lateral aspect of shape showed any differences among groups.

Head shape was analyzed using both relative warps scores and principal component scores. The dorsal aspect of head shape utilized 19 relative warps scores, all explaining over 0.5% variation (used as the cutoff) for a total of 96.69% of the total variation explained. At each time point (Day) a principal component analysis (PCA) was done on those 19 relative warps scores and only the significant principal component scores were used to test the models. For Day 480 of the dorsal aspect 12 PCA scores were used which explained 96.19% of the variation. For the lateral aspect, 17 relative warp scores were used which explained 96.56% of the variation. Lateral PCA of Day 480 provided 11 PCA scores accounting for 96.42% of the variation.

Analysis of head shape took into account the previous results before proceeding. Day 0 models indicated significant differences among populations. To attempt to account for these initial differences, the Miller population was removed from the analysis and examined separately. The reasoning behind this decision was three-fold. First, Miller snakes had larger head sizes for a given body size for the entire duration of the study regardless of treatment (Figure 6 and 7), this difference in size would confound any possible patterns of response to prey type. Secondly, previous work in this system suggested that the Miller population was unique and should be considered a separate entity (Smith, 2006). In fact, after removing Miller snakes, the POP was no longer significant. And lastly, Litter was added as a random effect in the shape models to account for the variation among litter-mates. The Miller population consisted of 20 snakes from a single litter, confounding the two variables and necessitating their removal. This

left a total of 45 individuals, 25 from Gill and 20 from Kuhn. Prey size treatments consisted of 18 snakes in the whole-rodent treatment, and 27 snakes in the force-fed treatment.

There was no significant difference between populations (Gill & Kuhn) or between food types (R & T) at Day 0 for the dorsal aspect of head shape. The selected model showed that only the covariate of DCS was significant (MANCOVA: Pillai's Trace = 1.39, $F_{42,90}$= 1.8599, P = 0.0073; DCS: $F_{14,28}$=7.325, P <0.0001). Lateral head shape at Day 0 provided similar results (Pillai's Trace = 1.12, $F_{33,99}$ = 1.7897, P= 0.0147), but the covariate only approached significance (LCS: $F_{11,31}$= 1.9476, P = 0.0712). Litter was left out of the comparisons with both the Food and POP variables due to a loss of degrees of freedom, but separate models indicated that Gill and Kuhn populations did not significantly differ at Day 0.

Day 480 yielded no significant differences in dorsal or lateral head shape between populations. Since degrees of freedom were a concern, population was modeled separately from prey type. Models included the covariate of centroid size, POP, and the continuous variable of prey consumed. Dorsal head shape MANCOVA for populations differences was not significant, even the covariate was not significant (Pillai's Trace = 1.006, $F_{36,96}$= 1.3465, P = 0.1278). The lateral head shape MANCOVA model was significant (Pillai's Trace $F_{33,99}$= 1.6799, P = 0.0264). Again the covariate was not significant, but prey consumed was ($F_{11,31}$= 2.2882, P = 0.0346).

Models testing for prey size differences included the covariate of centroid size, prey consumed, Food (R or T), and Litter as a random effect. The dorsal Day 480 model suggested that after accounting for variation among litters, prey size treatments were significantly different in shape ($F_{12,25}$ = 2.7377, P = 0.0162). The covariate of centroid size was also significant along with the model as a whole (CS: $F_{12,25}$ = 2.2586, P = 0.0415; Pillai's Trace = 2.863, $F_{96,256}$ =

1.4859, $P = 0.0076$), but the amount of prey consumed over the study was not significantly associated with dorsal head shape. Lateral head shape yielded similar results (MANCOVA: Pillai's Trace = 4.297, $F_{144,252}$=1.5992, $P = 0.0006$). For lateral head shape, once litter effects were accounted for, the model was significant for both the amount of prey consumed ($F_{16,20}$= 2.9054, $P = 0.013$) and prey size treatment ($F_{16,20}$= 3.2098, $P = 0.0076$). Miller populations yielded no significant comparisons between food treatments for either dorsal or lateral aspect.

Discussion

The size of prey items ingested by some snake populations can induce phenotypic plasticity in head shape. The results not only support the presence of environmental influence on trophic morphology, but also that not all litters respond to those influences in the same magnitude or direction. A difference in the responses of some litters to diet manipulations demonstrates that variation in plasticity is present.

Body size and mass variables were not significantly affected by the size of prey ingested. Even though the amount of prey consumed by the two prey size treatments differed significantly, the original design of the project created an overlap and there was no confounding affect between prey size and resource level that could not be accounted for. Population differences that were present at the beginning of the experiment were no longer statistically significant by the end. The Miller snakes were consistently the largest snakes in the study, but changes in SVL over time did not differ among populations or food treatments. The significant interaction between population and time for SVL and Mass ANOVAs was due to the initial difference between Kuhn and Miller populations. From Day 120 on, there was no difference in how food treatments or populations changed SVL or Mass. Therefore, growth rate differences were not present and did not play a role in the morphological differences.

DCS and LCS differences between Miller and the other two populations show that significant variation in trophic morphology may be present at birth (or very early in development). Miller snakes had larger heads for any given body size throughout the study. The observed pattern supports earlier investigations that demonstrated trophic morphology differences present in large snakes were also present in juvenile snakes (Smith, 2006). Our data are congruent with those from previous studies on the plasticity of snake morphology. Aubret and Shine (2009) documented differences in plasticity and canalization of relative head size in island Tiger snakes (*Notechis scutatus*). Island populations of Tiger snakes that had been isolated for extended periods of time were characterized by litters containing large neonates with relatively larger heads than mainland populations. These island populations were hypothesized to have adapted a larger relative head size in response selection for the ability to consume larger prey items. Similar results were found in some fish populations (Marcil et al., 2006), where cod populations responded differently to temperature influences on body shape.

Phenotypic plasticity is often considered to be the opposite of canalization (Whitman and Agrawal, 2009), but it is possible to canalize reaction norms (Scheiner, 1993) and often holding one trait constant in a variable environment requires plasticity in other traits. Some insects demonstrate canalized egg size, but a variable environment results in plasticity in clutch size (Stearns, 1992; Nylin and Gotthard, 1998), while other species show the reverse (canalization of clutch size) (Fox and Czesak, 2000). Examples such as these suggest that canalization may be accomplished through phenotypic plasticity or an initially plastic trait can become canalized (Whitman and Agrawal, 2009).

The Miller population may be under different selective pressures than the Gill and Kuhn populations. The Miller site is located near the western border of North Dakota, while the Gill

and Kuhn sites are located approximately 280 Km to the east in north-central South Dakota. There are several other possible causes for the observed difference in the Miller snakes. First, the Miller population consisted only of a single litter and so the observed response may be limited. Second, differences may be correlated with geographic differences in overall body size combined with the allometry of trophic structures (Forsman and Shine, 1997). Third, stochastic processes such as genetic drift or founder effects could have shaped population differences (Lande, 1979). Fourth, the Miller site was specifically chosen because of previous research (Smith and Collyer, 2008), presence of some unique phenotypic traits (Smith, 2006), and the presence of very large females that produce large litters (Smith, unpublished data).

Lastly, geographic differences in ecological conditions (food supply, temperature) could produce maternal effects. Possible parental/maternal effects are most likely responsible for the observed differences in the response by Miller snakes. Other reptile species have shown that larger mothers produce larger offspring (Lindeman, 2000). The Miller snake was the largest snake, so the difference in relative head size could be due to a greater overall investment in offspring size. Female map turtles (*Graptemys)* with larger alveolar surfaces produced offspring with relatively wider heads (Lindeman, 2000). Parental/maternal effects could also indicate transgenerational transfer and/or plasticity (Badyaev and Oh, 2008;Uller, 2008; Badyaev and Uller, 2009) Transgenerational transfer which initially begins with phenotypic retention or adaptive plasticity can set an evolutionary cycle in place that ends in the eventual genetic determination of the original trait (Badyaev, 2005; Badyaev, 2009; Badyaev and Uller, 2009). Some finches have shown the ability to alter sex ratios of their offspring, creating a sex-bias in ovulation sequence. This sex-bias is environmentally determined, with females possessing the plasticity to determine the sex of each egg in sequence. While newly established populations of

these finches require induction of this plasticity through environmental cues, native or long established populations do not require the environmental cues (Badyaev and Oh, 2008).

The possible parental/maternal effects witnessed in the Miller litter for the shape (see below) and size of trophic morphology may represent current transgenerational plasticity, a genetically assimilated production of large offspring, or a discrete allocation decision. Adaptive phenotypic plasticity may stimulate evolutionary diversification by generating novelty (West-Eberhard, 2003). In addition to the Tiger snake and finch examples already cited, adaptive plasticity has been cited as the evolutionary source for alternative mating tactics in dung beetles (Simmons et al., 2007). Traits initiated under phenotypic plasticity may contribute to diversification and even speciation. Often cited as Lamarckian evolution, phenotypic plasticity can lead to genetic assimilation and even speciation, but via Mendelian processes (see Whitman and Agrawal, 2009). The pathway from phenotypic plasticity to speciation may include phenotypic accommodation, genetic accommodation, the Baldwin effect, and genetic assimilation; but most certainly in not constrained to a single trajectory (West-Eberhard, 2003; Uller, 2008; Badyaev and Uller, 2009; Whitman and Agrawal, 2009). We lack the empirical data to draw any conclusions, but our results highlight the need for continued investigations into environmental induction of phenotypes, possible adaptive maternal effects, and the evolution of traits and populations.

Dorsal and lateral head shape differences between prey type treatments suggest that the Gill and Kuhn populations possess the ability to modify the shape of their trophic morphology in response to increased prey size. Dorsal head shape of snakes in the whole rodent treatment was characterized by a broader snout, or widening of the anterior portion of the head when compared to snakes in the force-fed treatment (Figure 12). Feeding on larger prey items appears to have

induced an overall broader head from the rostral scale back to the rictus of the jaw (Figure 13). Along with this broader shape, the supraocular scale has been shifted slightly posterior and closer to the midline of the body. The observed shift in ocular position may be due to the other morphological changes or an artifact of other morphological features our landmark placement did not detect. The shape difference between treatments relates to a positive or accelerative (Schuett et al., 2005) influence of larger prey size on the development of trophic morphology.

Gill and Kuhn snakes from the force-fed treatment were characterized by narrower heads and less pronounced quadrate bone (or the area where the quadrate bone is). Force-fed snakes exhibited a much more pronounced point to the head but a less pronounced posterior aspect. The posterior aspect slowly tapers off in force-fed snakes, compared to the more traditional spear-shape head of the snakes fed whole rodent. The qualitative shape differences between rodent and force-fed treatments fall in line with a use-induced change in shape (Padilla, 2001). It should be expected that snakes feeding on larger prey items should have broader and /or longer heads for a given body size (and in head size). Change in either or both of those dimensions would allow snakes to ingest larger prey items with less handling time (Arnold, 1983; Shine, 1991; Forsman and Lindell, 1993; Vincent et al., 2009).

Snakes from the Miller population seem to be an intermediate shape between the two extremes of the feeding treatments. There was no significant difference between food types for Miller snakes, but Figure 12 shows both treatment averages and how little morphological deformation there is. Sample sizes for the Miller population were smaller (n=20) than that of Gill and Kuhn combined (n=45), so consensus configurations may be driven by spurious individual differences. Miller snakes appear to have increased overall head size instead of altering the shape of trophic morphology.

Lateral head shape showed the same pattern among populations; with Miller snakes having an intermediate shape to the two food treatments of Gill and Kuhn snakes. Figure 14 shows the relationship of head shape among the feeding treatments and Miller population. Once again Miller snakes did not significantly differ in lateral head shape between rodent and force-fed, but both treatment mean configurations are shown. Unlike the dorsal aspect, the shape difference between feeding treatments of Gill and Kuhn snakes was not as predicted. Snakes fed whole rodents had a shallower head shape that was relatively shorter than those snakes that were force-fed (Figure 14). Snakes fed whole rodents appear to have their trophic morphology pulled in opposing directions from opposite corners, meaning the deformation in the snout is up and out, while the area around the quadrate is pulled down and in. Snakes in the force-fed treatment had a blunter snout accompanied by a slight shifting of the supraocular scale anteriorly. At the posterior aspect of the head, force-fed snakes had a deeper quadrate region will more of a blunt end (see Figure 15 for exaggerated differences).

Geographic and/or population differences in the size and shape of trophic morphology could have significant effects on the fitness of individuals. If stochastic differences in prey encounters and foraging success result in a significant difference in the trophic morphology of individuals, groups, or populations, then those individuals that successfully obtain larger prey items may be able to expand their prey base and exploit resources that are too large for other groups to consume. The resulting dimorphism in prey base and trophic morphology would result in an even greater divergence in not only trophic morphology, but overall size, reproduction, and fitness. Dimorphisms in trophic morphology are typically associated with differences between the males and females (Slatkin, 1984; Shine, 1991; Casselman and Schulte-Hostedde, 2004; Blanckenhorn, 2005; Bulté et al., 2008); but similar differences could arise with any group with

ecological divergence in diets. Increase in the size or shape of trophic morphology could give rise to an increase in the energy intake of those individuals. Increased energy allows for a greater allocation towards reproduction (Bulté et al., 2008). Larger females may produce larger offspring and/or have larger clutches (Andrews and Pough, 1985; Nagle et al., 1998). While males are considered to have less of a selective advantage than females in regards to increased size (Bulté et al., 2008), in species with male-male competition for mates, such as rattlesnakes, larger males would obtain more copulations and possibly increase their reproductive success (Shine, 1989; Duvall and Beaupre, 1998). Expanding on the reproductive role or dimorphic niche hypothesis (Slatkin, 1984; Shine, 1991), a trophic morphology induced by exposure to larger prey items may result in increased fitness for those individuals and possible divergence over time.

Previous work on snakes and possible plastic responses to prey size and resource level have generally concluded that plasticity in trophic morphology is not present (but see: Aubret et al., 2004; Aubret and Shine, 2009). Laboratory and field based projects have demonstrated multiple snake species exhibit alterations in growth rates that are significantly influenced by environmental factors (Madsen and Shine, 1993; Forsman, 1996a; Scudder-Davis and Burghardt, 1996; Bonnet et al., 2001), but rarely have those influences lead to differences in trophic morphology.

The majority of work done in this field has failed to quantify all three possible aspects of influence on trophic morphology. Prey size, resource level, and possible genetic factors interact to determine trophic morphology. Studies that have examined prey size before, have failed to account for resource level (Queral-Regil and King, 1998; Bonnet et al., 2001; Vincent et al., 2004). Only two previous studies have carefully controlled and/or accounted for all three possible influences on trophic morphology. Aubret *et al*. (2004) found Tiger snakes could

significantly alter their trophic morphology in response to prey size and that the response varied among populations. Schuett *et al* (2005) controlled for prey size, amount, and used only a single litter to reduce genetic variation. That study found snakes did not have the ability to alter their trophic morphology, a conclusion we would have matched if only Miller snakes were used.

Our study is only the second study of snake morphology so far to clearly demonstrate the plastic nature of trophic morphology. The observed treatment differences suggest that not only is plasticity present in the Prairie rattlesnake, but that all three factors (prey size, resource level, and litter) have varying influences on morphology. Prey size clearly affected head shape in Gill and Kuhn populations, and in the dorsal aspect in a predictable direction. The lack of change in the Miller population, combined with its larger relative head size suggests there is regional variation in this phenotype. Resource amount was significant in explaining variation in lateral head shape, but not dorsal. While it is possible it was a spurious result, the counter intuitive shape difference in the lateral aspect along with the fact that force-fed snakes consumed significantly more prey mass suggests that the lateral aspect of trophic morphology is more influenced by energetic intake than size of prey.

This study has improved upon previous research in several ways. First, the use of rodent homogenate instead of two different prey types, we avoided difference in energetic and nutrient content that may lead to differences in SDA and available metabolites (Secor and Faulkner, 2002; Zaidan III and Beaupre, 2003). Second, although many snakes refused meals and derailed the original design, our treatments still were able to account for both the amount of resources a snake ingested and the size of those food items. Third, uses larger samples sizes and multiple populations. Our increased sample size provided stronger statistical power to account for multiple influences. Fifth, by carrying out diet manipulations for 480 days, our study has the

longest duration of diet manipulations to date. Lastly, the use of geometric morphometrics instead of univariate linear measurements provides for a much more detailed quantification of trophic morphology.

Future improvements to studies on the plasticity of snake morphology should include investigations into possible differences in SDA costs and digestibility of homogenized rodent and whole rodents (Panizzutti et al., 2001). The use of homogenate was designed to minimize nutritional and energy content differences in prey, but no prior work was done looking at possible differences in the energetic costs associated with digestion and absorption of homogenized rodents. The documented shape differences in this study cannot be definitively partitioned to changes in soft tissue or bone. The use of radio-graphs (Schuett et al., 2005) or other imaging methods would not only provide images better suited for landmark placement, but also greater detail and contrast on the response of skeletal and muscular components of trophic morphology. Use of multiple types of images would provide the best and most accurate information about changes in morphology. The continued use of larger sample sizes and longer study durations will also help provide further insights. Studies should also continue to account for all three possible influences on trophic morphology and begin to test for their interactions.

In conclusion, this study provides some of the most definitive evidence that snakes have the ability to alter their trophic morphology in response to environmental cues. The plastic response in morphology is not universal and may be limited to certain populations/litters or absent in a proportion of individuals. The different response to prey sizes elucidates the two main strategies possible for selective advantages in trophic morphology. A population can either evolve with the ability to alter their trophic morphology in response to reliable environmental cues (in this case ingested prey items), or selection can favor increases in overall body and head

size at birth. A third option would be differences in the allometry of body size and head size, but that was not witnessed in this study. Our results also show there is some degree of predictability in shape change, and there is evidence to support the use-induced theory of predator inducible offense phenotypes (Padilla, 2001).

The concepts of adaptive phenotypic plasticity (Forsman and Shine, 1997), inducible offense (Padilla, 2001), and reciprocal plasticity (Kishida et al., 2006) encompass a wide range of ecological and evolutionary interactions which still need elucidation for many groups of organisms. Future work should continue to improve upon this and other studies, eventually moving out of the laboratory and into the field. The ultimate goal should be to find the underlying mechanisms of morphology change and the evolutionary forces that shaped a population's ability (or lack of) to respond to changes in the prey community and what those adaptations mean for population fitness.

Acknowledgements

I thank Dr. Steve Beaupre for his insights and input into this project as well as the logistic support he offered. I thank my dissertation committee: Drs. William Etges, Gary Huxel, and Giovanni Petris for their input and direction through the course of my degree. This project would not have been possible without the input from fellow lab members: Jason Ortega, Joe Agugliaro, James Van Dyke, Marshall McCue, and Lara Douglas. Suggestions on experimental design, setup, and data analysis from Drs. Mark Davis and Michael Collyer were instrumental in the completion of this project. Field collection was made possible through the permission of many private land owners and the help of Mark Davis and Joe Agugliaro. This project was conducted with the approval of the University of Arkansas IACUC committee (Protocol # 10039) and the primary author was supported by a Walton Distinguished Academic Fellowship and Graduate

Teaching Assistantship from the University of Arkansas. All snakes were obtained under the proper permits from the North Dakota Game and Fish (#GNF02388267 & GNF02599962) and the South Dakota Department of Game, Fish, and Parks (#37 & 51).

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FIGURES

FIGURE 1: Boxplot of LogSVL treatment averages over time (+/- 2SE). Feeding treatments of snakes fed whole rodents (R) or force fed through a tube (T) were measured every 120 days over the course of the 480 day experiment. Marks on the X-axis correspond to alternating treatments (R than T) for each day (0, 120, 240, 360, and 480).

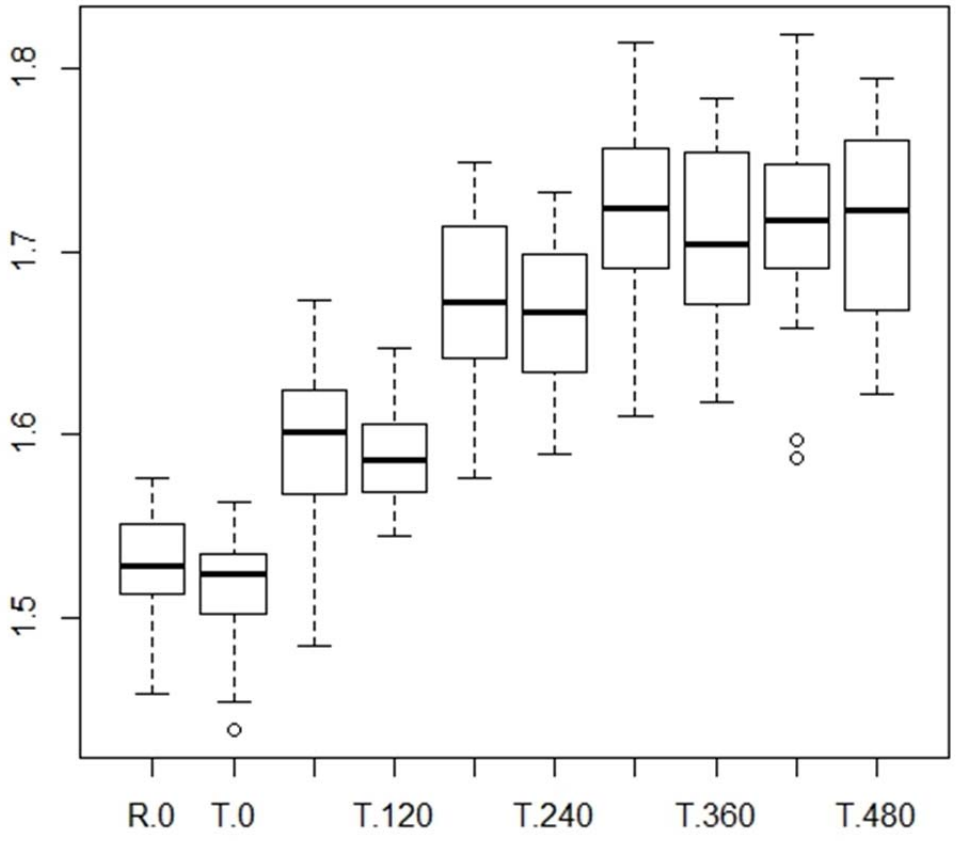


FIGURE 2: Boxplot of LogSVL mean values for each population (Gill – G, Kuhn – K, and Miller – M) over the course of the feeding study. Each population mean is given in sequence for every time interval. Error bars approximate a 95% C.I.

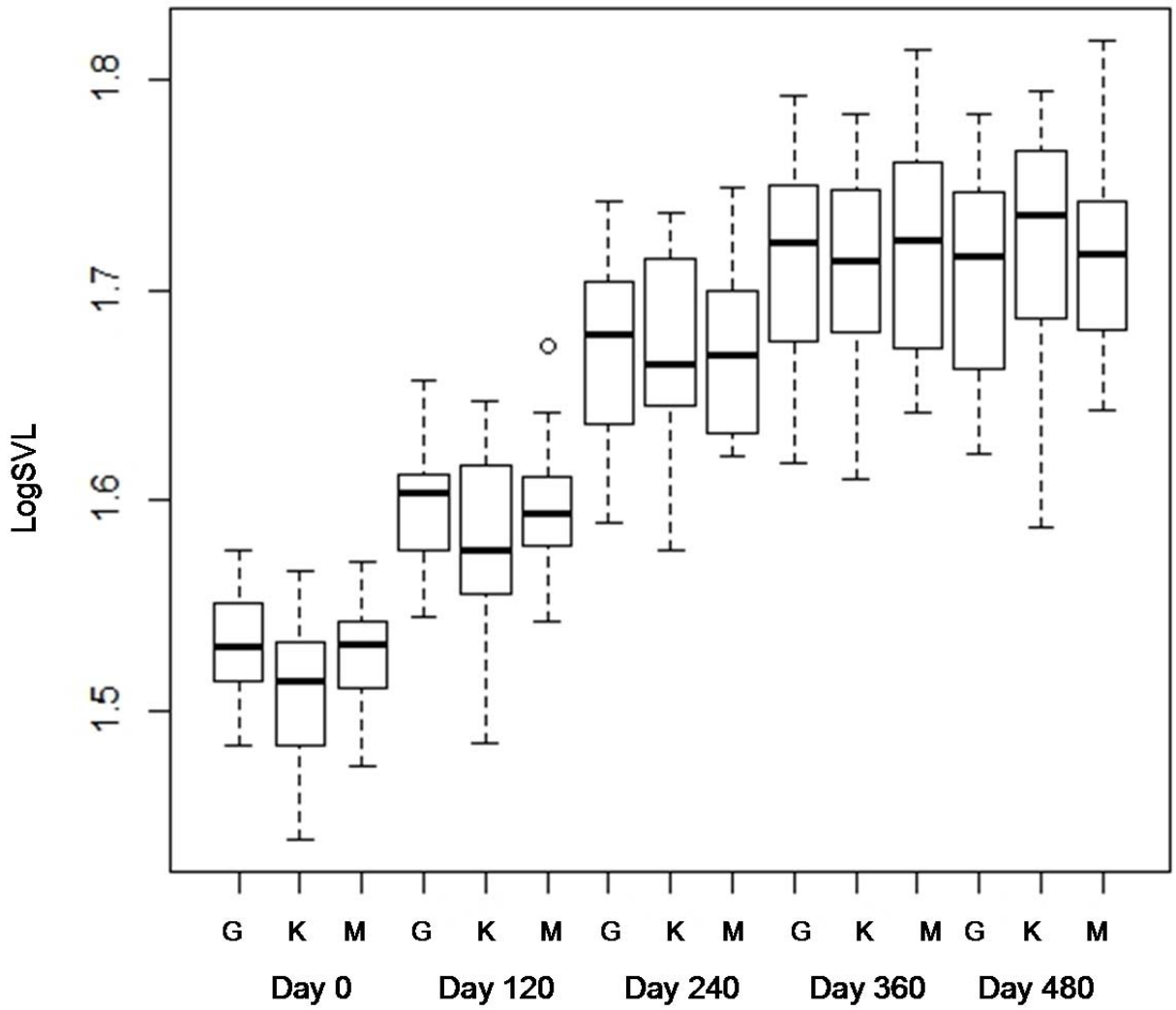


FIGURE 3: LogMass mean values for the feeding treatments over the course of the study. Snakes in the whole rodent (R) or force-fed (T) treatments did not significantly differ over time. LogMass values are on the Y-axis, while treatment and Day groupings are on the X-axis. Error bars approximate a 95% C.I.

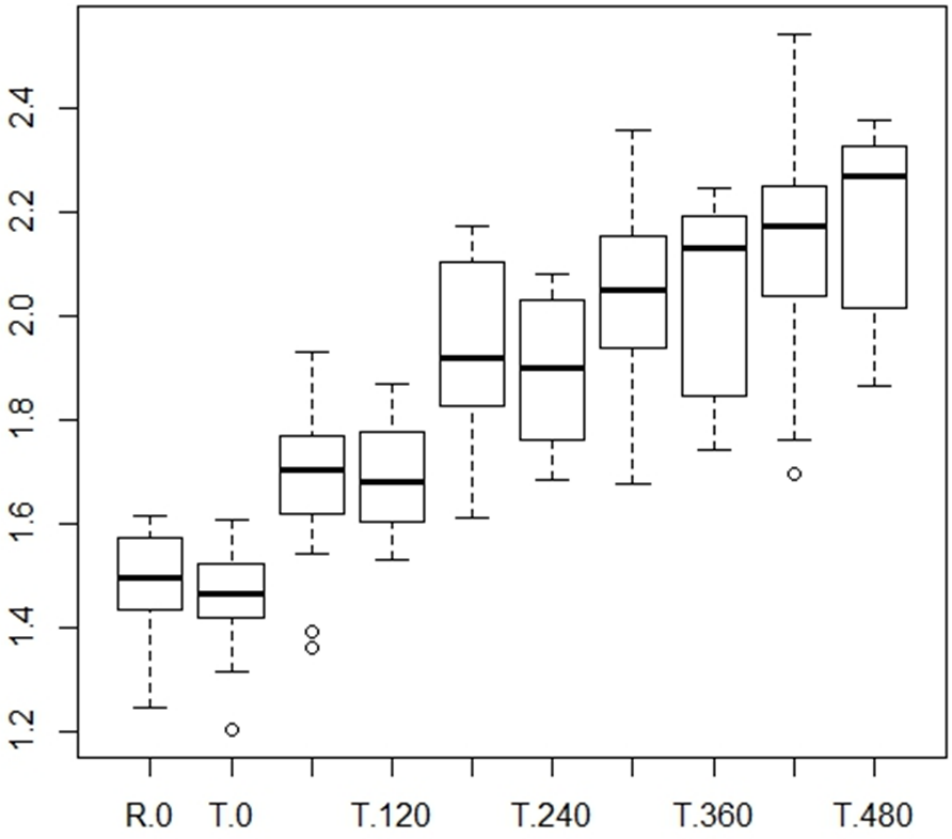


FIGURE 4: LogMass mean values for each population and Day grouping. Each population (Gill – G, Kuhn – K, and Miller – M) is shown in sequence over each time period. Error bars approximate a 95% C.I.

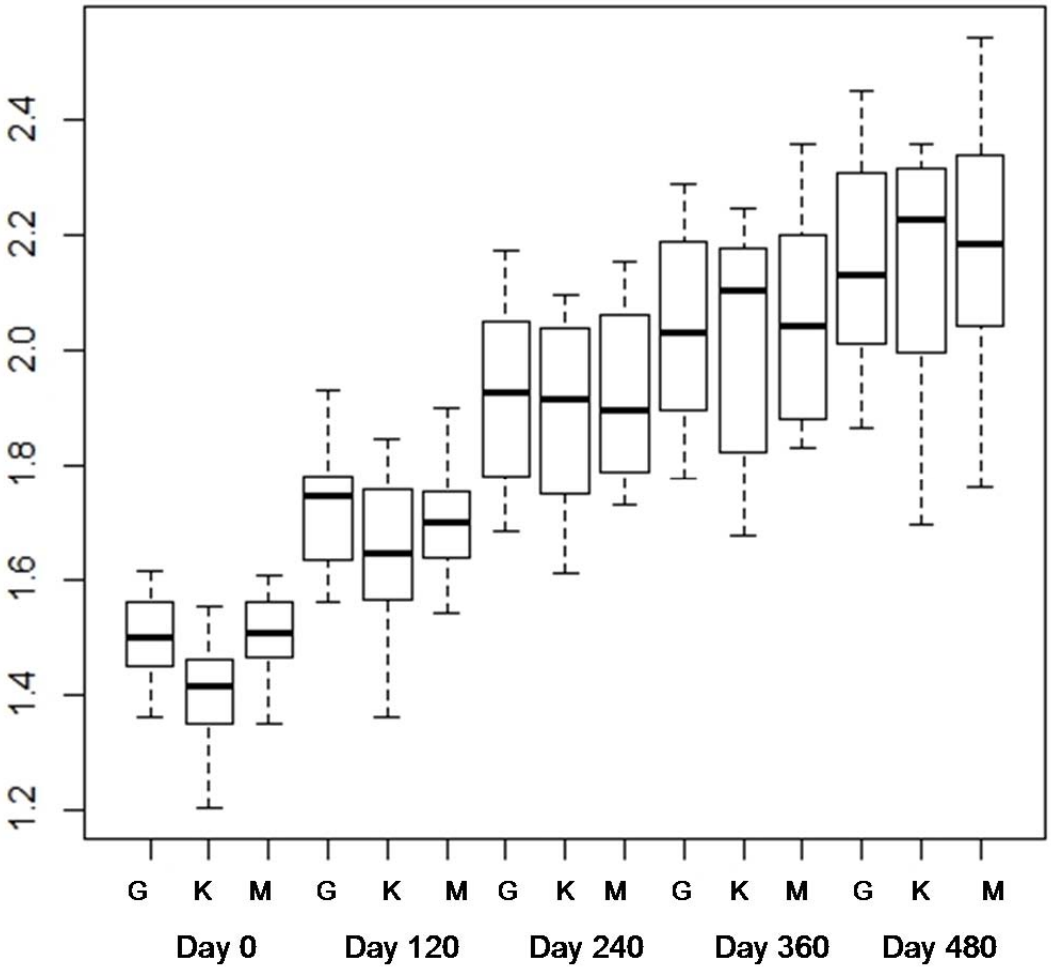


FIGURE 5: Dorsal centroid size (LogDCS) mean values for each prey size treatment over the course of the study. Whole rodent (R) and force-fed (T) snakes did not significantly differ in either centroid size aspect. X-axis shows treatment by Day groupings (R.0 = whole rodent treatment at Day 0). Error bars approximate a 95% C.I.

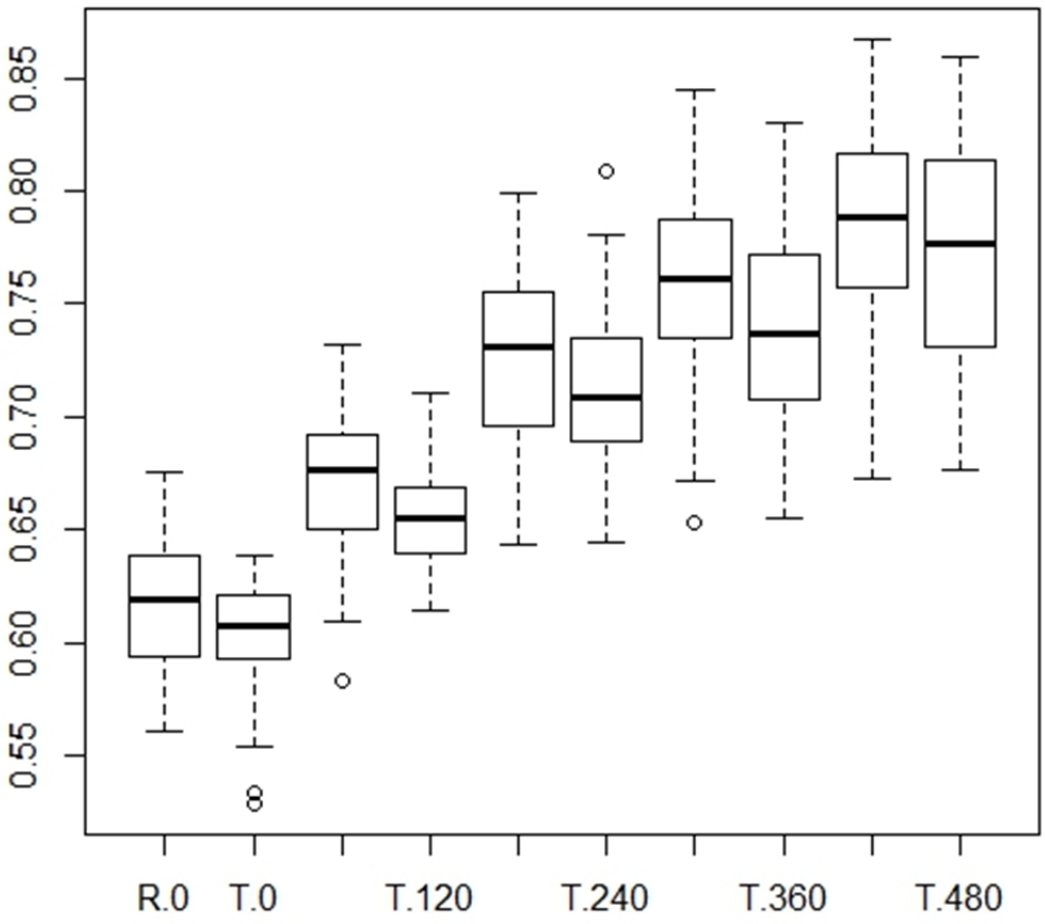


FIGURE 6: Plot of the separate regression lines for each population, showing the allometric relationship between body (LogSVL) and dorsal head size (LogDCS). Regression lines are not significantly different among populations, but the intercept values were. Miller snakes consistently had larger head sizes for a given body size.

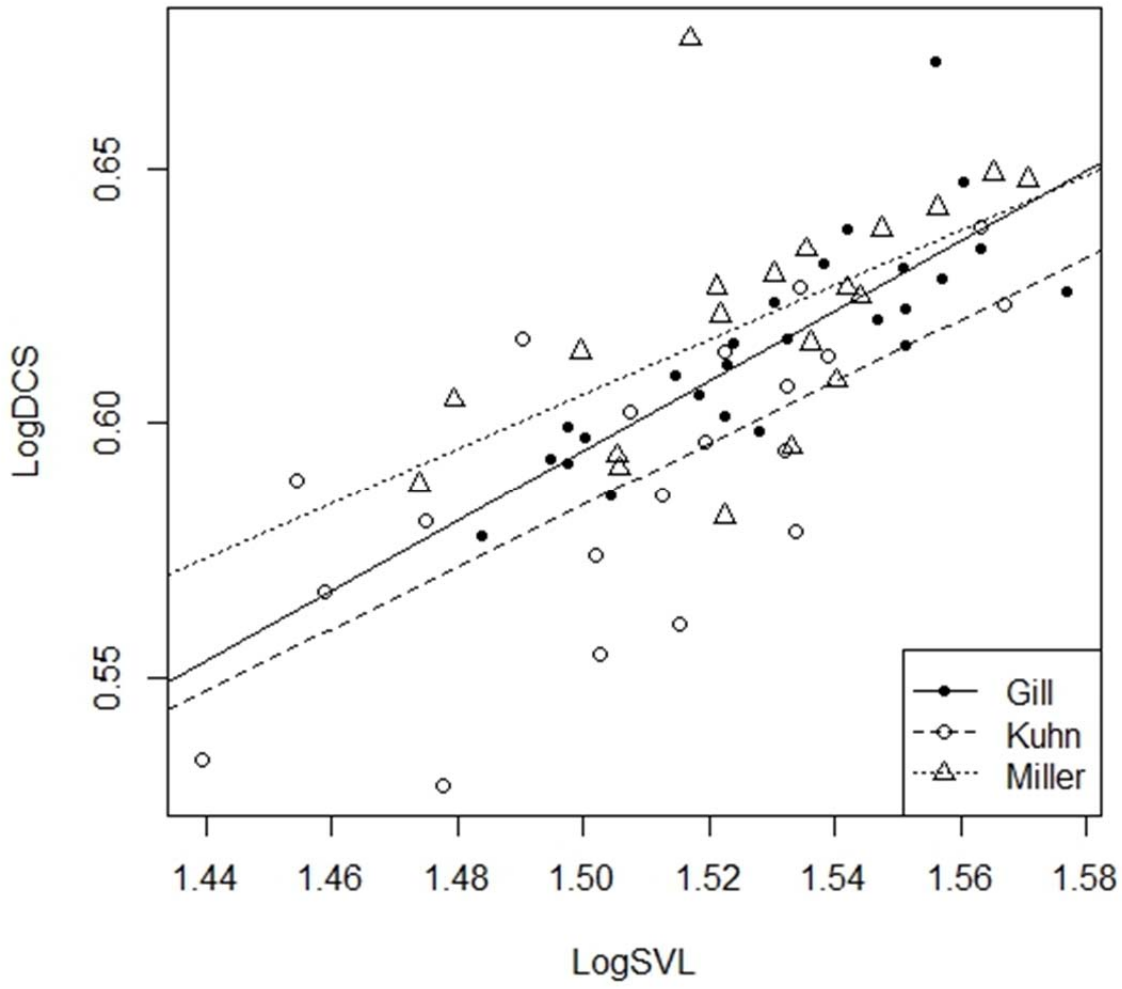


FIGURE 7: Plot of the allometric relationship between body size (LogSVL) and lateral head size (LCS). Separate regression lines are shown for each population, but there was no significant difference in slope among the treatments. Intercept values were significantly different, with Miller snakes having a higher value than the other two populations.

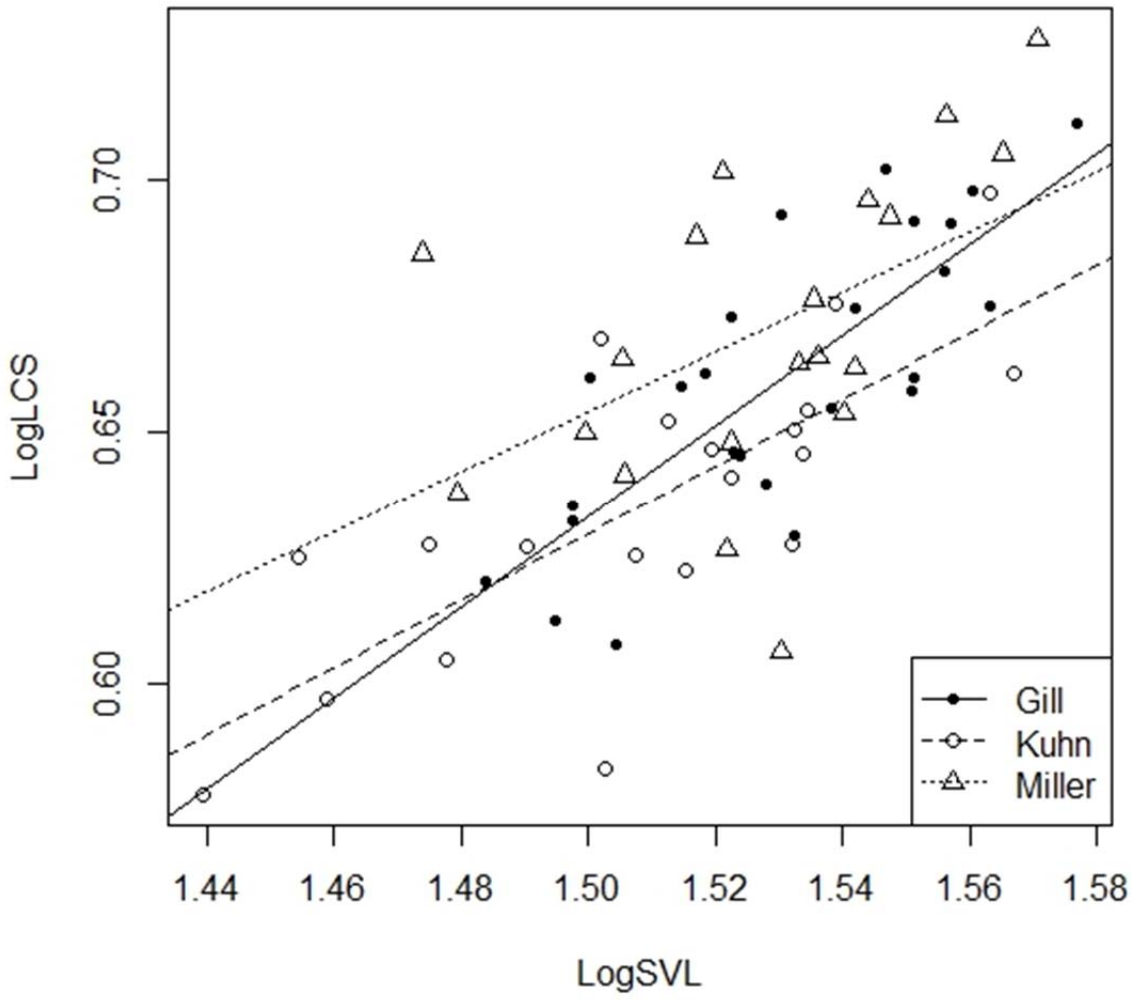


FIGURE 8: Graph plotting the mean Procrustes Distance for the dorsal aspect of trophic morphology at each time interval during the study. Mean population values are plotted with +/- 2 SE error bars. Procrustes Distance quantifies the magnitude of shape change from one time interval to the next (Day 0 to Day 120 = 120).

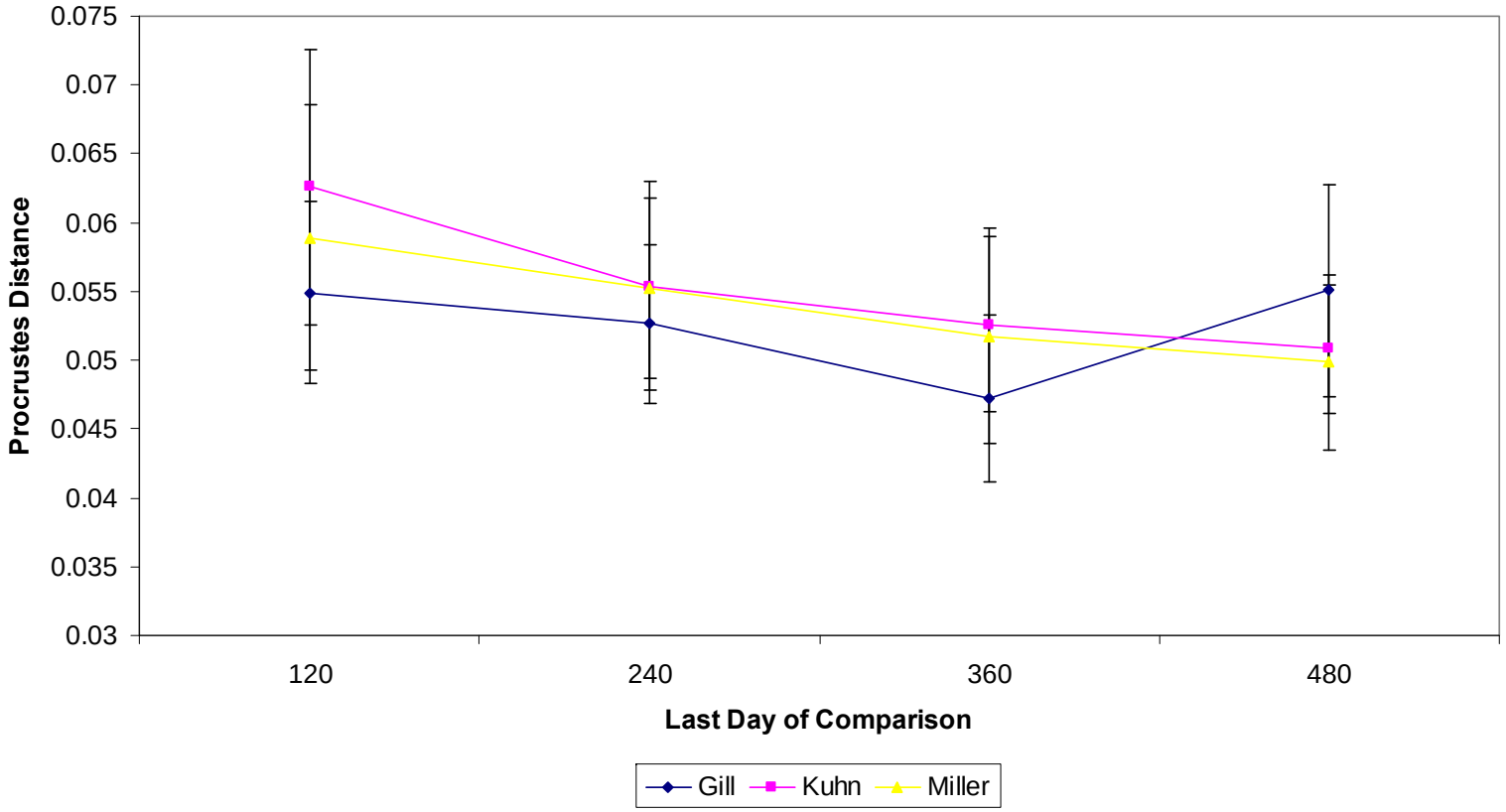


FIGURE 9: Mean treatment values of the magnitude of dorsal shape change (Procrustes Distance) for each time interval. Each point is the mean treatment value and error bars are +/- 2 SE. Time comparisons were always made over two consecutive intervals (Day 0 to 120 – denoted as 120 on the X-axis).

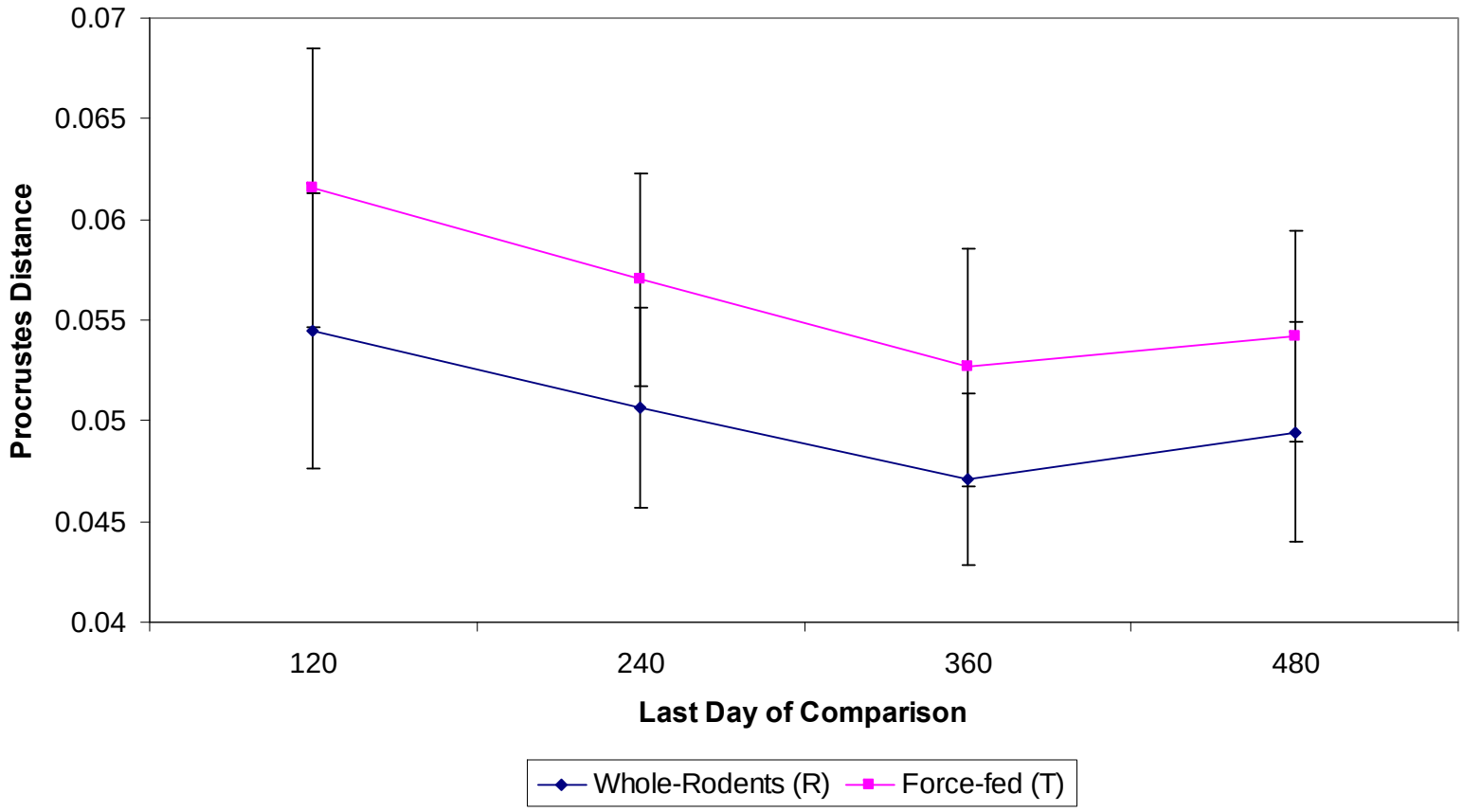


FIGURE 10: Plot of the magnitude of population shape change (Procrustes Distance) in the lateral aspect of trophic morphology for each of the four time comparisons during the study. Population mean values are plotted with +/- 2 SE error bars. There were no significant differences among populations in the magnitude of shape change.

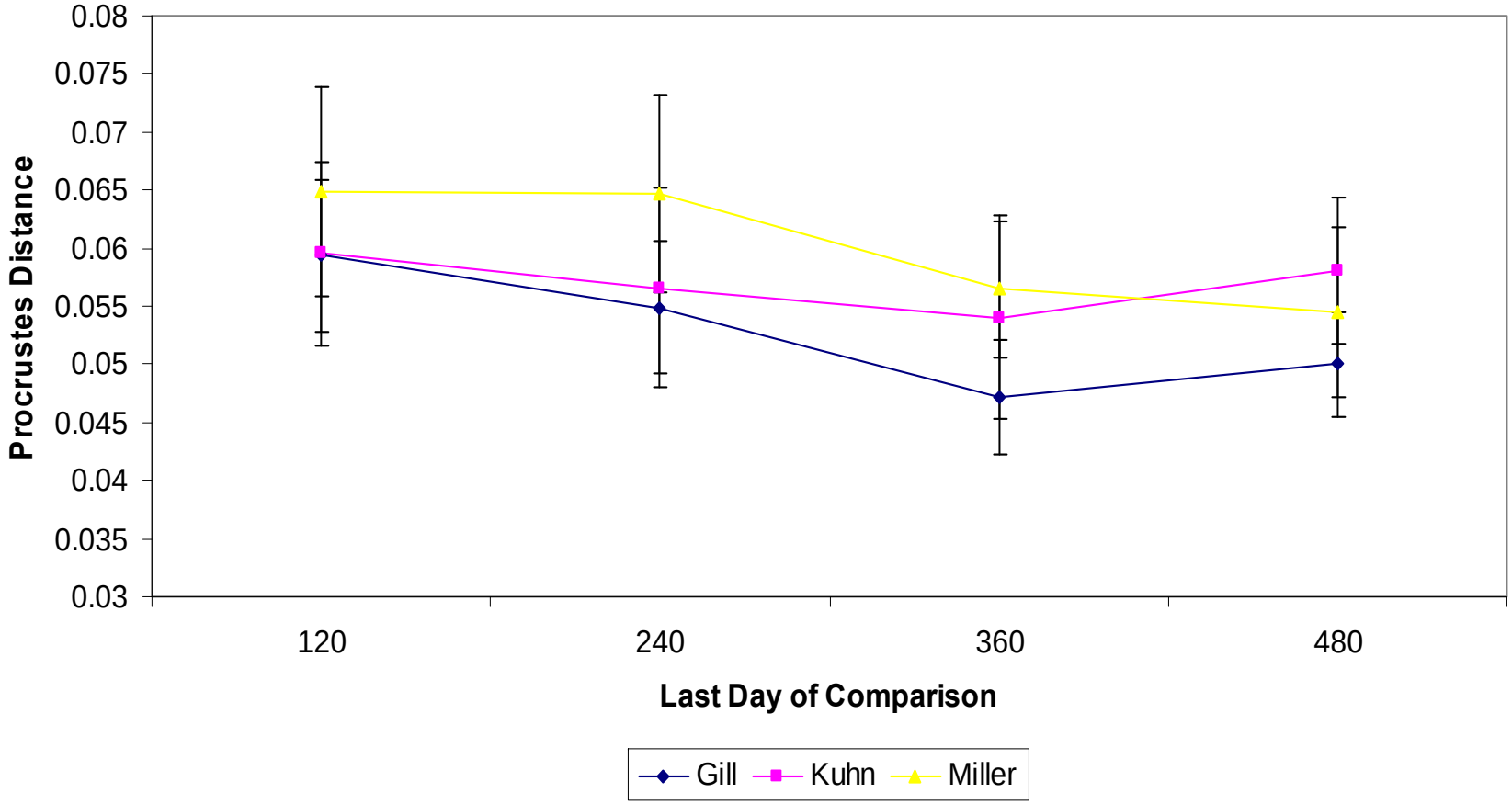


FIGURE 11: Treatment averages for the magnitude of shape change for the lateral aspect of trophic morphology. Treatment means are given with +/- 2 SE error bars. There were no significant differences among treatments at any time comparison.

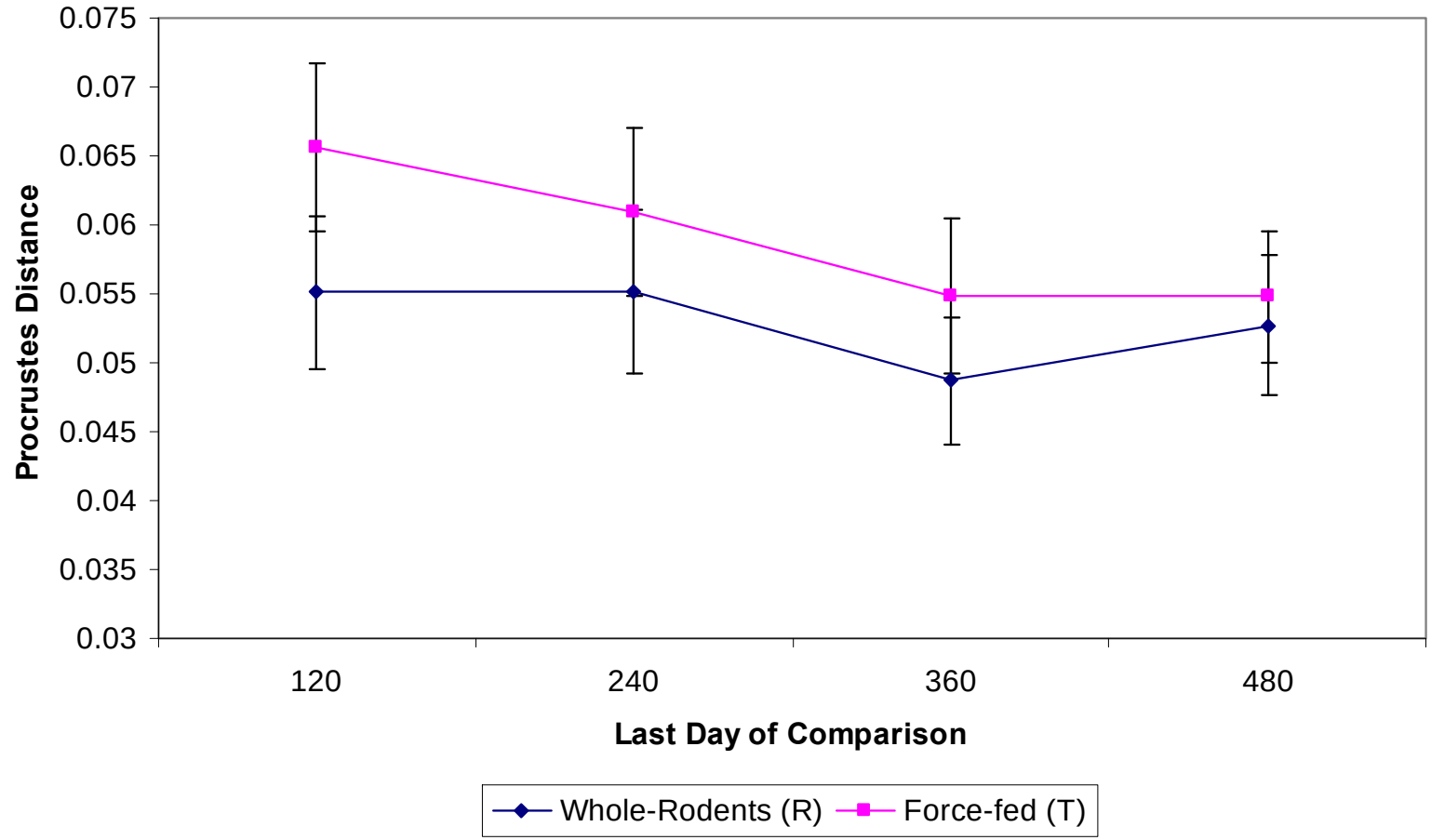


FIGURE 12: Dorsal deformation grids for the feeding treatments. Each gird shows LS Means configuration. The Whole-Rodent and Force-Fed configurations represent the mean shapes of snakes from the Gill and Kuhn population only. Miller snakes were separated due to population differences. Feeding treatments were significantly different from each other (Gill and Kuhn snakes only), while Miller snakes showed no significant treatment differences and appear to be intermediate in shape. Treatment differences are manifested in a broader head shape for snakes fed whole rodents, while those snakes force-fed had a narrower head with blunt termination of the head.

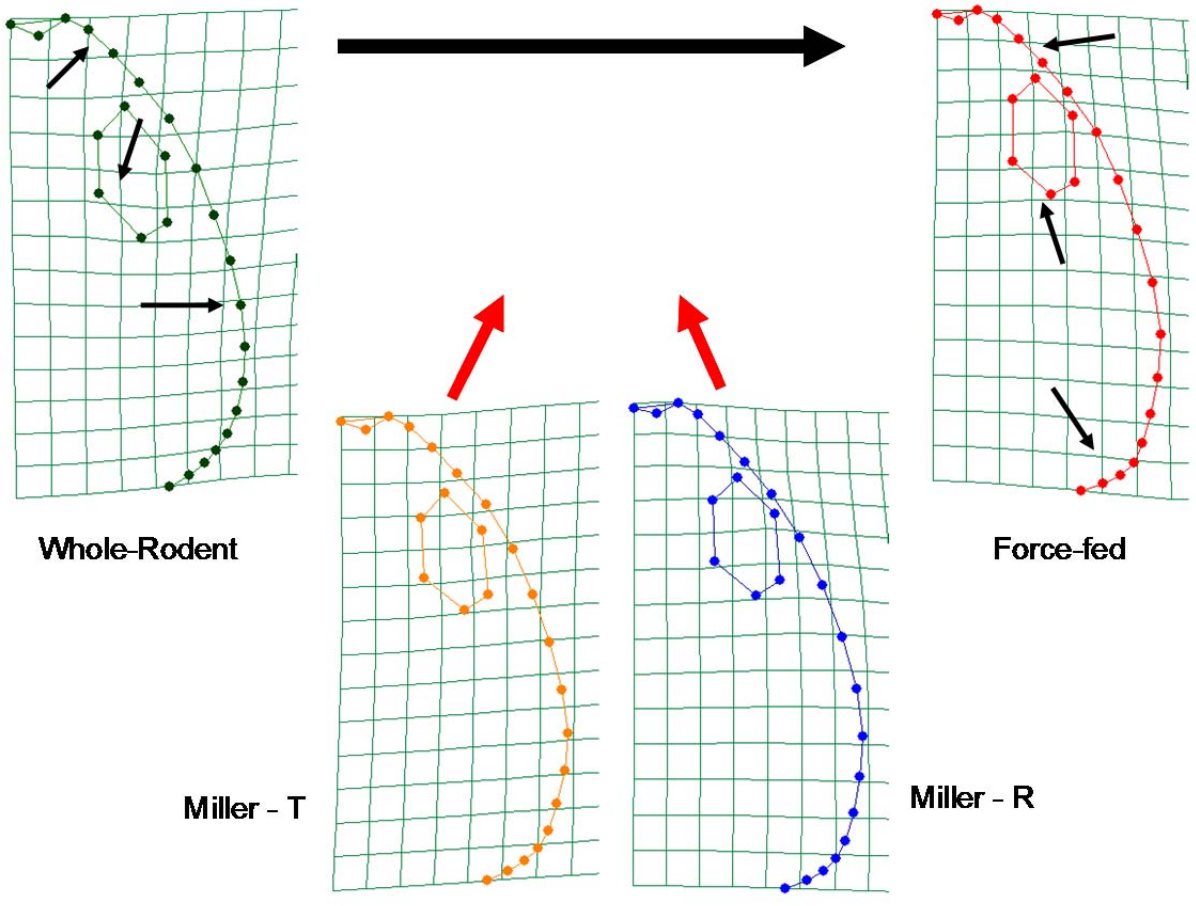


FIGURE 13: Overlay of the consensus configurations of the two feeding treatments (Gill and Kuhn snakes only), allowing for better visualization of shape differences. Lines are labeled with treatment (prey) types. Snakes fed whole-rodents had significantly broader heads than those force-fed through a catheter tube.

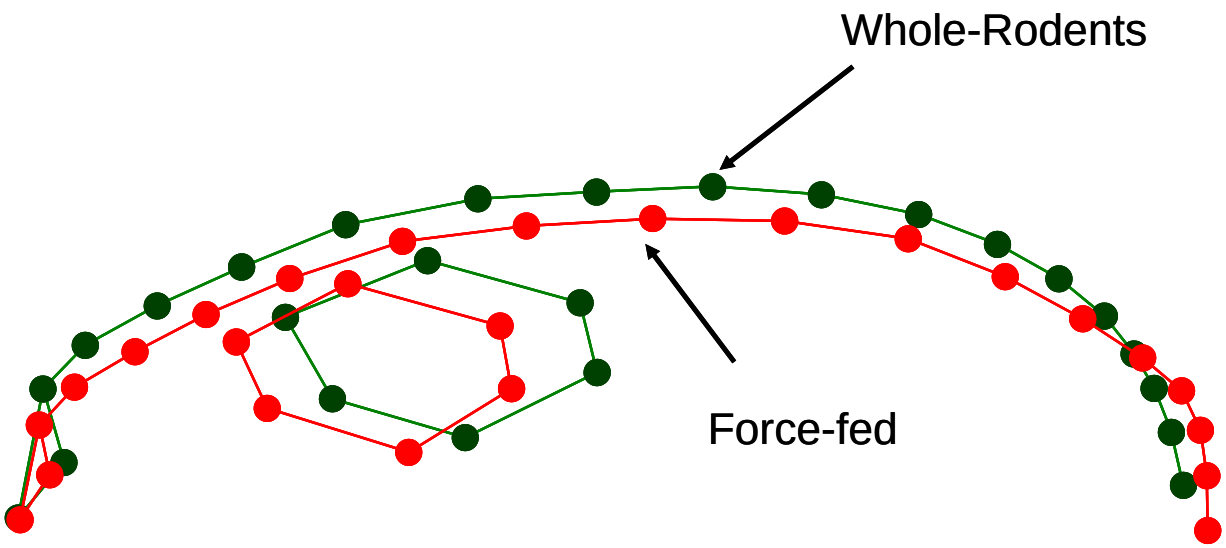


FIGURE 14: Lateral deformation grids showing the differences in shape between whole-rodent and force-fed treatments (Gill and Kuhn snakes only). Treatments were significantly different in lateral head shape at Day 480. Consensus configurations for each treatment from the Miller population are also shown. Snakes fed whole rodents exhibited a elongation of lateral head shape, with deformation girds pulling out at the extreme anterior and posterior ends. Snakes force-fed through catheter tubes showed a deeper lateral head shape, that was more compact and ended abruptly. Miller snakes showed no significant differences between treatments, and appear intermediate in shape to the two other configurations.

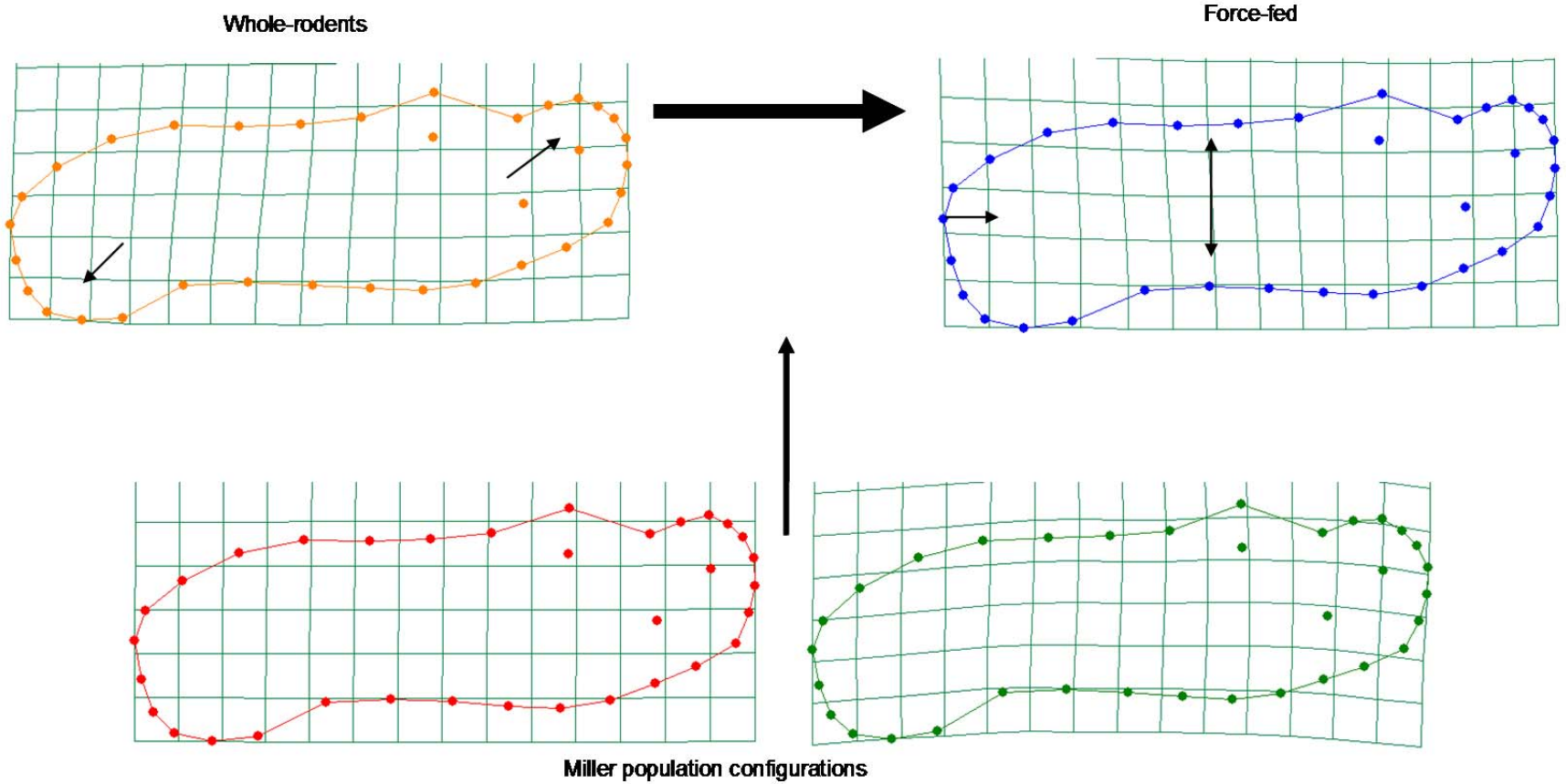
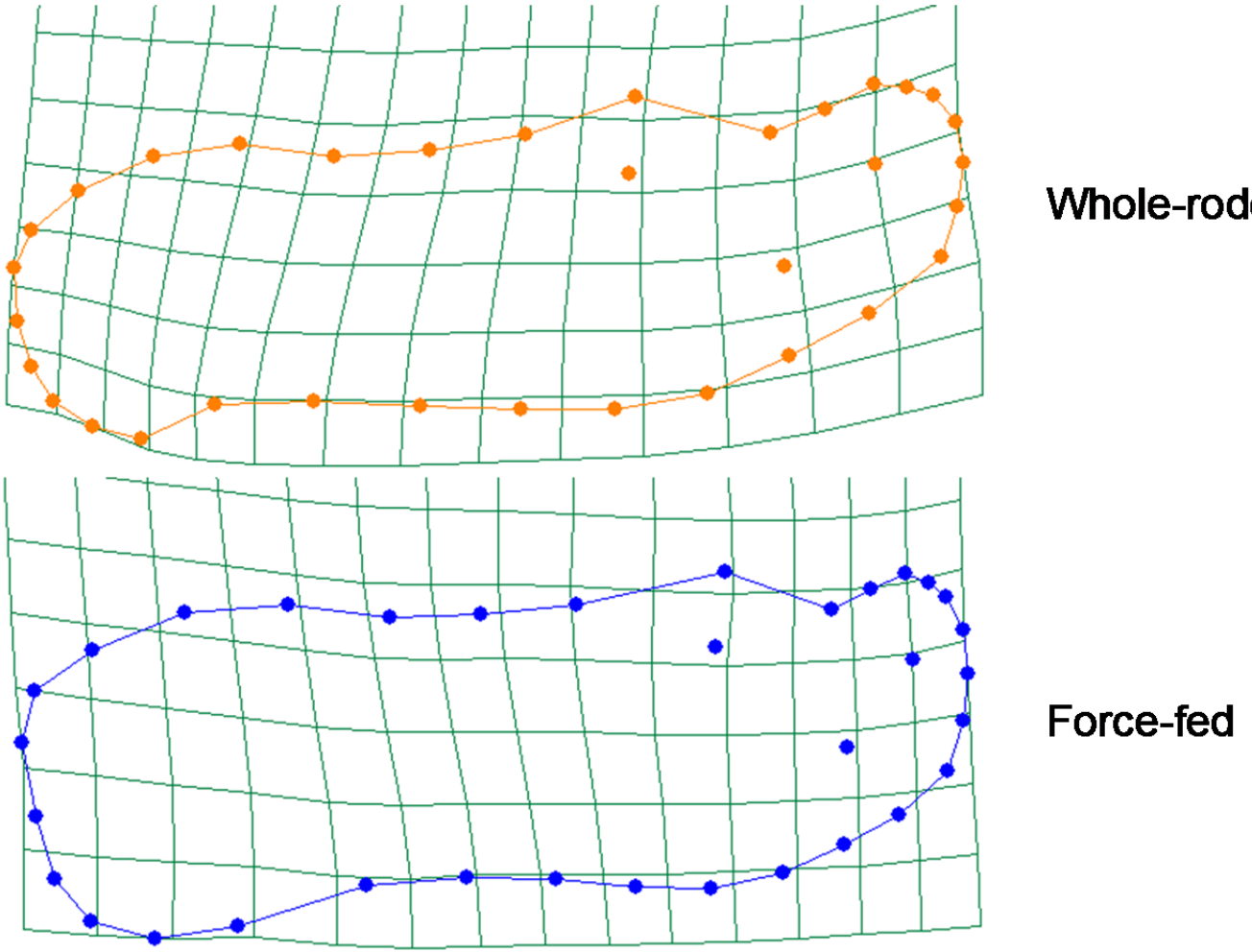

Whole-rodents
Force-fed
Miller population configurations

FIGURE 15: Exaggerated deformation grids of the consensus configurations from the Gill and Kuhn populations for each feeding treatment. True shape means are exaggerated by a factor of 5, but provide the ability to clearly detect shape differences and how shapes change from the overall consensus configuration.



TABLES

TABLE 1: A summary of the current literature on environmental and genetic influences over snake trophic morphology. Studies have focused primarily on resource level and sexual dimorphism in resource use and trophic morphology. To date no current pattern or trend has been found. Some studies have found significant effects of dietary factors on the trophic morphology of snakes, while others have shown little to no plasticity in response to dietary manipulations. Each study is further detailed within Chapter 1 and 4.

Citation	Species	Environmental Factor/Influence	Plasticity Present	Result Summary
Camilleri and Shine, 1990	*A. arafurae, D. punctulatus, P. porphyriacus, L. colubria*	Prey Type	YES	Sexual dimorphism in trophic morphology due to ecological divergence in diet
Forsman, 1996a	*Vipera berus*	Biomass/Resource level	NO	Resource level influences SVL, but not allometric relationship between SVL and trophic morphology
Queral-Regil and King, 1998	*Nerodia sipedon*	Prey Size	YES	Snakes that ingested larger prey items had longer relative jaw lengths. First study to suggest use-induced morphological change in snakes
Bonnet et al., 2001	*Bitis gabonica*	Resource level	NO	Relative body and trophic morphology sizes were affected by biomass, but in varied directions
Krause et al., 2003	*Thamnophis sirtalis*	Prey Size	YES	Sexual dimorphism in plasticity of trophic morphology to increases in prey size. Females more plastic than males
Aubret et al., 2004	*Notechis scutatus*	Prey Size	YES	Population differences in response to prey size. Island snakes were larger with bigger relative trophic morphology. Genotype by environment interaction present.
Schuett et al., 2005	*Boa constrictor*	Prey Size	NO	No response in trophic morphology to differences in prey size
Vincent et al., 2004	*Agkistrodon piscivorous*	Prey Type	YES	Ontogenetic shift in trophic morphology
Phillips and Shine, 2004	*P. porphyriacus, D. punctulatus, H. signata, T. mairii*	Prey Type	YES/NO	Those species at high risk to introduced species showed a change in trophic morphology, while those at low risk did not
Scudder-Davis and Burghardt, 1996	*Nerodia species*	Resource level	NO	Sexual dimorphism in growth rate, but no effect of resource level
Aubret and Shine, 2009	*Notechis scutatus*	Prey Size	YES	Different populations of snakes displayed varying degrees of plasticity in adjusting jaw length in response to increased prey size

CONCLUSION

The trophic morphology of Prairie Rattlesnakes (*Crotalus v. viridis)* exhibited changes in shape, size, and its allometric relationship to body size in response to prey size, population of origin, and starvation. Increased prey size induced a broadening of the head, resulting in wider and more elongated trophic morphology at a given head size. The direct response of trophic morphology to dietary manipulations reveals an environmental component to trophic morphology in snakes. Extended periods of starvation resulted in a decrease in the overall size of trophic morphology at a given body size. A decrease in the allometric slope implies that environmental factors, namely food deprivation, can significantly alter trophic morphology. Changes to the allometric relationship may or may not be reversible. Phenotypic plasticity in trophic morphology allowed treatments to respond to changes in prey size and resource level. Significant differences in the plasticity and response to dietary manipulations by the Miller litter suggest some geographic variation in trophic morphology. Observed litter differences could possibly be due to genetic factors influencing size and plasticity, or maternal effects. Patterns of morphological diversity among snake populations will provide insights into both the ecological and evolutionary processes at work.