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Adaptive digestive physiology in southern African snakes

MSc Dissertation

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Abstract

Snakes have often been proposed as ideal model organisms for studying digestive physiology. This is due to their easily–measureable and extreme changes in their digestive tracts in response to feeding, when compared to other vertebrates. Some species display extreme physiology regulation in response to feeding, a system known as digestive down-regulation. This regulation allows them to down-regulate their digestive tracts during their long fasting periods, which allows them to save energy. In response to feeding, they up-regulate their digestive tracts to a functional level, resulting in a significant increase in the size of the digestive organs during digestion. These changes have been found to be most noticeable in certain ambush foraging snakes. In contrast, actively foraging snakes appear to not display as extreme changes in response to feeding and keep their digestive tracts in a constant state of readiness. However it is not known if this pattern exists in all species and previous methods of classifying the digestive physiology have proven to be expensive, difficult and time consuming.

My study aimed to investigate if museum specimens could be used as a cheap and quick method of classifying the characteristics of the digestive physiology within a species. I measured the dimensions of several organs from museum specimens from 13 species of southern African snakes, as well as recorded the relative size of the meal and month of capture. I compared measurements between postprandial and fasting individuals from each species as well as between individuals from each species with the same feeding state to assess whether they were able to down-regulate their digestive systems when not digesting. While the different foraging strategies appeared to be linked to differences in organ morphology, the presence of down-regulation was not clear-cut. No significant differences in organ size between feeding states were found within each species, and very few significant differences were found between species. Few correlations with organ size to meal size or seasonality were found. This suggests that the museum specimens are not suitable for determining digestive physiology in snakes, probably due to the poor quality of the specimens. I therefore recommend the use of freshly obtained samples as a suitable comparison rather than the use of museum specimens.

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No ethical clearance or permits were required for this project. Permission to dissect specimens was obtained from the Ditsong Natural History Museum.

DECLARATION

I declare that this thesis is my own, unaided work unless specifically acknowledged in the text. It has not been submitted before for any degree or examination in any another university, nor has it been prepared under the aegis or with the assistance of any other body or organisation or person outside the University of the Witwatersrand, Johannesburg.

Signed:

Date:

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1. Introduction

Our understanding of various biological mechanisms has been achieved with the use of organisms that have exaggerated or convenient structures or mechanisms that have allowed for easy research and explanations, such as the genetics of *Drosophila* or the axons of squids (Secor & Diamond, 2000). Snakes have often been proposed as model organisms for research in various fields, particularly in ecology and physiology (Shine & Bonnet, 2000; Secor & Diamond, 2000). They also display high levels of diversity in many other traits, such as meal size and feeding frequency, which allows for novel comparisons across various taxa (Shine & Bonnet, 2000). Husbandry of snakes is simple requiring little maintenance or space in comparison to captive mammals (Shine & Bonnet, 2000). Studies into snake physiology have been particularly rewarding, due to the low Standard Metabolic Rate (SMR), long fasting periods and extreme physiological regulation when compared to other vertebrates (Secor & Diamond, 2000; Secor & Diamond, 1998). Their extreme physiological regulation means that any changes in metabolic rate can be easily detected and monitored, allowing for correlation to certain events. This makes them ideal for investigating influences on vertebrate physiology, such as investigations into the influences on vertebrate digestive physiology and how they are able to regulate any physiological changes.

Snakes display significantly larger changes in their digestive systems in response to a feeding event compared to most other terrestrial vertebrates (Secor & Diamond, 2000). As a result, the highly adapted digestive physiology of snakes has been extensively studied in recent years (Andrew, et al., 2015; Bovo, et al., 2014; Enok, et al., 2013). Certain species such as *Python bivitattus*, which are well known for extremely long fasting periods, have evolved a system of extreme physiological regulation that allows them to down-regulate their digestive tract to an inactive state between meals in order to conserve energy (Secor & Diamond, 2000). Research into down-regulation of the digestive tract has shown how the digestive tract of these animals is up-regulated in response to feeding, which builds up the digestive tract and associated organs in form and function in order to become active again and digest the meal (Secor, et al., 1994; Ott & Secor, 2007). The hypertrophy of these organs is fuelled by the snake's own fat reserves and results in a significant increase in the metabolic rate of the snake (Secor, et al., 1994). This makes snakes that are using down-regulation of the digestive tract ideal

subjects to understand digestive physiology in terrestrial vertebrates, as they display extreme changes in their digestive physiology in response to a meal, which can be easily studied (Secor & Diamond, 1998).

Snakes utilise specific foraging strategies that compliment their low metabolic rate and extreme physiological systems (Secor & Ott, 2007). Species of snakes can be grouped into two feeding strategies: sit-and-wait foraging snakes (e.g., *Crotalus cerastes*) spend most of their time in ambush, waiting for prey to come within striking range (Alexander & Marais, 2007; Branch, 1998). Sit-and-wait foraging snakes are therefore more commonly referred to as ambush foraging snakes. This results in ambush foragers feeding infrequently as they must wait for prey to come within striking range (Secor, 1995). This opportunistic approach results in extremely long periods of fasting and ambush foragers are necessarily generalist feeders, consuming a wide range of prey types and sizes (Alexander & Marais, 2007). Actively foraging snakes (e.g., *Masticophis flagellum*) actively search for prey resulting in more frequent meals compared to the ambush foragers (Huey & Pianka, 1981; Secor, 1995). They do not have to undergo fasting periods as long as those experienced by the ambush foragers (Secor & Diamond, 1999). This difference in feeding frequency has resulted in the evolution of differing digestive physiological systems which has maximized energetic benefits in relation to costs.

The different physiological systems found in species using different foraging strategies have been extensively studied in several species (Bessler & Secor, 2012; Bovo, et al., 2014; Secor & Diamond, 2000; Ott & Secor, 2007; Wall, et al., 2013). Studies have shown that many species of ambush foraging snakes undergo down-regulation of the digestive tract during their longer fasting periods (Secor & Diamond, 1998). However, active foragers that have been studied tend to keep their digestive tracts in a constant state of readiness, and so do not display as extreme physiological changes as species found to be using down-regulation of the digestive tract (Roe, et al., 2004; Secor & Diamond, 1999; Secor & Ott, 2007; Secor & Diamond, 2000; Crocker-Buta & Secor, 2014). While the differences in physiology has been well documented in certain species, it is still unclear if these patterns are widespread or that the link between feeding strategy and digestive physiology is pervasive (Secor & Ott, 2007; Secor & Diamond, 1998). Therefore further investigation into the

digestive physiology of other snakes is still required to confirm if foraging strategy and down-regulation is universally linked.

Past studies investigating the use of down-regulation in the digestive tract in snakes have relied on data collected from respirometers or from dissections from large numbers of freshly killed specimens (such as Bessler & Secor, 2012; Secor & Diamond, 1997; or Secor 1995). Both methodologies have their benefits and drawbacks. Traditional methods are time consuming and expensive, and the requirement of large numbers of freshly killed specimens (in a range of digestive states) requires intensive husbandry of the animals before they are killed. This methodology is also not ideal for threatened species. This has resulted in only a small number of species being investigated, which has limited the impact of these findings on snake ecology. Therefore, a simple and quick method of identifying species that are using down-regulation of the digestive tract is needed in order to expand our knowledge and application of down-regulation of the digestive tract.

I assessed the value of museum specimens for the diagnosis of down-regulation physiology in snakes. Museum specimens are readily available for dissection without the need for husbandry or field collections and could, potentially, serve as ready source of specimens for analysis, obviating the need to kill further specimens. I measured the organs of preserved specimens and used these measures in relation to data on gut content to assess if this method could be used to diagnose down-regulating snake species. Specimens from each species found with food in their digestive tracts that display significantly larger organs than specimens without food in their gut would be indicative of down-regulation, due to the hypertrophy of the organs in response to feeding. In contrast, organ sizes of non-down-regulators should have organs of similar size regardless of the presence of food in the gut. Since foraging strategy appears to have a strong correlation with down-regulation, I compared several species of ambush foraging and actively foraging species.

2. Literature review

2.1 Investigating differences in physiology based on foraging strategy in snakes

The relationship between foraging strategy and physiology has been extensive studied in reptiles (Andrew, et al., 2015; Bessler & Secor, 2012; Bovo, et al., 2014; Secor & Diamond, 1998). Reptiles make excellent subjects to investigate this relationship due to their extreme physiological regulation, when compared to other vertebrate species. These extreme changes are therefore easy to detect and study (Shine & Bonnet, 2000). Their low standard metabolic rate (SMR) and long fasting periods also help to make their physiology easy to investigate, as they respond with large metabolic changes to feeding that are easy to monitor and correlate to events. Snakes also show high levels of diversity in their foraging strategies and ecology that allow for large-scale comparisons across species and families (Huey & Pianka, 1981). These factors make them ideal subjects to investigate how physiology is affected in response to feeding.

Foraging strategy has an important impact on energy balance in snakes. Considering the low standard metabolic rate and long fasting periods seen in snakes, snakes need to be highly adapted in order to maintain a positive energy balance. They achieve a positive energy balance through specific foraging strategies and physiological systems that allow them to survive. Secor and Nagy (1994) clearly illustrated how snakes can achieved this using two different strategies: sit-and-wait foraging snakes (e.g., *Crotalus cerastes*) spend most of their time in ambush, waiting for prey to come within striking range. In comparison, actively foraging snakes (e.g., *Masticophis flagellum*) actively search for prey. Ambush foragers have a lower field metabolic rate (FMR) than actively foraging species, but only catch prey infrequently in comparison to active foragers (Secor & Nagy, 1994). As a result, they have developed different physiological systems that allow them to cope with low rates of ingestion (Secor & Nagy, 1994; Secor, 1995).

Previous studies have found differing physiological strategies in each foraging strategy (Bessler & Secor, 2012; Bovo, et al., 2014; Secor & Diamond, 2000; Ott & Secor, 2007). The studies have shown how many species of ambush foraging snakes display extreme down-regulation of their organs during fasting periods which allows them to lower their SMR while fasting. In contrast, the active foragers that have been studied have not used as extreme regulation between fasting and

postprandial states as the ambush foraging snakes (Roe, et al., 2004; Secor, 1995; Secor & Diamond, 1999; Secor & Ott, 2007). However previous studies have only focused on a few species (primarily *P. bivitattus* and *C. cerastes*) and so it is not known if down-regulation of the digestive tract is typical of ambush foragers (Secor & Ott, 2007; Secor & Diamond, 1998). Therefore further investigation into other species and the relationship between their digestive physiology and foraging strategy are needed in order to assess if there is a link between down-regulation of the digestive tract and ambush foraging.

2.2 Foraging strategy and physiology of ambush foraging snakes

Ambush foraging snakes are characterised by a stocky body and cryptic coloration. This is particularly evident for members of the Viperidae and Pythonidae, with their thick and camouflaged bodies (Alexander & Marais, 2007; Branch, 1998). Their cryptic colouration allows them to lie motionless in a suitable ambush position undetected (Branch, 1998). Since ambush foragers must passively wait for prey to approach, capture rates tend to be low (Secor & Nagy, 1994; Ott & Secor, 2007) and they thus are usually generalist feeders since infrequent meals mean they cannot afford to be choosey (Krivan & Eisner, 2003; Lillywhite, et al., 2002). Selective pressures that resulted in generalist diets have also selected for the ability of ambush foragers to consume large meals. Pythons are often seen preying on antelope that are significantly larger than themselves (Alexander & Marais, 2007; Bates, et al., 2014).

Since prey encounter rates tend to be low, ambush foragers tend to feed infrequently and are often forced to survive long fasting periods (*Python bivitattus* have been known to survive for up to two years without feeding) (Secor, et al., 1994). In some species, this has led to the evolution of down-regulation of the digestive tract whereby the gut and supporting organs undergo atrophy, with the consequent formation of fat, after a meal is digested. The atrophy of organs results in these snakes using less energy due to the reduced size of the organs. This significantly lowers SMR during fasting, allowing them to survive for long periods without food (Secor & Diamond, 1998; Secor & Diamond, 1999). After the ingestion of food, the digestive tract is rapidly up-regulated, using the snake's fat

reserves to build the digestive tract back up to a functional level (Secor & Diamond, 1995; Overgaard, et al., 2002).

During up-regulation, several organs enter a state of hypertrophy. Significant increases in triglyceride concentrations in the blood after feeding have been recorded in *P. bivitattus, C. cerastes* as well as in *C. durissus*, indicating that the snakes use their own fat reserves, rather than the ingested meal, to fuel hypertrophy (Bovo, et al., 2014; Secor & Diamond, 1995). The increase in metabolic rate in response to feeding is significant (*P. bivitattus* have been recorded increasing their metabolic rate by 44 times their SMR), making the hypertrophy of the organs extremely expensive and so requires a large amount of stored energy in order to digest a meal. Secor and Diamond (1995) described the full extent of this trend when they recorded a 78% decline in pO and a rise in pCO_2 due to the significant increases in metabolism. This means that the up-regulation of the digestive tract requires a large amount of energy to digest a meal (a process known as specific dynamic action, or SDA). The hypertrophic state causes significant increases in the mass of digestive and cardiovascular organs in order to increase the rate of digestion and assimilation (Secor & Diamond, 1995; Secor, et al., 1994).

Secor *et al.* (1994) illustrated how there are several changes at a cellular and tissue level during hypertrophy. The mucosal layer in the intestinal tract doubled in mass over a 24 hour period in order to assist with absorption and assimilation (Secor & Diamond, 1999). The cell composition of the mucosal layer is also changed, with a significant increase in transport epithelial cells by up to 22 times their fasting levels, which allows for faster uptake of nutrients (Secor, 1995; Secor, et al., 1994). Changes also occur in the blood chemistry of the snake during digestion. Bovo *et al.* (2014) clarified this in their study on *Crotalus durissus terrificus*, which shows significant increases in the haemoglobin's affinity for oxygen during digestion. This is to provide enough oxygen to the increased form and function seen in the digestive tract, as well as the associated organs. Secor *et al.* (2001) also found that there is a rapid increase in the concentrations of certain micronutrients (such as plasma folate, a water-soluble form of vitamin B) in *P. bivitattus.* These changes in the blood chemistry allow for rapid uptake and transport of oxygen to the tissues. This contributes to the sharp increase in the metabolism during digestion (Bovo, et al., 2014; Enok, et al., 2013; Lourdais, et al., 2014). Similar

chemical changes are also seen in the stomach, where the pH drops rapidly due to increased production of gastric juices in response to the distension of the stomach tissues by a meal (Enok, et al., 2013; Norgaard, et al., 2016).

After digestion has been completed, the digestive tract and its associated organs undergo further complex changes that restore the organs back to their fasting state (Bessler & Secor, 2012; Ott & Secor, 2007; Overgaard, et al., 1999). The mass that was added to the organs during hypertrophy is reabsorbed and returned to the snake's fat reserves (Secor & Diamond, 1995; Secor & Diamond, 1998). The pH of the stomach begins to rise again as well, as the gastric juices are slowly removed from the stomach (Bessler & Secor, 2012; Enok, et al., 2013; Norgaard, et al., 2016). The reduction in organ size allows the snake to reduce its metabolic rate, as it does not require as much energy to maintain its organs in a state of readiness. This allows them survive for extremely long periods between meals (Secor & Diamond, 2000).

Due to the large changes in organs and tissues observed in previous studies, I expected to find similar changes in the species examined in this study. Species suspected of using down-regulation of the digestive tract (such as *Python natalensis* and *Bitis arietans*) should show large difference in organ sizes based on their feeding state, with individuals that are in a postprandial state having larger organs than individuals in a fasting state. The Southern African Python (*P. natalensis*) is a close relative of the well-studied *P. bivitattus* and *Python sebae*, and so is similar in build and foraging strategy (Secor & Diamond, 2000; Bates, et al., 2014). I therefore expected that *P. natalensis* will display similar changes to its digestive physiology during fasting. Similarly, the Puff Adder (*B. arietans*) would be a suitable comparison to *C. cerastes*, as they are both members of the Viperidae family and I expected that southern African vipers also use down-regulation.

2.3 Foraging strategy and physiology of active foraging snakes

Active foraging snakes are identified by their long slender bodies and highly active lifestyles (Alexander & Marais, 2007; Branch, 1998). Typical examples of actively foraging snakes can be seen in the Elapidae and Colubridae. Actively-foraging snakes seek out suitable prey items, and generally feed more frequently than ambush foragers (Alexander & Marais, 2007; Branch, 1998). They also

tend to be more selective in their choice of prey, often choosing smaller prey items that are easier to handle (such as fledgling chicks in a nest rather than a fully grown bird). As a result of this foraging strategy, they do not need to tolerate long fasting periods (Secor, 1995). However this means that they generally have higher energy costs and so require a different physiological strategy to that seen in ambush foragers (Secor, 1995).

Frequent meals also mean that it does not pay active foragers to down-regulate their digestive systems, as start-up costs would likely exceed savings, as they would have to undergo it more regularly (Roe, et al., 2004; Secor & Diamond, 1998; Secor & Ott, 2007). Thus, they typically do not show significant changes in organ size or blood chemistry in response to feeding (Secor & Diamond, 1998). This also results in higher maintenance costs in order to keep the digestive tract in a state of readiness. The high maintenance costs combined with the active lifestyle results in a significantly higher energy expediture than ambush foragers. This was illustrated further by Lourdais *et al.* (2014) who found that active foragers have significantly higher haematocrit levels than ambush foragers. They accredit this difference to the active lifestyle and high levels of organ maintenance in these snakes, and so require the extra haematocrit in order to transport more oxygen to their tissues.

Due to the high levels of maintenance seen in their digestive tracts, I expected to see very little difference in organ size between feeding states in species classified as active foragers (such as *Boaedon capensis* or *Dispholidus typus*). Individuals found in a postprandial state should not display significant difference in organ sizes to individuals found in a fasting state. Southern Africa has a high diversity of actively-foraging snakes that will make suitable comparisons to the ambush foraging species, particularly from the Elapidae, Colubridae and Lamprophiidae families (Alexander & Brooks, 1999; Roe, et al., 2004; Branch, 1998; Alexander & Marais, 2007).

2.4 Other influences on digestive physiology in snakes

While foraging strategy has been extensively studied in snakes, there are several other factors that may cause variation in the metabolic response to feeding. Some studies found varying results regarding some organs and their change in mass. Secor (1995) as well as Secor and Diamond (2000) found that kidneys and hearts did not increase significantly in Burmese Pythons in response to

feeding. This apparent contradiction to other studies on the species may indicate that there are other influences on SDA responses. Factors such as meal size, meal type, nutrient content, and seasonality have all been shown to influence the scope of the SDA response (Ayers & Shine, 1997; Alexander & Brooks, 1999; Naya, et al., 2011; Secor & Faulkner, 2002; Grobmann & Starck, 2006).

Secor and Faulkner (2002) investigated the effect of many of these factors on SDA in their study on the Marine Toad (*Rhinella marina*). Body temperature was found to influence the length of time needed for digestion in *R. marina*, but did not appear to influence the amount of energy required to digest the meal (Secor & Faulkner, 2002). Meal size affects the duration and peak of SDA, with larger meals causing larger and longer SDA peaks. The peak of the SDA in *R. marinus* as well as the duration of the SDA also increased linearly as the meal size increased, as larger meals require more energy and time in order to digest them. The relationship between meal size and SDA is less clear in snakes, as there does not appear to be a linear relationship. Ott and Secor (2007) found that three-fold increase in meal size produced a 3.2-fold increase in total SDA costs compared to smaller meals in various boas and pythons, as well as a significant increase in organ size. Tein-Shung *et al.* (2008) also showed that arboreal Pit Vipers (*Trimeresurus stejnegerii*) have significantly larger SDA peaks on larger meals, but that a doubling of meal size only produced an SDA peak 48% larger than the smaller meal. However all of these studies showed that larger meals increase the time taken to fully digest a meal, as larger meals require longer exposure to the digestive enzymes in order to fully digest it.

The relationship between meal size and digestion may be more complicated in species using down-regulation (Secor & Diamond, 1997). Secor and Diamond (1997) found that *P. bivitattus* only showed significantly increased organ size with large meals (meals larger than 25% of the snakes' body mass). This indicates that *P. bivitattus* is able to digest smaller meals without having to significantly increase the mass of its organs. This should not be possible with down-regulation of the digestive tract, as the digestive tract could not be active enough to digest. Other studies (Secor, 1995; Secor & Diamond, 1995; Lignot, et al., 2005) that have measured significant increases in organ mass during digestion have mostly used meal sizes over 25% of the snake's body mass, and so would show increased organ size according to the meal size limit described by Secor and Diamond (1997), and so

may not have considered this as a factor. Therefore, the influence of meal size on digestive physiology requires further investigation.

Seasonality may also influence the scope of the SDA by forcing the organism to shut down its digestive tract during lean seasons. Tracy and Diamond (2005) illustrated this effect in hibernating Chuckwalla lizards (*Sauromalus ater*). Chuckwallas are found at different elevations, which have different growing seasonal lengths, due to the highly isolated climatic conditions found around their habitat. Chuckwallas that live at lower elevations with shorter growing seasons showed significantly higher nutrient uptake rates than Chuckwallas who live at higher elevations with longer growing seasons. Chuckwallas at lower elevations also showed higher levels of digestive physiology (including nutrient transport and assimilation) between seasons. The increase in digestive physiology allows the Chuckwallas at lower elevations to digest and assimilate meals quickly during their shorter growth season and reduce their organs in a similar manner to snakes using down-regulation. All of the Chuckwallas also showed significant differences between hibernating and active states. Therefore Tracy and Diamond (2005) indicate that seasonality may have an evolutionary influence on digest physiology.

Factors, such as meal types, size and seasonality may therefore influence this study. Individuals consuming larger meals would be expected to show larger relative organ sizes than individuals with smaller meals. Seasonality may also produce a similar effect to down-regulation on all reptile species, as the digestive tract may reduce in size during winter months to accommodate for the reduction in feeding, regardless of foraging strategy. Since museum specimens are acquired from various sources, there is no consistency in their meals and seasons. Therefore, these factors may influence the measurements taken from the museum specimens and may explain variations seen between individuals.

2.5 Investigating the influence of foraging strategy on digestive physiology using museum specimens

Previous studies investigating the changes in digestive physiology in snakes have focused on intense dissection and respirometry, often following a similar methodology as discussed in Secor *et al.*

(1994).. The Majority of previous literature (Cox & Secor, 2007; Lignot, et al., 2005; Ott & Secor, 2007) has used large numbers of captive snakes which are harvested and measured over a period of two weeks after feeding. The snakes are fasted for three weeks at the start of the experiment to ensure all individuals are in a fasting state at the start of the experiment. Several snakes are harvested before feeding in order to establish a control value from fasting individuals. The snakes are then fed a meal of roughly 25% of the snake's body mass (although other studies varied in meal size). Closed-system respirometer values are then taken over the following two weeks to establish change in metabolic rate. During this time, several snakes are killed and dissected daily in order to take organ measures at corresponding time periods. Wet and dry mass of various organs are measured. These studies have used anything from 10 individuals (Secor, 1995) to 75 individuals (Secor, et al., 1994).

The methodology used in previous studies mostly involves the use of captive specimens in large numbers. This creates a problem for future studies that may want to examine new species, but cannot be reared in captivity. The methodology would also not work on threatened species, as investigators would not be able to harvest a suitable sample size. The killing of a large number of individuals may also be considered unethical. It is also time consuming and expensive, considering the snakes need to be housed for an extended period of time. This has led to a restriction in the number of species that have been examined, with a distinct bias towards *P. bivitattus* (Castoe, et al., 2013; Enok, et al., 2013; Overgaard, et al., 1999; Secor, 1995), and therefore restricts further our knowledge of how snake digestion can influence their ecology. Therefore a simpler and cheaper method of testing for down-regulation in other snake species is needed. I proposed the use of museum specimens as a suitable substitute.

The aim of my study is to evaluate whether museum specimens can be used to unambiguously detect down-regulation in snakes. This will be done through anatomical and histological sampling to detect atrophy and hypertrophy in organs associated with digestion in fasting and postprandial states. Ambush and active foragers will be compared to assess whether down-regulation of the digestive tract is a widespread response to infrequent feeding in snakes. This information can then be used to develop a quick and reliable method of diagnosing down-regulation in future studies. The information from my study can then be used to investigate several other species whose current digestive

physiology is unclear based on the current literature, due to their unusual ecology. For example, the Twig Snake (*Thelotornis capensis*) is a member of the Colubridae family (which typically display active foraging strategies), but is classified as an ambush forager as it lies on low-hanging branches of trees and bushes and strikes at passing lizards, frogs and small mammals (Shine, et al., 1996). However, it has a body plan more typical of an actively foraging snake (Shine, et al., 1996; Alexander & Marais, 2007).

3. Aims and objectives

My study aimed at investigating the of the use of organ measurements taken from museum specimens as a method for diagnosing down-regulation in snakes. In order to evaluate the methodology, I had two aims to consider:

3.1 To be able to identify southern African species suspected of using digestive down-regulation

- *Comparison of organs between postprandial individuals and fasting individuals within species*

Measurements of organ dimensions in relation to their overall body size will demonstrate any changes in the digestive tract in response to feeding. I expected to find that ambush foragers show relatively large differences between recently fed individuals and fasting individuals. Active foragers are expected to show slight changes, but no significant differences, between fed individuals and fasting individuals.

- *Comparison of organs from postprandial individuals of different species*

Comparisons between species can be used to assess any relationship between foraging strategy and digestive physiology. Recently-fed individuals are expected to show increased organ mass and volume. However ambush foragers are expected to show significantly larger organs than active foragers during digestion due to their extreme up-regulation and costly SDA.

- *Comparison of organs from fasting individuals of different species*

Comparisons between active and ambush foragers in fasting states will confirm the presence of the extreme down-regulation. Ambush foragers are therefore expected to show significantly smaller organ masses and volumes during fasting compared to the active foragers.

3.2 Assess the influences of meal size, seasonality and foraging stategy on postprandial physiological changes

- *Identifying differences in the digestive tract based on meal size*

Since meal size has been shown to influence the factorial scope of the SDA, there should be a corresponding change in relative organ size. Measurements of various organs from individuals within the same species with difference meal sizes may indicate if snakes are able to regulate their metabolic response to a meal. This should then be detectable as a difference in organ size with meal size in the museum specimens. Ambush foragers are expected to show a strong linear relationship between meal size and organ size. Active foragers are not expected to show a significant difference in organ size with different meal sizes due to the constant maintenance of their digestive tracts.

- *Identifying differences in the digestive tract based on seasonality*

Due to the seasonal fluctuations in the availability of food as well as the reduced activity during winter, down-regulation may be modulated by season, even in active foragers. Seasonality may then produce a similar effect to the down-regulation seen in ambush foragers. A significant reduction in organ size would be expected in all snakes during winter months. Active foragers are expected to show significantly smaller organ sizes during fasting in winter months compared to the summer months. Ambush foragers are not expected to show any changes between seasons, as their digestive tracts should be in their atrophic state already due to the fasting periods during winter.

- *Comparison of organs between active and ambush foragers*

The comparison of various organ measurements between active and ambush foragers will confirm if the previously observed changes in organ size in other species are also occurring in southern African snakes. These comparisons will allow me to develop a simple method of diagnosing down-regulation by examination of certain organs. I will also use this to confirm if snakes do form two distinct digestive physiology groups based on their foraging strategy.

4. Materials and methods

4.1 Study species

The species investigated in this study are listed in Table 1. All specimens were supplied by the Ditsong Natural History Museum. All species were initially classified as either active foragers or ambush foragers according to Branch (1998), Bates *et al.* (2014) and Alexander and Marais (2000). I selected species that are considered to be typical examples of each strategy and are common. The number of individuals dissected was limited by the number of specimens available and the Museum's permission.

Table 1. A table listing the examined species according to their foraging strategy. Sample size is included in brackets.

Active foragers	Ambush feeders
Boaedon capensis (10)	*Python natalensis* (9)
Psammophis mossambicus (10)	*Bitis gabonica* (8)
Psammophylax rhombeatus (10)	*Bitis atropos* (9)
Pseudaspis cana (9)	*Bitis arietans* (10)
Dendroaspis polylepis (9)	*Thelotornis capensis* (9)
Naja annulifera (10)	
Dispholidus typus (10)	
Causus rhombeatus (9)	

4.2 Procedure

Individuals of each species were chosen according to their body size and feeding state. Individuals that had clearly eaten recently (seen as bulging in their abdominal region) and fasting individuals of each were chosen. Individuals of similar body size were preferably chosen when possible to limited variability during measurements. The snout-vent length (SVL) of each specimen was measured in millimetres using a tape measure. The month in which the snake was collected was recorded from the labels on each specimen. This was used to evaluate seasonality.

Each specimen was dissected to reveal the gastrointestinal tract and circulatory system. Once exposed, the length and breadth of the heart, liver, right kidney and stomach was measured using a tape measure and calipers. The longest possible length of the organ was organ, while an average breadth was calculated from three separate measures. The contents of the stomach and intestine were

removed and examined before measurements. Any food found in the gastrointestinal tract were examined and scored out of 10 (with 1 being a very small meal and 10 being an extremely large meal) according to the relative size of the snake. The degree of digestion was scored on a scale of 0 to 10 (with 0 being no digestion occurring and 10 being nearly completely digested). Individuals with very little digestion were not considered further as postprandial individuals as the digestive tract may not have had enough time to response to the meal.

Sections were cut from the proximal section of the duodenum to histologically measure the thickness of the mucosal layers. The sections were cut into 10 mm long sleeves and preserved in 70% ethanol. These were then dehydrated in a series of alcohol solutions (70%, 95% and 100% solutions) before being fixed in paraffin wax. 8 µm cross sections were sliced using a microtome and fixed to a glass slide. The cross sections were then stained with Eosin and Haematoxylin. The Eosin and Haematoxylin stain was freshly prepared before staining. The thickness of the mucosal layer was examined using a light microscope (Carl Zeiss Axio Imager M2). Micrograph images were captured and analysed using ZEN computer software to measure the mucosal thickness. Three measures of each sample were taken and averaged. Specimens that did not survive the preparation process were discarded and not measured.

4.3 Statistical analysis

Data were transformed by converting organ measures to proportional measurements according to SVL of each corresponding individual. Differences in organ size within a species between fasting and postprandial individuals were tested using an unpaired Student's t-test, using initial SVL as the correctional factor. The change in organ size within each species was calculated using the difference between the mean values of the fasting and postprandial measurements. A Student's t-test was then used to detect any differences in organ change between foraging strategies. Differences between foraging strategies were detected using a two-way ANOVA, with foraging strategy and feeding state as the factors of interest. Significant ANOVA results were analysed using a Tukey HSD post-hoc test. Species were included in the post-hoc test to analyse differences between species. Pearson's correlations were run within each species to test for the relationship between organ size and meal size

as well as for the relationship between organ size and seasonality. Seasonality was determined by considered the month of capture of each specimen as the months from mid-winter (with June and July considered one month away, and December and January considered 6 months away). Only fasting specimens were used to examine the relationship between organ size and seasonality, due to the lack of postprandial specimens found during winter months (no specimens were found to have fed between the end of April to the end of August).

All statistical analysis was conducted using Statistica 8.0. Significance considered at the $p < 0.1$ level, due to the one-tailed nature of the study. A Bonferroni correction was applied when comparing species due to the large number of tests (13 species with two measures for each of the four examined organs as well as the additional intestinal mucosal measure, therefore $m = 117$). This adjusted the probability levels to $p = 0.00085$ when considering at the $p = 0.1$ level.

5. Results

5.1 Comparison between feeding state and foraging strategy

The two-way analysis of variation conducted on the influence of foraging strategy and feeding on organ size revealed fewer than expected significant differences (Fig. 1 and Fig. 2). No significant difference was found between the foraging strategies in the length of the liver, regardless of feeding state ($F_{1,75} = 2.00$, $p > 0.1$; Fig.1 A). However liver width revealed highly significant differences based on foraging strategy and feeding state ($F_{1,75} = 6.21$, $p < 0.1$; Fig. 1 B), with ambush foraging species having wider livers than the active foraging species while in a postprandial state, but a narrower liver while fasting.

Heart length ($F_{1,75} = 0.50$, $p > 0.1$) and heart width ($F_{1,75} = 3.79$, $p > 0.1$) revealed no significant interaction between foraging strategy and feeding state, although there were significant differences between several species (Fig. 2, panel A and B). Stomach length ($F_{1,75} = 0.81$, $p > 0.1$) and stomach width ($F_{1,75} = 1.50$, $p > 0.1$) also revealed no significant interactions (Fig. 1, panel D and E). Kidney length revealed no significant interaction ($F_{1,75} = 1.19$, $p > 0.1$; Fig. 2 C), but kidney width revealed a significant difference ($F_{1,75} = 4.55$, $p < 0.1$; Fig. 2 D), with ambush foragers have significantly wider kidneys than active foragers, regardless of feeding state. Intestinal mucosal layers were found to have no significant differences between fasting individuals ($F_{1,75} = 2.26$, $p > 0.1$).

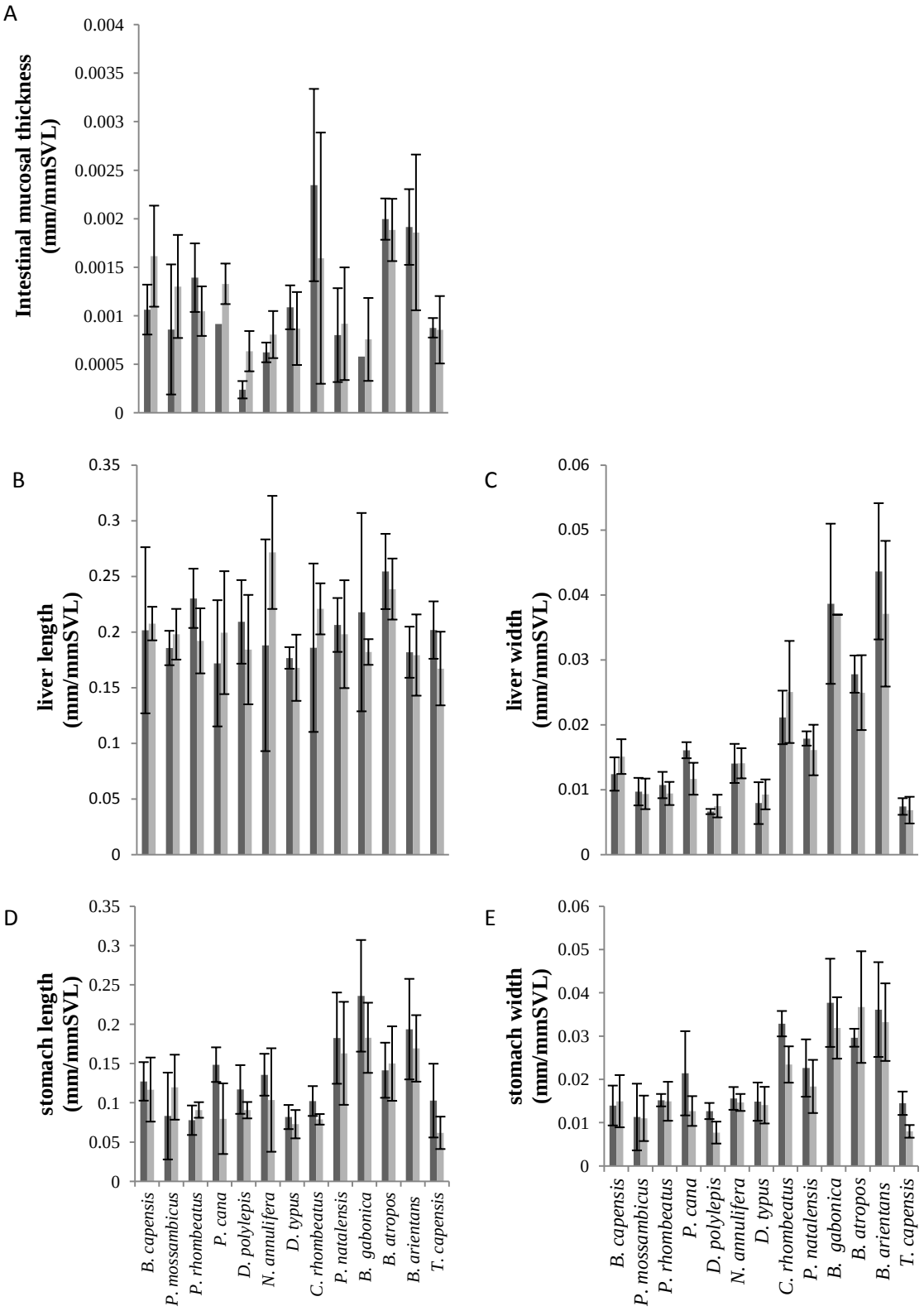

Figure 1. A series of graphs showing the mean organ measurements from the gastrointestinal tract for the study species between fasting and postprandial states. All measurements are shown relative to Snout-Vent Length. The dark bars represent the postprandial feeding state, while the lighter bars represent the fasting state. Standard deviation is also shown as error bars.

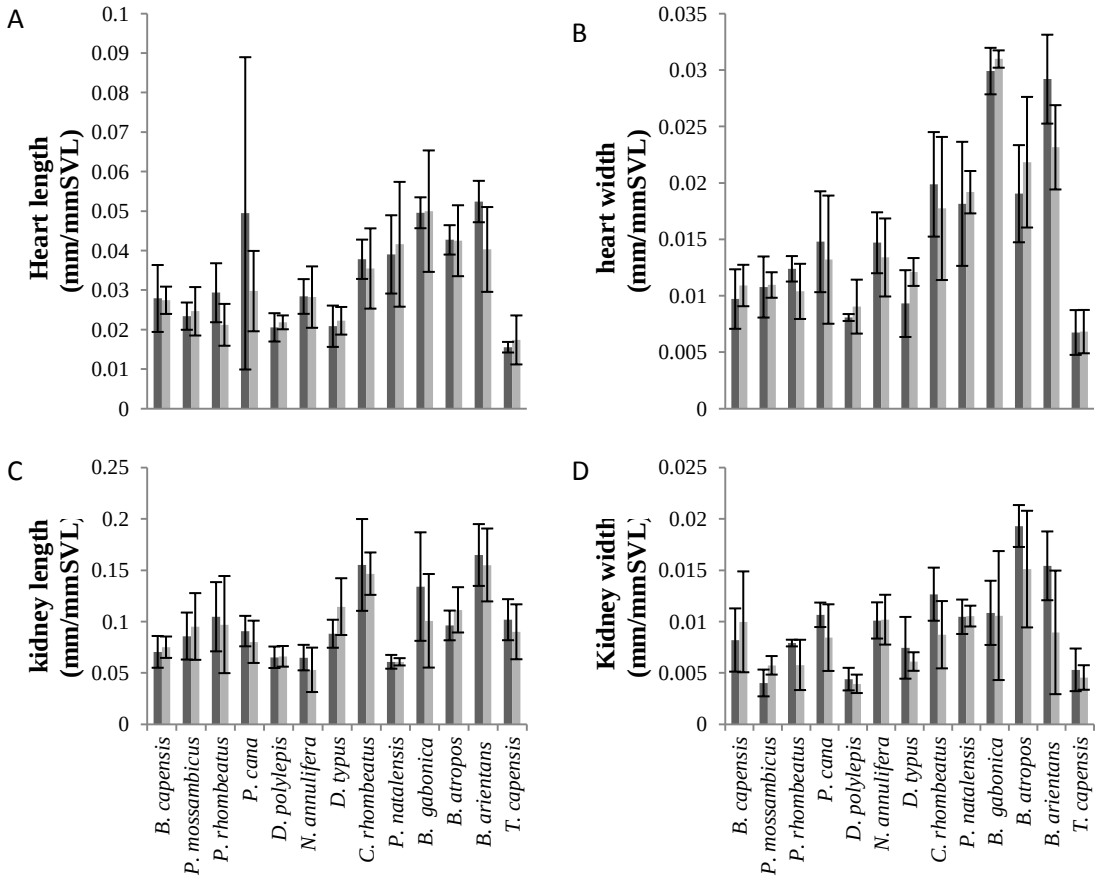

Figure 2. A series of graphs showing the mean associated organ measurements for the study species between fasting and postprandial states. All measurements are shown relative to Snout-Vent Length. The dark bars represent the postprandial feeding state, while the lighter bars represent the fasting state. Standard deviation is also shown as error bars.

5.2 Within species differences between fasting and postprandial individuals

T-tests comparing organ size between fasting and postprandial individuals in each species revealed no significant differences once the Bonferroni correction was applied (Table 2).

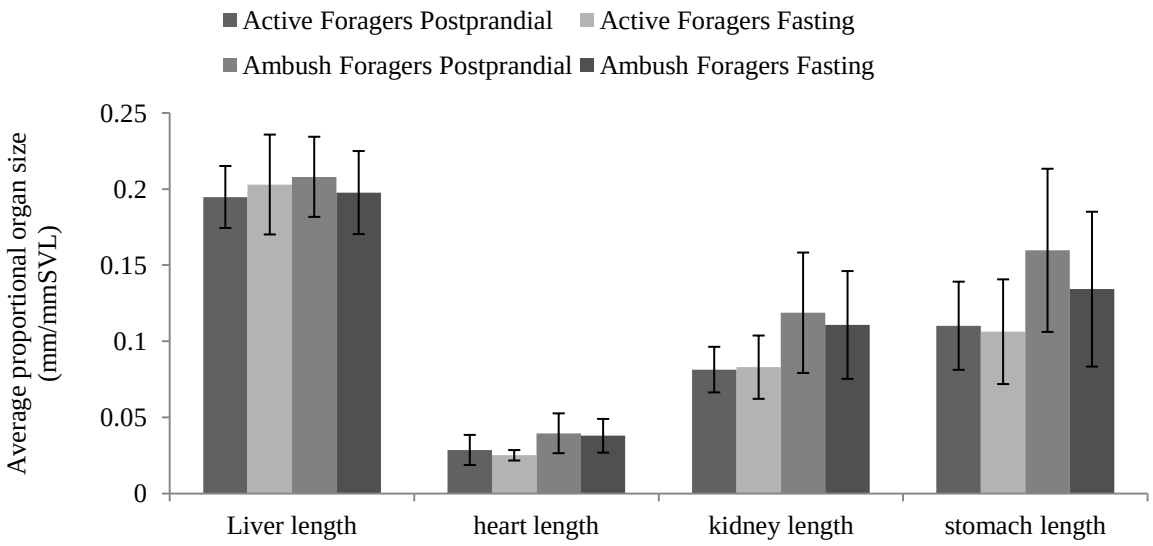

Figure 3. A bar graph comparing the average organ length measurements of the different foraging strategies within each feeding state. All values are shown relative to Snout-Vent Length. Standard deviation between species is displayed as error bars.

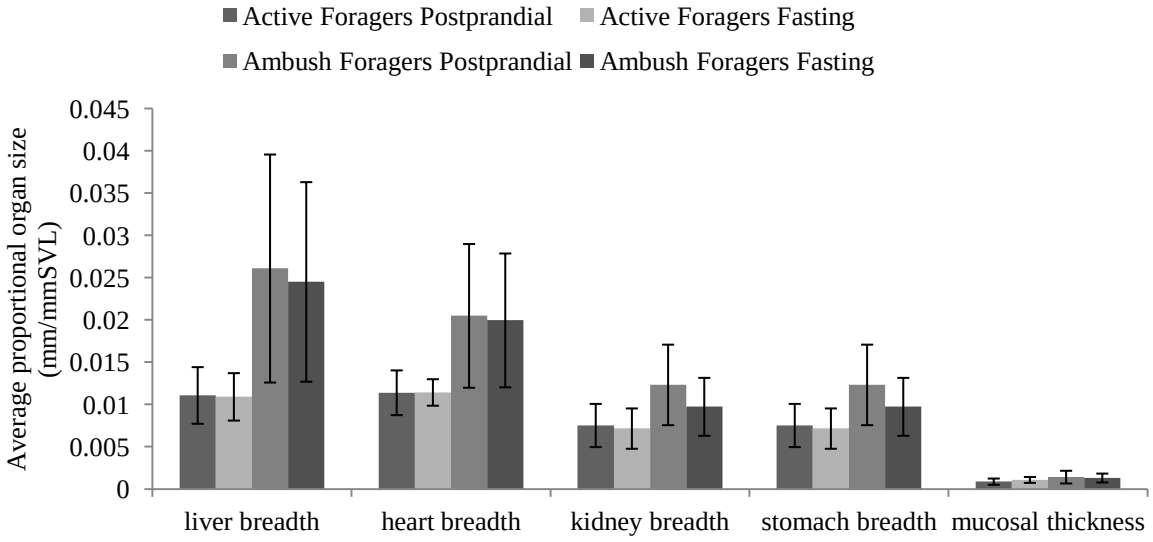

Figure 4. A bar graph comparing the average organ breadth measurements as well as mucosal thickness of the different foraging strategies within each feeding state. All values are shown relative to Snout-Vent Length. Standard deviation is displayed as error bars.

5.3 Influence of meal size on organ size in postprandial individuals.

Correlations between meal size and organ size revealed no significant trends (Table 3). No correlations were found once the Bonferroni Correction was applied. However several species showed high R^2 values, which accounts for much of the variation seen in the data, such as the increase in liver length seen in *P. cana*. However this was not significant with the small sample size.

5.4 Influence of seasonality on organ size

Seasonality (defined as months from mid-winter, which was taken as the 1st of July) appears to have a little correlation with organ size in most species, as shown in table 4. Only two species showed significant correlations with seasonality. The length of the kidneys in *C. rhombeatus* showed a significant negative correlation with seasonality ($p < 0.00085$). The width of the liver in *B. arietans* also showed a significant negative correlation with seasonality ($p < 0.00085$), although the rate of change was very low (0.03 mm / mm SVL/month), indicating that liver width was fairly constant. However several species showed high R^2 values, which accounts for much of the variation seen in the data, such as the increase in liver length seen in *B. arientans*. However this was not significant with the small sample size.

Table 2. Student's t-tests comparing proportional organ sizes between fasting and postprandial individuals within species. Probability values are also shown. Probability was considered at p = 0.1 level (p = 0.00085 with a Bonferroni Correction). Results shown by a dash (-) indicate that no regression was possible due to a lack of points. Regressions indicating a value of 1.000 were also not considered further due to a lack of data.

		Liver length		**liver width**		**heart length**		**heart width**		**stomach length**		**stomach width**		**kidney length**		**kidney width**		**mucosal thickness**	
Species	**N**	**t value**	**p value**	**t value**	**p value**	**t value**	**p value**	**t value**	**p value**	**t value**	**p value**	**t value**	**p value**	**t value**	**p value**	**t value**	**p value**	**t value**	**p value**
Boaedon capensis	10	-0.16	0.88	-1.34	0.25	-1.10	0.35	-0.77	0.50	0.32	0.77	-0.47	0.67	-1.40	0.26	-1.29	0.29	-0.84	0.56
Psammophis mossambicus	10	-1.12	0.33	0.20	0.85	-0.06	0.95	0.12	0.91	-1.15	0.33	0.23	0.83	-0.13	0.91	-1.61	0.21	-1.47	0.24
Psammophylax rhombeatus	10	4.87	0.01	1.15	0.31	2.90	0.06	1.82	0.17	-1.91	0.15	-0.26	0.81	-0.08	0.94	1.16	0.33	4.28	0.05
Pseudaspis cana	9	-0.58	0.60	2.49	0.09	0.85	0.46	1.64	0.20	2.35	0.10	1.36	0.27	1.28	0.29	1.86	0.16	-	-
Dendroaspis polylepis	9	0.67	0.55	-1.17	0.33	-1.16	0.33	-0.87	0.45	2.17	0.12	2.34	0.10	-0.19	0.86	1.20	0.32	-9.84	0.06
Naja annulifera	10	-1.50	0.21	-0.01	0.99	0.03	0.97	0.63	0.56	-0.48	0.65	0.82	0.46	0.87	0.43	-0.06	0.96	-	-
Dispholidus typus	10	0.61	0.57	-0.56	0.61	-0.45	0.68	-1.92	0.13	0.86	0.44	0.25	0.82	-2.90	0.04	0.84	0.45	-	-
Causus rhombeatus	9	-1.47	0.24	-1.66	0.19	0.23	0.84	0.30	0.78	3.17	0.05	4.19	0.02	-0.41	0.72	1.29	0.33	0.28	0.81
Python natalensis	9	0.98	0.40	1.02	0.38	0.14	0.90	0.05	0.96	0.35	0.75	0.64	0.57	-0.47	0.69	0.22	0.86	-0.53	0.64
Bitis gabonica	8	-	-	-	-	-	-	-	-	0.57	0.63	0.82	0.50	18.63	0.03	0.80	0.51	-	-
Bitis atropos	9	0.39	0.72	1.25	0.30	0.01	0.99	-0.80	0.48	-0.24	0.82	-1.19	0.32	-0.63	0.57	1.05	0.37	0.06	0.96
Bitis arietans	10	0.11	0.92	1.87	0.14	1.83	0.16	1.94	0.15	0.60	0.59	0.10	0.93	-0.25	0.83	1.91	0.15	-	-
Thelotornis capensis	9	2.12	0.12	0.66	0.56	-0.73	0.52	-0.11	0.92	2.42	0.09	5.69	0.01	0.74	0.51	1.04	0.38	0.02	0.99

Table 3. Correlation analysis comparing proportional organ sizes with meal sizes within a species. Probability values are also shown. Probability was considered at p = 0.1 level (p = 0.00085 with a Bonferroni Correction). Results shown by a dash (-) indicate that no regression was possible due to a lack of points. Regressions indicating a value of 1.000 were also not considered further due to a lack of data.

		Liver length		**liver width**		**heart length**		**heart width**		**stomach length**		**stomach width**		**kidney length**		**kidney width**		**mucosal thickness**	
Species	**N**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**
Boaedon capensis	5	0.01	0.96	0.62	0.27	0.38	0.69	0.03	0.78	0.05	0.72	0.21	0.44	0.70	0.07	0.60	0.12	0.10	0.60
Psammophis mossambicus	5	0.17	0.48	0.25	0.39	0.01	0.88	0.19	0.46	0.27	0.48	0.16	0.59	0.02	0.83	0.25	0.39	0.01	0.92
Psammophylax rhombeatus	4	0.35	0.41	0.71	0.16	0.03	0.82	0.04	0.79	0.55	0.26	0.06	0.75	0.01	0.95	0.21	0.54	0.27	0.65
Pseudaspis cana	4	**0.91**	0.04	0.11	0.67	0.02	0.86	0.80	0.10	0.19	0.56	0.17	0.58	0.41	0.35	0.18	0.58	-	-
Dendroaspis polylepis	4	0.21	0.54	0.87	0.06	0.11	0.67	0.01	0.99	0.09	0.69	0.83	0.09	0.12	0.66	0.30	0.45	0.15	0.75
Naja annulifera	4	0.07	0.72	**0.95**	0.03	0.04	0.80	0.06	0.75	0.47	0.31	0.05	0.77	0.29	0.47	0.02	0.85	1.00	-
Dispholidus typus	5	0.30	0.34	0.49	0.19	0.04	0.07	0.01	0.90	0.36	0.29	0.01	0.98	0.01	0.93	0.13	0.55	**0.97**	0.10
Causus rhombeatus	4	0.20	0.55	0.91	0.04	0.73	0.14	0.46	0.32	0.00	0.99	0.45	0.33	0.85	0.07	0.72	0.15	**0.97**	0.10
Python natalensis	4	0.28	0.47	0.39	0.37	**0.94**	0.03	0.79	0.11	0.24	0.51	0.53	0.28	1.00	-	1.00	-	0.11	0.67
Bitis gabonica	3	0.56	0.46	0.55	0.47	0.40	0.56	0.03	0.89	0.85	0.26	**0.95**	0.14	**0.97**	0.10	0.54	0.48	-	-
Bitis atropos	4	0.02	0.88	0.02	0.85	0.09	0.69	0.83	0.09	0.79	0.11	0.13	0.63	0.57	0.24	0.35	0.41	0.03	0.81
Bitis arietans	5	0.04	0.74	0.38	0.27	0.28	0.35	0.01	0.90	0.00	0.96	0.00	0.97	0.14	0.53	0.02	0.81	0.03	0.81
Thelotornis capensis	5	0.88	0.06	0.84	0.08	**0.90**	0.05	0.66	0.19	0.14	0.62	0.29	0.46	0.79	0.11	0.42	0.35	0.94	0.15

Table 4. Correlation analysis comparing proportional organ sizes with months from mid-winter within a species. Probability values are also shown. Probability was considered at p = 0.1 level (p = 0.00085 with a Bonferroni Correction). Results shown by a dash (-) indicate that no regression was possible due to a lack of points. Regressions indicating a value of 1.000 were also not considered further due to a lack of data.

		Liver length		**liver width**		**heart length**		**heart width**		**stomach length**		**stomach width**		**kidney length**		**kidney width**		**mucosal thickness**	
Species	**N**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**	**R^2 value**	**p value**
Boaedon capensis	4	0.65	0.19	0.16	0.60	0.86	0.07	0.03	0.84	0.02	0.86	0.14	0.62	0.16	0.60	0.14	0.62	1.00	-
Psammophis mossambicus	2	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-
Psammophylax rhombeatus	4	0.31	0.45	0.23	0.52	0.00	0.93	0.07	0.73	0.02	0.87	0.68	0.17	0.15	0.61	0.30	0.45	0.54	0.26
Pseudaspis cana	4	0.86	0.07	0.04	0.80	0.22	0.53	0.01	0.91	0.17	0.59	0.52	0.28	0.04	0.81	0.60	0.23	-	-
Dendroaspis polylepis	4	0.87	0.07	0.00	0.97	0.38	0.39	0.83	0.09	0.09	0.70	0.00	0.93	0.45	0.33	0.00	0.98	**0.92**	0.18
Naja annulifera	4	0.33	0.42	0.08	0.72	0.74	0.14	0.83	0.09	0.02	0.86	0.06	0.75	0.16	0.60	0.18	0.57	1.00	-
Dispholidus typus	5	0.59	0.13	0.59	0.13	0.28	0.36	0.12	0.57	0.08	0.64	0.17	0.49	0.36	0.29	0.49	0.19	0.03	0.89
Causus rhombeatus	5	0.21	0.43	0.10	0.61	0.16	0.51	0.19	0.46	0.26	0.38	0.06	0.69	**0.99**	0.00	0.73	0.15	0.22	0.42
Python natalensis	4	0.58	0.24	0.34	0.42	0.00	0.98	0.16	0.60	0.37	0.39	0.42	0.35	0.30	0.46	0.02	0.85	0.67	0.18
Bitis gabonica	3	0.19	0.72	0.25	0.67	0.03	0.90	0.28	0.65	**0.95**	0.15	0.00	0.99	**0.92**	0.18	0.25	0.67	-	-
Bitis atropos	4	0.59	0.23	0.05	0.79	0.22	0.53	0.69	0.17	0.00	0.99	0.81	0.09	0.53	0.27	0.09	0.70	0.53	0.48
Bitis arietans	3	0.14	0.76	**0.99**	0.00	0.04	0.87	0.56	0.46	0.95	0.14	0.01	0.95	0.62	0.42	0.53	0.48	-	-
Thelotornis capensis	3	0.10	0.79	0.78	0.31	0.79	0.30	0.74	0.34	**0.99**	0.00	**0.96**	0.12	**0.92**	0.18	0.05	0.86	1.00	-

6. Discussion

6.1 Diagnosing down-regulation in future studies

The main aim of this study was to test a quick and reliable method of diagnosing the digestive physiological system in snakes without the use of expensive respirometers or the need for captive maintenance and euthanasia of the large number of specimens. While down-regulation of the organs was not clearly diagnosable using the methods tested in my study, several differences between ambush and active foragers were evident. While there may be small differences in digestive physiology between foraging strategies, these differences were not as clear-cut or significant as expected. Thus, the use of museum specimens to diagnose down-regulation of digestive systems may not be a suitable methodology, due to the degradation of the specimens during the preservation process.

Examination of the liver revealed the most consistent differences between active forager and ambush foragers. While the results do not support the conclusion that any of the species use Down-regulation, measurements from the liver appear to be the closest to the expected results based on previous literature (Ott & Secor, 2007; Secor & Diamond, 2000).On average, ambush foragers had relatively larger differences in livers size between feeding state compared to the active foragers livers between feeding states; with the changes nearly double that of the active foragers. The length and width of the liver increased in almost every ambush foraging species. Therefore, comparisons of the liver between fasting and postprandial individuals within a species may be the best indicator of ambush foraging and may even be indicative of down-regulation of digestive physiology. Thus, species that display consistently large increases in liver size when food is present in the stomach may be diagnosed as using a down-regulation physiology, but differences were not as large as expected. These species would warrant further studies to confirm if they are using down-regulation.

While there was little significant difference seen within each species, the data suggests that the ambush foragers may be displaying a stronger response to feeding as most ambush foragers displayed an increase in organ size in response to ingestion of a meal, although further studies are required to confirm this. *Bitis arietans* was the only species that was found to have an increase in every organ measurement (even if it was not significantly).The species appearing to display the

closest results to previously measured active foragers was *Psammophis mossambicus*, as it was found to have the smallest changes, once ranked with the other species, indicating that it regulates it's organs to keep them constant and active. The confusion surrounding the influence of the ambush foraging strategy on the physiology of *Thelotornis capensis* can also be addressed to a certain degree. *Thelotornis capensis* displayed increases in most of its organs in response to feeding. This pattern is similar to what one would expect in an ambush forager, and so I would classify it as having an ambush forager's digestive physiology despite having a body from more indicative of an actively foraging species.

6.2 Confirming the use of Down-regulation in southern African snakes

The presence of down-regulation in ambush foraging snakes was not clearly evident using the methods in this study. Therefore the use of museum specimens to diagnose down-regulation may not be appropriate. While there were few significant differences within species, ambush foragers did show consistently larger organs than active foragers while in a postprandial state. This may indicate that there are differences in physiology between active and ambush foraging snakes, but do not confirm the use of down-regulation. While the ambush foragers did show an increase in organ size in response to feeding, the changes were not as extreme as reported in the literature for other ambush foragers (such as *P. bivitattus* or *C. cerastes*). This may indicate that the use of museum specimens is not suitable to detect the hypertrophy of organs in ambush foraging snakes.

Differences in organ size within each species between fasting and postprandial groups could be used to indicate the extent of the physiological changes occurring in response to feeding. However, no significant differences within each species indicate that the expected changes in organ size cannot be measured using museum specimens. The lack of significant difference in organ size between feeding states did not fit with my initial hypothesis that ambush foraging snakes would show significant increases in organ size due to the hypertrophy of the digestive tract during digestion. The active foragers did seem to support my initial hypothesis, as the active foragers showed relatively constant organ sizes throughout. However the lack of difference in organ size between feeding states

may be due to the poor quality of the museum specimens rather than due to a lack of physiological regulation.

The lack of significant differences between active and ambush foragers, within each feeding state, does not support my hypothesis that many ambush foragers are using down-regulation. Very few significant differences were found between postprandial individuals. This is further confirmed by the lack of differences in the fasting individuals, as there were very few significant differences were found between active and ambush foragers. Most of the significant differences also revealed that the ambush foragers had larger organs than active foragers during fasting periods, which did not support my hypothesis. This may again indicate that museum specimens are not appropriate for the detection of changes in organ size described in the literature surrounding down-regulation.

The intra-specific comparisons of this study do not follow the expected trends in organ size in response to feeding. This is clearly evident in the data regarding *P. natalensis*, as they should be showing similar results to studies that used other species of the *Python* genus, such as *P. bivitattus* or *P. sebae* (Secor & Ott, 2007; Secor & Diamond, 2000; Secor, 1995). Most studies focusing on ambush foraging snakes found that the mass of various organs increased significantly within a day of feeding. This gain in mass was most pronounced in the stomach and intestines, which appeared to double in mass within 24 hours of receiving a meal in nearly every study (Secor, et al., 1994; Secor & Diamond, 1995). The lack of similarity between the findings of my study to previous findings indicates that the methodology used here may not be suitable.

Some previous studies comparing changes in organ size between different foraging strategies also found few differences, similar to the results found in this study. Secor and Diamond (2000) compared the organs of four species of actively foraging snakes and four species of ambush foraging snakes. They found that there was no significant difference in organs sizes during fasting between ambush and active foragers. In the postprandial state, they found that most organs had increased in mass, but not significantly in any of the active foragers. The findings from my study for the ambush foragers did not fit with Secor and Diamond (2000) as they found significant increases in organ sizes in response to feeding, whereas I found no significant increase. My study also found few significant

differences between the species in postprandial states. This again indicates that fresh samples may prove to be more accurate than museum specimens for organ measures.

The results shown here do not necessarily rule out the use of down-regulation in ambush foragers, as further analysis of their metabolic rates may be useful in clarifying their physiology. Overgaard *et al.* (2002) concluded in their study of *P. bivitattus* that the extremely large costs of digestion are not correlated with the increased mass and function of the organs and must be due to other physiological processes. They came to this conclusion after they found that *P. bivitattus* displayed no significant difference in peak SDA values with short fasting periods, when compared to SDA values from longer fasting periods. These results is unexpected as the SDA value after a short fasting period should be significantly lower than the SDA peak from a longer fasting period as the digestive tract has not had time to begin atrophy of the digestive tract and so should be ready for a meal already. This means that the hypertrophy of the organs may not be responsible for the high SDA values seen in ambush foragers. Thus, species examined may still be exhibiting similar physiological responses to feeding, but the organ measures may not be capable of measuring it. Therefore, further studies are required before down-regulation can be ruled out as the digestive physiology of southern African ambush foraging snakes.

The lack of significant difference between fasting and postprandial states in ambush foraging snakes with museum specimens may be due to the cellular mechanisms that control the hypertrophy of the organs in response to feeding. Andrews *et al.* (2015) as well as Castoe *et al.* (2013) found that the rapid changes in gene expression in response to feeding in *P. bivitattus* caused a significant increase in cell mitosis (hyperplasia) as well as cell hypertrophy, which accounts for the increased organ mass. The hypertrophy of the cells is caused by the expression of protein synthetic pathways that promote the uptake of water and nutrients (Riquelme, et al., 2011; Andrew, et al., 2015; Castoe, et al., 2013). The presence of a strong ethanol solution used by museums as a preservative may then cause the hypertrophic cells to then dehydrate again, and so may shrink in both mass and volume. This would cause any organ measurements to result in smaller than expected measurements and so would not adequately describe the hypertrophy of the organs during digestion, even with the increased

number of cells from hyperplasia. This would explain why the museum specimens examined did not display the extreme changes as observed in previous studies.

My study had several distinct flaws that were unavoidable. Many of these limitations were imposed at the dictate of the Ditsong Natural History Museum. They insisted that I was only able to dissect 10% of the usable specimens per species. This restricted the sample size of many of the species (particularly *P. natalensis* and *B. gabonica*) to fewer than 10 samples. I was also not able to measure the mass of the various organs during the dissections, as I would have had to completely remove each organ in order to measure it. The Museum asked me to remove mass measurements from the study in the interest of preserving their specimens. Neglect of samples may also be contributing factor, as there was little to no record of any maintenance in recent years. The 70% ethanol that the samples had been preserved in had not been changed in many years and so may have contributed to the degradation of many of the samples and skewed my results. This is particularly evident in the measures of intestinal mucosal thickness, where many samples did not survive the lab processing required to prepare a slide. Many of the samples were too brittle to fix to a slide and so could not be measured.

Several corrections to this methodology may be useful in future studies. Comparisons to freshly obtained samples may also prove to be useful, as we can then calculate a correction factor from repeated measures on each individual for changes in tissue during the preservation process. Mass measurements of the dissected organs as well as the whole snake should also be taken as a further measurement. Mass measurements can also then be correlated to snout-vent length and organ dimensional measures and then be used to calculate organ mass based on the dimensional measures. This would allow future studies to then estimate mass measurements without removing the organs and damaging the museum specimen.

In conclusion, the lack of significant difference between fasting and postprandial individuals within each species indicates that the use of museum specimens to diagnose down-regulation is not suitable. Certain similarities seen when comparing species within each feeding group (for example, the Viperidae species all showed significantly wider livers than active foragers, regardless of feeding state) indicates that differences seen between species may be due to different organ morphology and

ancestry rather than due to feeding responses. Therefore it is not clear that any of the species examined in this study are using down-regulation and further studies are required before a definitive classification can be made. Further studies involving changes in metabolism, changes in organ mass as well as comparisons to freshly obtained samples should prove to be more conclusive.

6.3 The influence of meal size on postprandial changes

I detected no strong relationship between meal size and organ size in any of the examined species. I feel this may be due to the poor state of the museum samples rather than due to any significant trend. This would then also explain why no significant correlations were found in the ambush foragers, despite our initial hypothesis that ambush foragers should show a linear relationship between meal size and organ size. This is evident with *B. arietans*, which showed an increase in all its organs in response to feeding (albeit not significantly) as expected, but showed no correlation to meal size. This indicates that museum specimens may not be suitable to measure the relationship between digestive physiology and meal size.

There were several possible flaws with the measurements of the meal size. Estimates of meal size were scored out of ten according to the size of the snake. However this became difficult when meals were partially digested and made my estimates of the meal size unreliable. It was also difficult to address if a snake had eaten recently and had already digested the meal. If a snake had recently digested a meal, I may have classified it as a fasting individual despite it being in a postprandial state. The only organ that appears to be consistent when considering museum specimens with regards to meal size was the liver, with four species tending towards significant correlations between meal size and liver size, when considered at the $p = 0.1$ level. The only species that appeared to be strongly influenced by meal size was *T. capensis*, which showed correlations between meal size and its liver, heart and intestines when considered at the $p = 0.1$ level. This may be due to their extremely narrow bodies which allow them to camouflage amongst branches well. A strong response to meal size may be advantageous for them as they can keep their bodies as narrow as possible during digestion, and so allow them to stay as camouflaged as possible.

It is not clear how the results for meal size compare with the existing literature surrounding the influence of meal size on organ size. While several studies have examined the effects of meal size on SDA and digestive efficiency, very few have looked at how organ size varies with meal size. Secor and Diamond (1997) found that *P. bivitattus* only showed significantly increased organ size with extremely large meals (meals larger than 25% of the snakes' body mass). This indicates that the snake was able to digest the smaller meal efficiently without having to significantly increase the mass of its organs. Other studies (Secor, 1995; Secor & Diamond, 1995; Lignot, et al., 2005) that have measured significant increases in organ mass during digestion have mostly used meal sizes over 25% of the snake's body mass, and so would show increased organ size according to the meal size limit described by Secor and Diamond (1997). As a result, the authors may not have considered this as a factor. This may explain the results found in my study, as the ambush foraging snakes examined in my study may have been capable of digesting the meal without significantly increasing its organ size. It may also be possible that the meal was not large enough to elicit a significant response and so no significant difference would be detected.

Several studies have found that SDA and digestive efficiency increases with meal size (Secor & Faulkner, 2002; Cox & Secor, 2007; Tein-Shun, et al., 2008), indicating that the extremely large SDA recorded in ambush foragers must be due to other energetic costs and not associated with the assumed increase in organ size. This is confirmed by Overgaard *et al.* (2002), who also found that the increase in metabolic rate during digestion did not correlate with the increase in organ size and so also concluded that the increased metabolic rate must be due to other physiological processes (Overgaard, et al., 2002). Therefore it may be possible that changes in organ size may not correlate with the increased SDA. Therefore, while evidence presented by my study does not support the hypothesis that the change in organ size in response to a meal is influenced by the meal size, further studies with more accurate measures of meal size are required to clarify the influence of meal size on postprandial responses.

6.4 The influence of seasonality on postprandial changes

I did not detect any significant correlations between seasonality and organ size in most species. The lack of correlation did not support my hypothesis regarding the seasonally modulated digestive regulation in active foragers. However, this again may be due to the quality of the museum specimens rather than due to any significant trend. The poor quality of specimens and small sample size suggests that the museum specimens may not be suitable to measure changes due to seasonality. This may explain why I did not find any significant correlations between the active foragers and seasonality. I also cannot use this evidence to support the hypothesis that ambush foragers do not show seasonal regulation.

There is strong evidence in the current literature to support the hypothesis that most snakes will have down-regulated digestive tracts during winter months. Numerous studies (Alexander, et al., 2012; Toledo, et al., 2003; Greene, et al., 2013; Zaidan & Beaupre, 2003) have found that digestive time increases as the temperature decreases in snakes. The longer digestion time may then cause a meal to putrefy before it can be digested and assimilated. Alexander and Brooks (1999) discussed the risk of putrefying food in the gut as a possible reason for why actively foraging *Hemachatus haemachatus* refused to eat during winter months, as *H. haemachatus* showed significantly lower rates of food acceptance during winter months even when temperature was controlled. Naya *et al.* (2011) and Tracy and Diamond (2005) also found that their study species of lizards displayed larger organ sizes during summer months. This supports the hypothesis that snakes should therefore be showing down-regulation during winter months in order to save energy due to the lower temperatures and lack of food. Therefore it is clear that the results from this study do not fit with previous studies.

The poor quality of the preservation process with the museum specimens may have influenced the organ measurements. There may also be a bias in sampling, as there were fewer specimens found during winter months, and so there may be a bias against winter measurements. Furthermore, many of the labels had been lost from several of the specimens and so I could not record the month in which they had been captured. Therefore, there were too few data for analysis of several of the species such as *B. atropos*. This was disappointing, considering the high altitude and extreme seasonal variation seen with *B. atropos*'s habitat. I would recommend further studies be conducted on

the other species, using fresh samples. In conclusion, it appears that museum specimens are not suitable to measure changes in digestive physiology in response to seasonality.

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