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A Comparison of Adrenal in Toad-Eating and Nontoad-Eating Snakes

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A COMPARISON OF ADRENAL GLANDS IN TOAD-EATING AND NONTOAD-EATING SNAKES

by

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ABSTRACT

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Toads are chemically defended by bufadienolides, a class of cardiotonic steroids lethal to most predators, including many snakes. Bufadienolides bind to Na+K+-ATPase, inhibiting their ability to transport ions. In cardiocytes, this inhibition cause arrhythmia and severely increased contraction strength, which, if prolonged, lead to death. However, several snakes are resistant to bufadienolides and consume toads with no ill effects. Adrenal glands produce hormones that are important for the maintenance of Na+K+-ATPase, and may therefore play an important role in countering the negative effects of bufadienolides. Indeed, the toad-eating specialist *Heterodon platirhinos* has been known to possess enlarged, and sexually dimorphic, adrenal glands. I hypothesized that toad-eating snakes have modified adrenal glands that play a role in the snakes' resistance to bufadienolides and that sexual dimorphism in adrenal gland size is a general characteristic of bufophagous snakes. I used phylogenetically independent taxa to investigate adrenal morphology in bufophagous and non-bufophagous species. I compared adrenal mass among species and found that the allometric relationship between adrenal mass and body size is significantly different in bufophagous and non-bufophagous snakes, and that these differences also exist between sex in bufophagous species. One bufophagous species, *Natrix natrix*, fell out of the pattern seen in the others,

having adrenal glands more similar to nonbufophagous species. In addition to the morphological comparisons, I compared tissue proportions in histological sections of the adrenal glands but found no significant differences there.

This thesis is dedicated to my dearest friends,

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and

Deborah C. Fry,

all of whom have and always will provide me with unyielding guidance and support throughout my life.

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CHAPTER I

INTRODUCTION

Bufophagous Snakes

Consumption of toads, or bufophagy, is unusual among snakes due to the lethal effects of the bufadienolide toxins found in the skin, and especially the parotoid glands, of toads (Licht and Low, 1968). There are, however, several snakes that have evolved resistance to the toxins and are capable of consuming toads with no apparent ill effects. The ability to consume toxic prey presents several opportunities to a predator. One obvious benefit is reduced competition for food. Another, more functional, benefit is to utilize the consumed toxins for defense against predation. Indeed, recent work on the Japanese snake *Rhabdophis tigrinus* has revealed that individuals sequester bufadienolides from the toads they consume into specialized nuchal glands on the backs of their necks specifically for defense (Hutchinson et al., 2007). Furthermore, females can deposit the toxins in their yolk or transmit them to the embryos during oviducal passage to produce chemically defended offspring (Hutchinson et al., 2008). The only other known form of sequestered chemical defense in snakes exists in the common garter snakes (*Thamnophis sirtalis*), which accumulate substantial quantities of tetrodotoxin (TTX), especially in their livers, from newts they consume (Williams et al., 2004). However, this form of defense does not involve a specialized organ such as that seen in *R. tigrinus*. Individuals of *T. sirtalis* exhibit reduced locomotor performance shortly after

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ingestion of tetrodotoxin, and are therefore potentially susceptible to predators during that time. The accumulation of TTX in their tissues presumably affords some protection against predators.

Few studies have investigated the physiological mechanism that allows bufophagous snakes to overcome the lethal effects of bufadienolides. Several observations have been made, however, including the enlargement of adrenal glands in *Heterodon platirhinos* (Smith and White, 1955; Spauer and Smith, 1971). Another pattern seen repeatedly among bufophagous species is "death-feigning" behavior. *H. platirhinos* exhibits bradycardia when death-feigning (McDonald, 1974), and it has been suggested that this behavioral pattern might be a physiological result of dealing with bufadienolides (McDonald, 1974).

Due to the scarcity of comprehensive dietary studies it is difficult to assess the exact number of specialized bufophagous snakes worldwide. Nonetheless, a few species are well known to consume toads as a primary component of their diet. In North America, the most widely studied species is the eastern hognose snake *Heterodon platirhinos* (Cooper and Secor, 2007). Other congeners, *H. nasicus* and *H. simus*, also feed largely on toads, although the latter species have a more varied diet (Carr, 1940; Kroll, 1976; Platt, 1969). Some species of the North American natricid genera *Nerodia* and *Thamnophis* consume toads occasionally, though not frequently (Mushinsky and Hebrard, 1977; Hirai, 2004; Tanaka and Toa, 2002; Abbadié-Bisogno et al., 2003). In Central and South America, several species of the genera *Liophis*, *Lystrophis*, *Waglerophis* and *Xenodon* consume toads (Baptista de Oliveira et al., 2000; Vitt, 1983;

Michaud and Dixon, 1989), with *W. merremii* feeding predominantly on toads (Vitt, 1983). In Europe and eastern Asia, the grass snake, *Natrix natrix,* is the only snake reported to consume toads on a regular basis (Gregory and Isaac, 2004). Interestingly, Madsen (1983) demonstrated that toads represent a much higher proportion of the diet of female *Natrix natrix* than of males. Gregory and Isaac (2004) attribute this pattern to differences in head size between males and females and note that, although females prey on toads more frequently, both sexes consume toads. In eastern Asia, members of the genus *Rhabdophis* consume toads (Hirai, 2004; Hutchinson et al., 2007; Hutchinson et al., 2008). In Africa, night adders (*Causus*) are toad specialists (Greene, 1997), and several members of the genera *Naja* and *Hemachatus*, which range from Africa throughout the Middle East, to southeast Asia, also consume toads (Shine et al., 2007; Luiselli et al., 2002).

Cardiotonic Steroids

Cardiotonic steroids include a wide variety of animal- and plant-derived toxins that affect cardiac contractility by binding to and disabling the sodium-potassium pump, or sodium-potassium ATPase (Na^+,K^+-ATPase; NKA). Humans have exploited this phenomenon for over three thousand years, using these compounds to treat heart conditions (Krenn and Kopp, 1997; Schoner, 2002). All cardiotonic steroids share the general structure of a steroidal body with a lactone ring (Dvela et al., 2007). Plant-derived cardiotonic steroids such as digitalis and ouabain, which contain a five-carbon lactone ring, are known as cardenolides and are widely used in contemporary

pharmaceuticals. Animal-derived cardiotonic steroids, which contain a six-carbon lactone ring, are known as bufadienolides. These are best known from the toads (Bufonidae), but they also occur in two species of fireflies (Lampyridae; Krenn and Kopp, 1998; Dvela et al., 2007; Eisner et al., 1978). Interestingly, over thirty years ago, a clinical study was conducted in which subjects who were not given cardiotonic steroids tested positive for their presence, suggesting that they exist naturally in mammals (Besch et al., 1976). Subsequent studies established that endogenous cardiotonic steroids were indeed present in the adrenal cortex and that they were structurally similar to the amphibian bufadienolide marinobufagenin (Iqbal et al., 1991; Dmitrieva et al. 2000). This discovery shed new light on the biological significance of cardiotonic steroids in drugs, and their pharmacological effect is now regarded as a hormonal effect at concentrations tenfold or higher than the baseline physiological level (Dvela et al., 2007).

The Sodium Potassium Pump

The sodium-potassium pump is a vital transmembrane protein found in all cells. It is responsible for the transport of sodium (Na^+) ions out of the cell and potassium (K^+) ions into the cell, creating a strong concentration gradient that provides the energy necessary for the membrane to transport metabolites and nutrients (Croyle et al., 1997). The pump also regulates cell volume and action potential in muscle and nerve cells (Croyle et al., 1997). NKA is composed of two major proteins, the α and β subunits. These can exist in multiple isoforms, many of which are tissue-specific (Rose and Valdes, Jr., 1994).

In the heart, NKA acts as an indirect regulator of contractions by maintaining intracellular calcium (Ca^{2+}) levels. In a properly functioning cell, Ca^{2+} is pumped into the sarcoplasmic reticulum and then released in controlled levels to muscle fibers, where the calcium ions bind to the protein complex troponin, causing a change in conformation of the actin and myosin filaments to produce a contraction (Barry and Bridge, 1993). When NKA is disabled, the membrane stops transporting Na^+ out of the cell. The resulting elevated intracellular Na^+ level activates the sodium-calcium exchanger, (Ca^{2+}/ $3Na^+$ exchanger; NCS), which compensates for high Na^+ concentrations by importing more Ca^{2+}, causing an increase in Ca^{2+} levels (Reuter et al., 2002; Barry and Bridge, 1993). To regain Ca^{2+} equilibrium, the sarcoplasmic reticulum releases more Ca^{2+} to muscle fibers at a faster rate (Barry and Bridge, 1993). In myocardial cells, the increase in Ca^{2+} released by the sarcoplasmic reticulum enhances contractile strength, and the faster release causes arrhythmia. In cases where NKA function is disabled for a prolonged period, these symptoms can lead to cardiac failure (Rose and Valdes, Jr., 1994).

The α subunit of NKA contains a binding site specific to cardiotonic steroids, and variation at this site among isoforms can influence its affinity for these molecules. Isoforms found in human NKA, for example, are a thousand times more sensitive to cardiotonic steroids than those of rats or mice (Price and Lingrel, 1988). Between species that exhibit such differences, NKA has been found to be nearly identical except for structural changes in the cardiotonic-steroid binding site on the α subunit. These differences among isoforms can exist within species as well, due to factors such as age,

ion concentration, hormone concentrations, and pathological conditions (Rose and Valdes, Jr., 1994; McDonough et al., 1992).

Adrenal Glands in Snakes

Unlike mammals, the adrenal glands of reptiles exhibit a consistent morphological association with the reproductive organs (Hebard and Charipper, 1955). Adrenal glands receive arterial blood from the dorsal aorta, and venous blood is drained from the glands through the vena cava. Adrenal nerve supply is derived from sympathetic ganglia that are found chiefly on the dorsal surface of the gland, closely associated with arterial blood vessels (Hebard and Charipper, 1955).

In reptiles, the adrenal glands are composed of catecholamine-producing chromaffin tissue, homologous to the adrenal medulla in mammals, and corticosteroid-producing interrenal tissue homologous to mammalian cortex. The morphology of chromaffin tissue varies according to taxon, but in most squamates it consists of a dorsal cap and isolated islets of cells located near blood vessels (Rupik, 2002). Cells of the dorsal cap synthesize norepinephrine (noradrenaline), whereas the islet cells, located among the interrenal cords, synthesize epinephrine (adrenaline; Rupik, 2002). The interrenal tissue synthesizes the corticosteroids aldosterone, corticosterone, and 18-hydroxycorticosterone (Norris, 1997).

The interrenal cells of snakes have abundant and very well-developed smooth endoplasmic reticulum (ER), as well as lipid inclusions (Hebrard and Charipper, 1955). Histochemical stains have indicated that the lipid inclusions are composed of a mixture of

neutral fats, cholesterol and cholesterol esters, and there is an inverse ratio between the numbers of mitochondria and lipid inclusions (Hebrard and Charipper, 1955). In interrenal cells, these lipids are synthesized into corticosteroids by mitochondria with the help of the smooth ER (Norris, 1997).

Aldosterone is a mineralocorticoid hormone, the main function of which is to maintain normal sodium-potassium balance. Aldosterone also controls NKA by inducing a long-term increase in the gene expression of NKA proteins (Therien and Blostein, 2000). It does so by binding to, and thereby activating, a specific intracellular mineralocorticoid hormone receptor, which in turn induces transcription by binding to a DNA-hormone response element (Ikeda et al., 1991). When aldosterone increases Na^+ influx via the Na^+/H^+ exchange mechanism, it provides an early signal to increase the rate of NKA mRNA synthesis and subsequent gene expression. Ikeda et al. (1991) conducted a series of controlled experiments where controlled concentrations of aldosterone were added to a medium containing cardiac cells. The cells exposed to aldosterone experienced a three-fold increase in α-1 and β-1 NKA protein mRNA within six hours and reached steady state levels in 12 hours, suggesting that aldosterone causes an increase in NKA content per cell. They also found that after removal of aldosterone, the steady state level of NKA mRNA decreased to baseline levels within six hours, demonstrating that the presence of aldosterone is required for the maintenance of NKA mRNA levels. Furthermore, by measuring the fluorescence intensity distribution of anti-α sites in the cardiocytes, they determined that increases in NKA mRNA lead to an increase in actual NKA. This study strongly suggests that aldosterone can potentially

counter the NKA-disabling effects of bufadienolides on cardiocytes by increasing NKA synthesis.

Corticosterone is the primary glucocorticoid hormone produced in the reptilian adrenal gland. Substantially less work has been done on the effects of corticosterone than on aldosterone, but studies indicate that their effects are similar. Lyoussi and Crabbé (1996) showed that when corticosterone is added to renal cells, NKA activity increases, as measured by the amount of phosphate released from ATP when binding to NKA. Interestingly, the addition of corticosterone resulted in a greater increase in NKA activity than did the addition of aldosterone (Lyoussi and Crabbé, 1996).

Adrenergic agents such as epinephrine and norepinephrine are known to modulate sodium pump activity through several mechanisms. When mice are subjected to various stressors, they respond with increases in blood pressure, adrenal weight, and catecholamine concentrations in chromaffin cells (Axelrod and Reisine, 1984). Svoboda et al. (1986) demonstrated that both catecholamines increase NKA activity in the brain and brown adipose tissue without the presence of NKA inhibitory factors. However, Cook et al. (1983) showed that myocardial cells subjected to cardiotonic steroids showed no change in NKA activity when norepinephrine was added, suggesting that adrenergic agents may not directly counteract bufadienolides. Catecholamines do, however, have the ability to stimulate ACTH release from the pituitary gland by directly acting on its anterior lobe, which, in turn, stimulates the synthesis of corticosteroids in interrenal tissue (Axelrod and Reisine, 1984). It may be that the role adrenergic agents play to counteract bufadienolides, is simply to help to stimulate corticosteroid release.

Because adrenal glands produce hormones that help to maintain the function of NKA, it is reasonable to expect that selection to counteract the negative pharmacological effects of bufadienolides could result in the evolution of adrenal enlargement, such as the condition reported for *Heterodon platirhinos*. The chapters that follow constitute an investigation into the comparative morphology and histology of adrenal glands in bufophagous and nonbufophagous snakes. Chapter II provides evidence that adrenal mass scales differently with body size in these two groups, and Chapter III reveals that the effects of adrenal mass cannot be explained simply on the basis of one adrenal tissue type (chromaffin or interrenal) being larger than the other.

CHAPTER II

ADRENAL MASS COMPARISON IN BUFOPHAGOUS AND NONBUFOPHAGOUS SNAKES

Introduction

About 50 years ago Smith and White (1955) reported that the bufophagous (toad-eating) snake *Heterodon platirhinos* (Xenodontindae) possesses markedly enlarged adrenal glands (Smith and White, 1955). Subsequently, Spaur and Smith (1971) showed that the degree of enlargement is sexually dimorphic, with males possessing significantly larger adrenal glands. Additionally, recent observations have suggested that the bufophagous Japanese snake *Rhabdophis tigrinus* (Natricidae) exhibits these same features (Figure 2.1; Savitzky, Mori, and Hutchinson, unpublished). These observations suggest that bufophagy may be related to enlargement, and possibly sexual dimorphism, of adrenal glands. Various authors have since attempted to explain the enlarged adrenal glands of *Heterodon* as either a requisite for, or a consequence of, feeding on toads (Smith and White, 1955; Spaur and Smith, 1971; Licht and Low, 1968). However, these studies have typically considered few taxa and have not controlled for phylogenetic patterns of variation. Therefore, they have not convincingly shown that adrenal enlargement is a general trait associated with a bufophagous diet.

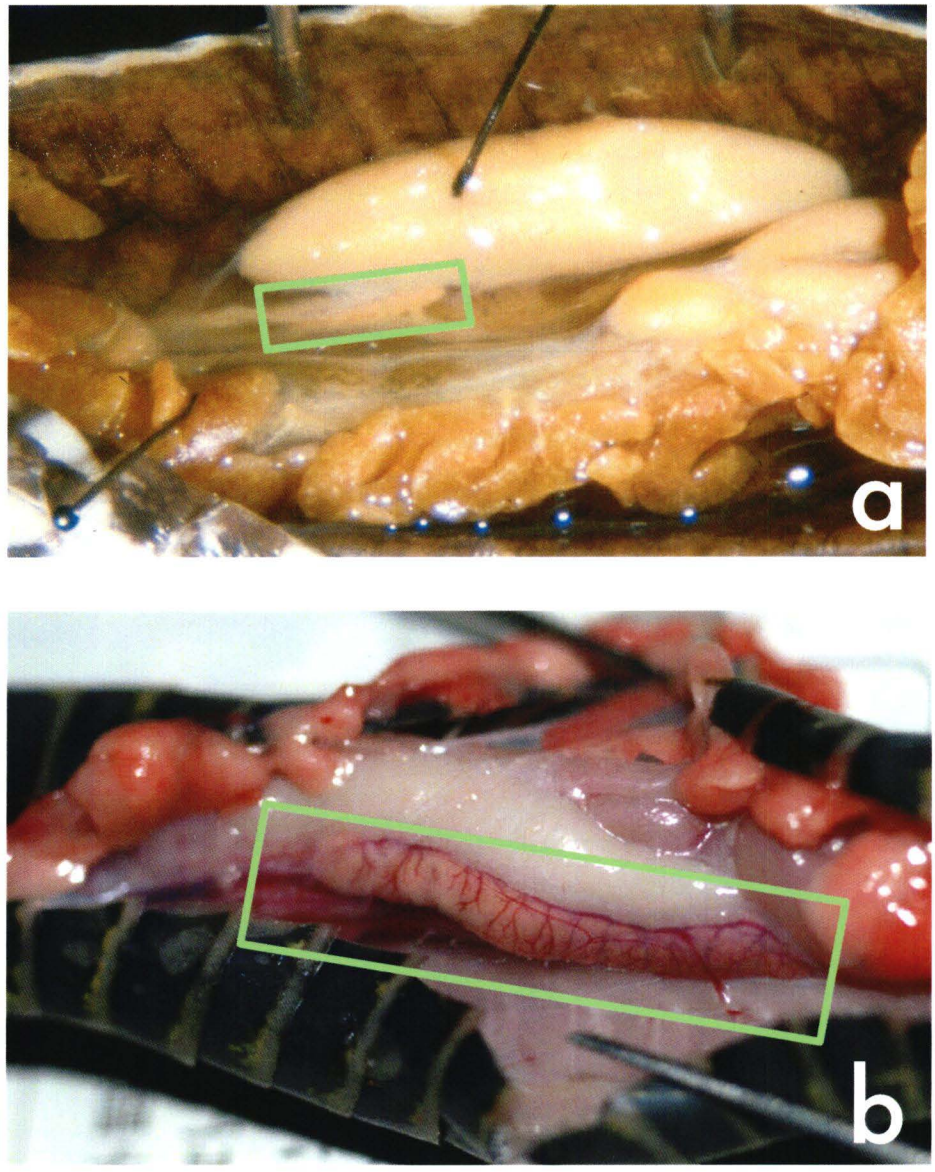

Figure 2.1. The adrenal glands (in green boxes) of (a) the nonbufophagous natricid snake *Nerodia sipedon* and (b) the bufophagous natricid *Rhabdophis tigrinus,* showing the difference in adrenal size. The photos have been reproduced at the same testis length for comparative purposes. (Photos by A. H. Savitzky)

Adrenal glands produce a variety of hormones that influence the activity of NKA and the maintenance of cardiac function, and might therefore play a significant role in resistance to bufadienolides (see Chapter 1). I hypothesized that bufophagous snakes have larger adrenal glands than nonbufophagous snakes, presumably to counteract the negative effects of bufadienolides. I tested this hypothesis by comparing relative adrenal mass among several groups of phylogenetically independent species. My objectives were (1) to determine whether adrenal enlargement has evolved repeatedly in bufophagous snakes and (2) to determine whether sexual dimorphism in adrenal mass exists in other species, either in all snakes or in bufophagous species alone. The results of this analysis provide new insights into the mechanisms underlying bufadienolide resistance in snakes.

Methods

Correcting For Phylogenetic Effects

I generated a phylogenetic tree (Figure 2.2) comprised of 64 terminal taxa of colubroid snakes, using sequences from three mitochondrial genes: 12S, 16S, and cytochrome *b* (Table 2.1). The taxa were selected to include known bufophagous species in several lineages, as well as related nonbufophagous taxa, as a means of identifying phylogenetically controlled pairs of species with different diets. One outgroup taxon was selected (*Acrochordus granulatus*), based on previous phylogenetic analyses (Sander, et al., 2010; Slowinkski and Keogh, 2000; Wüster, et al., 2008; Zaher et al., 2009). DNA sequences were acquired from GenBank, accession numbers are listed in Table 1. Sequences were edited and aligned in Geneious Pro, ver. 4.5.3 (Biomatters Ltd., Auckland, New Zealand), followed by manual refinement in MacClade, ver. 4.08

(Sinauer Associates, Inc., Sunderland, MA, USA). The aligned sequences were run by the best fit model (GTR+I+G), as inferred by jModelTest, ver. 0.1.1 (Posada, 2008) in MrBayes, ver. 3.1.2 (Huelsenbeck and Ronquist, 2003), using Bayesian inference to yield trees. Markov chain Monte Carlo searches were performed for 10 million generations, with every one thousandth sample being retained. From the resulting tree, four phylogenetically independent species pairs were selected for comparison, each consisting of one bufophagous and one nonbufophagous species (Figure 2.2).

Table 2.1. List of taxa used in the phylogenetic analysis and the GenBank sequence accession numbers.

	Family	Taxa	12S	16S	Cyt *b*
1	Achrocordidae	*Acrochordus granulatus*	AF544738.1	AF544786.1	AF217841.1
2	Colubridae	*Amphiesma sauteri*	AF402622.1	—	AF402905.1
3		*Carphophis amoenus*	AY577013.1	AY577022.1	AF471067.1
4		*Contia tenuis*	AY577021.1	AY577030.1	AF471095.1
5		*Diadophis punctatus*	AF544765.1	AY577023.1	AF471094.1
6		*Erythrolamprus aesculapii*	AF158462.1	AF158531.1	—
7		*Farancia abacura*	AY577016.1	—	U69832.1
8		*Farancia erythrograma*	AY577017.1	—	—
9		*Heterodon nasicus*	AY577018.1	AY577027.1	—
10		*Heterodon platirhinos*	AY577019.1	AY577028.1	—
11		*Heterodon simus*	AY577020.1	AY577029.1	AF217840.1
12		*Hypsiglena slevini*	—	—	EF078499.1
13		*Hypsiglena torquata*	—	—	AF471038.1
14		*Liophis amarali*	GQ457807.1	GQ457747.1	—
15		*Liophis breviceps*	AF158464.1	AF158533.1	—
16		*Liophis elegantissimus*	GQ457808.1	GQ457748.1	—
17		*Liophis jaegeri*	GQ457809.1	GQ457749.1	—
18		*Liophis juliae*	AF158445.1	AF158514.1	—
19		*Liophis lineatus*	—	—	—
20		*Liophis meridionalis*	GQ457810.1	GQ457750.1	—
21		*Liophis miliaris*	AF158409.1	AF158480.1	—
22		*Liophis reginae*	AF158433.1	AF158501.1	—
23		*Liophis typhlus*	AF158410.1	AF158481.1	—
24		*Lystrophis dorbignyi*	GQ457812.1	GQ457752.1	—
25		*Lystrophis histricus*	GQ457813.1	GQ457753.1	—

Table 2.1. Continued.

	Family	Taxa	12S	16S	Cyt *b*
26		*Natrix maura*	AF402623.1	—	AY487690.1
27		*Natrix natrix*	AY122682.1	AF158530.1	AF471059.1
28		*Natrix tessellata*	—	—	AY487587.1
29		*Rhabdophis nuchalis*	AF402624.1	—	AF402907.1
30		*Rhabdophis subminiatus*	AF544776.1	—	—
31		*Rhabdophis tigrinus*	AF236679.2	—	AF471051.1
32		*Rhadinaea flavilata*	—	—	AF471078.1
33		*Rhadinaea fulvivittis*	—	—	EF078539.1
34		*Thamnophis butleri*	AF402640.1	—	AF402923.1
35		*Thamnophis couchii*	AF402653.1	—	
36		*Thamnophis cyrtopsis*	AF402641.1	—	AF402924.1
37		*Thamnophis elegans*	AF402642.1	—	AF305638.1
38		*Thamnophis ordinoides*	AF402644.1	—	AF402927.1
39		*Thamnophis proximus*	AF402645.1	—	AF402928.1
40		*Thamnophis radix*	AF402651.1	—	—
41		*Thamnophis sirtalis*	—	—	AF305681.1
42		*Waglerophis merremii*	GQ457840.1	—	—
43		*Xenochrophis flavipunctatum*	AF544780.1	AF544809.1	—
44		*Xenochrophis punculatus*	—	—	AF471079.1
45		*Xenochrophis vittatus*	EF395871.1	EF395846.1	—
46		*Xenodon neuwiedi*	GQ457841.1	GQ457779.1	AF236814.1
47		*Xenodon severus*	Z46449.1	Z46474.1	—
48		*Xenodon weneri*	AF158468.1	AF158538.1	—
49	Elapidae	*Hemachatus haemacatus*	U96797.1	—	AF217821.1
50		*Naja atra*	—	EF413645.2	EF206656.1
51		*Naja haje*	—	—	DQ897735.1
52		*Naja kaouthia*	EU624235.1	EF413641.2	AF217835.1
53		*Naja katiensis*	—	—	DQ897750.1
54		*Naja melanoleuca*	U96801.1	—	DQ897732.1
55		*Naja mossambica*	—	—	AF399747.1
56		*Naja naja*	Z46453.1	Z46482.1	—
57		*Naja nigricollis*	EU624237.1	EU624271.1	DQ897747.1
58		*Naja nivea*	EU624238.1	EU624272.1	—
59		*Naja nubiae*	—	—	AF399751.1
60		*Naja pallida*	—	—	AF399748.1
61		*Naja siamensis*	—	—	AF155214.1
62	Viperidae	*Causus defilippi*	AF057186.1	AF057233.1	AY223556.1
63		*Causus resimus*	AF544763.1	AF544790.1	AY223555.1
64		*Causus rhombeatus*	DQ305409.1	DQ305432.1	DQ305455.1

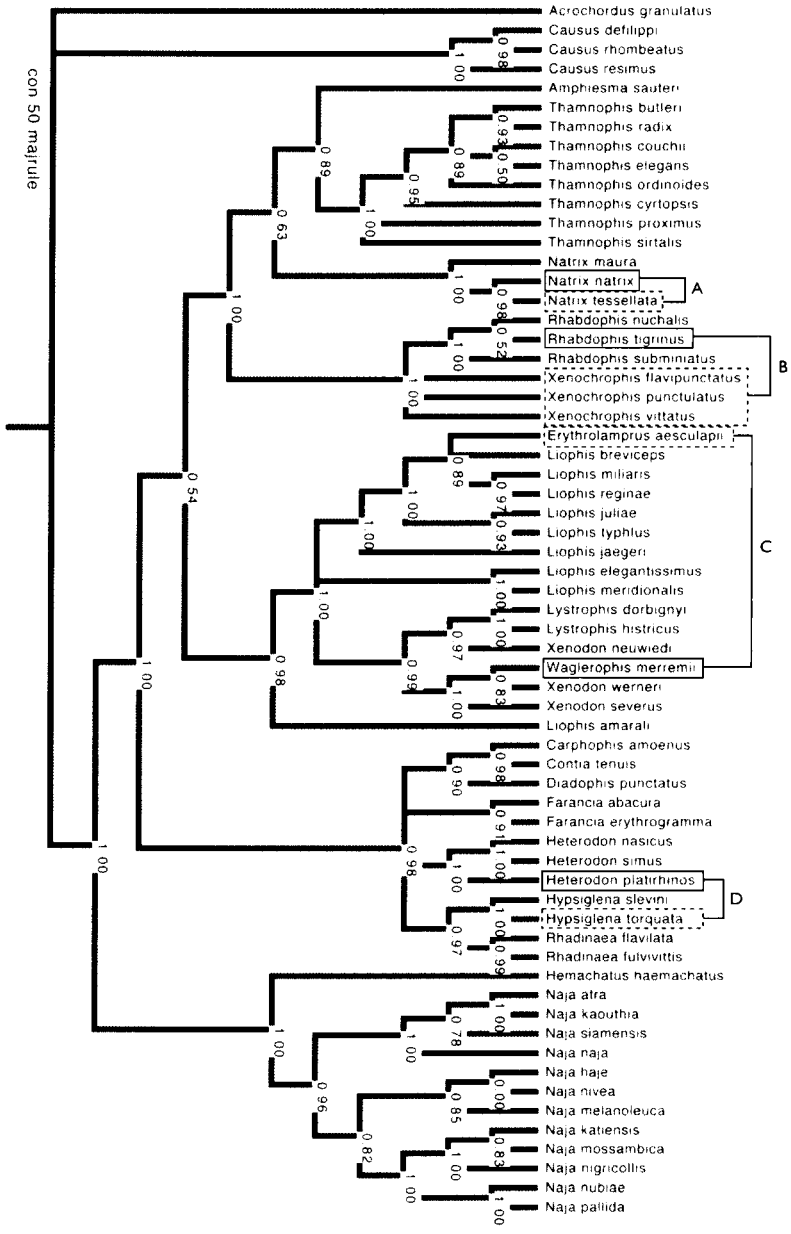

Figure 2.2. Phylogenetic tree from which taxon pairs were selected for comparison. Species pairs A-D represent taxa selected for the adrenal mass comparison. Solid boxes represent bufophagous species and dashed boxes represent nonbufophagous species. Gene sequences for *Xenochrophis piscator* were not available on GenBank so other members of its genus were used to gauge its position on the tree (B).

Sample Collection

Specimens were examined from the collections of the National Museum of Natural History (USNM), Field Museum of Natural History (FMNH), California Academy of Science (CAS), and American Museum of Natural History (AMNH); catalogue numbers are listed in Table 2.2. Presumably adult specimens were selected on the basis of body size and the condition of preservation. For each specimen, the snout-vent length (SVL) and total length were recorded. Next, the right and left adrenal glands were carefully removed, cleaned of membranes, blotted, and then weighed on an analytical balance (Denver Instrument model PI-225D, +/- 0.00001g). For each species, adrenal glands from 20 individuals (10 males and 10 females) were collected, with the exception of *Waglerophis merremii*, for which only 10 suitable individuals (5 males and 5 females) were available.

Table 2.2. List of specimens used in adrenal mass comparison with museum catalogue numbers and associated data.

	Catalogue #	Species	Sex	SVL (mm)	L. Adrenal Mass (g)	R. Adrenal Mass (g)	Total Adrenal Mass
1	**USNM 561161**	*Heterodon platirhinos*	male	557	0.0934	0.08389	0.17729
2	**USNM 516610**	*Heterodon platirhinos*	male	439	0.04086	0.04447	0.08533
3	**USNM 516608**	*Heterodon platirhinos*	male	453	0.05473	0.06601	0.12074
4	**USNM 516619**	*Heterodon platirhinos*	male	349	0.0067	0.01244	0.01914
5	**USNM 516604**	*Heterodon platirhinos*	male	504	0.1365	0.11394	0.25044
6	**AMNH 121559**	*Heterodon platirhinos*	male	520	0.10365	0.12205	0.2257
7	**AMNH 121568**	*Heterodon platirhinos*	male	563	0.08735	0.10313	0.19048
8	**AMNH 158669**	*Heterodon platirhinos*	male	400	0.10665	0.08902	0.19567

Table 2.2. Continued.

	Catalogue #	Species	Sex	SVL (mm)	L. Adrenal Mass (g)	R. Adrenal Mass (g)	Total Adrenal Mass
9	**AMNH 158675**	*Heterodon platirhinos*	male	415	0.05149	0.05474	0.10623
10	**AMNH 128616**	*Heterodon platirhinos*	male	524	0.06794	0.05231	0.12025
11	**USNM 516611**	*Heterodon platirhinos*	female	495	0.01129	0.01125	0.02254
12	**USNM 515103**	*Heterodon platirhinos*	female	651	0.0460	0.03466	0.08066
13	**USNM 497392**	*Heterodon platirhinos*	female	749	0.05612	0.07808	0.1342
14	**USNM 516621**	*Heterodon platirhinos*	female	659	0.05474	0.06147	0.11621
15	**USNM 516620**	*Heterodon platirhinos*	female	464	0.01584	0.01607	0.03191
16	**AMNH 121560**	*Heterodon platirhinos*	female	667	0.05872	0.12205	0.18077
17	**AMNH 121562**	*Heterodon platirhinos*	female	369	0.00809	0.01735	0.02544
18	**AMNH 107608**	*Heterodon platirhinos*	female	527	0.02102	0.01512	0.03614
19	**AMNH 65467**	*Heterodon platirhinos*	female	688	0.02430	0.02406	0.04836
20	**AMNH 128617**	*Heterodon platirhinos*	female	860	0.07502	0.10804	0.18306
21	**USNM 246464**	*Hypsiglena torquata*	male	324	0.00149	0.00148	0.00297
22	**USNM 246460**	*Hypsiglena torquata*	male	356	0.00202	0.00250	0.00452
23	**USNM 165361**	*Hypsiglena torquata*	male	245	0.00028	0.00038	0.00066
24	**USNM 246892**	*Hypsiglena torquata*	male	241	0.00032	0.00035	0.00067
25	**USNM 209475**	*Hypsiglena torquata*	male	301	0.00078	0.00085	0.00163
26	**USNM 132762**	*Hypsiglena torquata*	male	309	0.00161	0.00172	0.00333
27	**AMNH 158687**	*Hypsiglena torquata*	male	336	0.00136	0.00132	0.00268
28	**AMNH 158696**	*Hypsiglena torquata*	male	292	0.00083	0.00117	0.002
29	**AMNH 112219**	*Hypsiglena torquata*	male	268	0.00056	0.00016	0.00072
30	**AMNH 112221**	*Hypsiglena torquata*	male	239	0.00031	0.00035	0.00066
31	**AMNH 63732**	*Hypsiglena torquata*	male	305	0.00080	0.00075	0.00155
32	**USNM 244649**	*Hypsiglena torquata*	female	385	0.00275	0.00203	0.00478
33	**USNM 247755**	*Hypsiglena torquata*	female	335	0.00106	0.00150	0.00256
34	**USNM 222765**	*Hypsiglena torquata*	female	331	0.00090	0.00096	0.00186
35	**USNM 222768**	*Hypsiglena torquata*	female	289	0.00068	0.00072	0.0014
36	**USNM 240874**	*Hypsiglena torquata*	female	396	0.00333	0.00328	0.00661
37	**AMNH 158685**	*Hypsiglena torquata*	female	298	0.00088	0.00062	0.0015
38	**AMNH 158694**	*Hypsiglena torquata*	female	335	0.00141	0.00175	0.00316
39	**AMNH 158693**	*Hypsiglena torquata*	female	285	0.00156	0.00153	0.00309

Table 2.2. Continued.

	Catalogue #	Species	Sex	SVL (mm)	L. Adrenal Mass (g)	R. Adrenal Mass (g)	Total Adrenal Mass
40	**AMNH 158697**	*Hypsiglena torquata*	female	298	0.00112	0.00095	0.00207
41	**AMNH 158698**	*Hypsiglena torquata*	female	274	0.00056	0.00078	0.00134
42	**AMNH 158685**	*Hypsiglena torquata*	female	298	0.00088	0.00062	0.0015
43	**AMNH 158694**	*Hypsiglena torquata*	female	335	0.00141	0.00175	0.00316
44	**AMNH 158693**	*Hypsiglena torquata*	female	285	0.00156	0.00153	0.00309
45	**AMNH 158697**	*Hypsiglena torquata*	female	298	0.00112	0.00095	0.00207
46	**AMNH 158698**	*Hypsiglena torquata*	female	274	0.00056	0.00078	0.00134
47	**AM 2009-032**	*Rhabdophis trigrinus*	male	427	0.00700	0.00910	0.0161
48	**AM 2009-034**	*Rhabdophis trigrinus*	male	684	0.05740	0.06660	0.124
49	**AMNH 24588**	*Rhabdophis trigrinus*	male	503	0.02450	0.02784	0.05234
50	**AMNH 28324**	*Rhabdophis trigrinus*	male	583	0.04897	0.05005	0.09902
51	**AMNH 17436**	*Rhabdophis trigrinus*	male	471	0.03536	0.05095	0.08631
52	**2008-001**	*Rhabdophis trigrinus*	male	669	0.14458	0.13098	0.27556
53	**2008-011**	*Rhabdophis trigrinus*	male	704	0.08497	0.09035	0.17532
54	**2008-012**	*Rhabdophis trigrinus*	male	619	0.02967	0.03967	0.06934
55	**2008-019**	*Rhabdophis trigrinus*	male	585	0.07908	0.08413	0.16321
56	**2008-020**	*Rhabdophis trigrinus*	male	604	0.05039	0.04707	0.09746
57	**2008-002**	*Rhabdophis trigrinus*	female	825	0.06735	0.06476	0.13211
58	**2008-008**	*Rhabdophis trigrinus*	female	598	0.01225	0.01044	0.02269
59	**2008-013**	*Rhabdophis trigrinus*	female	776	0.02480	0.03005	0.05485
60	**2008-022**	*Rhabdophis trigrinus*	female	645	0.00979	0.01465	0.02444
61	**AM 2009-033**	*Rhabdophis trigrinus*	female	750	0.02070	0.02020	0.0409
62	**AM 2009-035**	*Rhabdophis trigrinus*	female	730	0.02260	0.02720	0.0498
63	**AMNH 23941**	*Rhabdophis trigrinus*	female	685	0.02874	0.04063	0.06937
64	**AMNH 29989**	*Rhabdophis trigrinus*	female	728	0.01483	0.02969	0.04452
65	**AMNH 28327**	*Rhabdophis trigrinus*	female	616	0.01358	0.02816	0.04174
66	**AMNH 28328**	*Rhabdophis trigrinus*	female	665	0.01823	0.01647	0.0347
67	**USNM 562770**	*Xenochrophis piscator*	male	375	0.00511	0.00391	0.00902
68	**USNM 562766**	*Xenochrophis piscator*	male	361	0.00486	0.00579	0.01065
69	**USNM 562764**	*Xenochrophis piscator*	male	355	0.00313	0.00338	0.00651
70	**USNM 562767**	*Xenochrophis piscator*	male	540	0.00929	0.01270	0.02199

Table 2.2. Continued.

	Catalogue #	Species	Sex	SVL (mm)	L. Adrenal Mass (g)	R. Adrenal Mass (g)	Total Adrenal Mass
71	**USNM 562763**	*Xenochrophis piscator*	male	372	0.00485	0.00534	0.01019
72	**AMNH 28244**	*Xenochrophis piscator*	male	457	0.01783	0.01664	0.03447
73	**AMNH 28240**	*Xenochrophis piscator*	male	439	0.01529	0.01389	0.02918
74	**AMNH 28155**	*Xenochrophis piscator*	male	465	0.00664	0.00821	0.01485
75	**AMNH 28177**	*Xenochrophis piscator*	male	350	0.00586	0.00653	0.01239
76	**AMNH 28154**	*Xenochrophis piscator*	male	425	0.01023	0.01074	0.02097
77	**USNM 562771**	*Xenochrophis piscator*	female	520	0.00760	0.00720	0.0148
78	**USNM 562769**	*Xenochrophis piscator*	female	451	0.00466	0.00470	0.00936
79	**USNM 562765**	*Xenochrophis piscator*	female	554	0.00566	0.00811	0.01377
80	**USNM 562768**	*Xenochrophis piscator*	female	645	0.01040	0.01395	0.02435
81	**USNM 562760**	*Xenochrophis piscator*	female	540	0.00928	0.00975	0.01903
82	**AMNH 28247**	*Xenochrophis piscator*	female	524	0.00980	0.01544	0.02524
83	**AMNH 28241**	*Xenochrophis piscator*	female	500	0.00624	0.01325	0.01949
84	**AMNH 34043**	*Xenochrophis piscator*	female	575	0.01790	0.02484	0.04274
85	**AMNH 28249**	*Xenochrophis piscator*	female	594	0.00705	0.01514	0.02219
86	**AMNH 28254**	*Xenochrophis piscator*	female	519	0.00683	0.00430	0.01113
87	**FMNH 195869**	*Waglerophis merremii*	male	525	0.04280	0.04180	0.0846
88	**FMNH 195890**	*Waglerophis merremii*	male	522	0.02980	0.02550	0.0553
89	**FMNH 42200**	*Waglerophis merremii*	male	560	0.03110	0.03790	0.069
90	**CAS 49348**	*Waglerophis merremii*	male	493	0.02947	0.03720	0.06667
91	**CAS 49327**	*Waglerophis merremii*	male	514	0.04212	0.04689	0.08901
92	**FMNH 195868**	*Waglerophis merremii*	female	798	0.03090	0.03260	0.0635
93	**FMNH 195867**	*Waglerophis merremii*	female	671	0.03250	0.04590	0.0784
94	**USNM 342417**	*Waglerophis merremii*	female	913	0.07180	0.07135	0.14315
95	**USNM 165590**	*Waglerophis merremii*	female	484	0.00836	0.01273	0.02109
96	**USNM 253588**	*Waglerophis merremii*	female	789	0.04071	0.04335	0.08406
97	**USNM 165557**	*Erythrolamprus aesculapii*	male	568	0.00377	0.00364	0.00741
98	**USNM 210989**	*Erythrolamprus aesculapii*	male	531	0.00262	0.00242	0.00504
99	**USNM 219070**	*Erythrolamprus aesculapii*	male	569	0.00437	0.00507	0.00944
100	**USNM 158097**	*Erythrolamprus aesculapii*	male	436	0.00215	0.00257	0.00472
101	**USNM 158096**	*Erythrolamprus aesculapii*	male	456	0.00186	0.00224	0.0041

Table 2.2. Continued.

	Catalogue #	Species	Sex	SVL (mm)	L. Adrenal Mass (g)	R. Adrenal Mass (g)	Total Adrenal Mass
102	**AMNH 55618**	*Erythrolamprus aesculapii*	male	554	0.00525	0.00137	0.00662
103	**AMNH 52291**	*Erythrolamprus aesculapii*	male	756	0.00295	0.00423	0.00718
104	**AMNH 53367**	*Erythrolamprus aesculapii*	male	767	0.00686	0.00750	0.01436
105	**AMNH 53826**	*Erythrolamprus aesculapii*	male	664	0.00400	0.00954	0.01354
106	**AMNH 55164**	*Erythrolamprus aesculapii*	male	635	0.00455	0.00540	0.00995
107	**USNM 222354**	*Erythrolamprus aesculapii*	female	724	0.00822	0.00951	0.01773
108	**USNM 321113**	*Erythrolamprus aesculapii*	female	871	0.01060	0.01311	0.02371
109	**USNM 165560**	*Erythrolamprus aesculapii*	female	537	0.00246	0.00369	0.00615
110	**USNM 165559**	*Erythrolamprus aesculapii*	female	630	0.00531	0.00531	0.01062
111	**USNM 165558**	*Erythrolamprus aesculapii*	female	464	0.00190	0.00100	0.0029
112	**AMNH 52197**	*Erythrolamprus aesculapii*	female	618	0.00724	0.00894	0.01618
113	**AMNH 53470**	*Erythrolamprus aesculapii*	female	837	0.01285	0.02300	0.03585
114	**AMNH 55287**	*Erythrolamprus aesculapii*	female	659	0.00759	0.00797	0.01556
115	**AMNH 62222**	*Erythrolamprus aesculapii*	female	680	0.00848	0.01333	0.02181
116	**AMNH 62220**	*Erythrolamprus aesculapii*	female	635	0.00314	0.00378	0.00692
117	**FMNH 26814**	*Natrix natrix*	male	555	0.008	0.0065	0.0145
118	**FMNH 30518**	*Natrix natrix*	male	557	0.00580	0.00770	0.0135
119	**FMNH 62452**	*Natrix natrix*	male	592	0.01090	0.01980	0.0307
120	**FMNH 217649**	*Natrix natrix*	male	481	0.00750	0.00340	0.0109
121	**FMNH 234288**	*Natrix natrix*	male	563	0.00950	0.01100	0.0205
122	**CAS 182663**	*Natrix natrix*	male	546	0.00754	0.00979	0.01733
123	**CAS 182664**	*Natrix natrix*	male	524	0.00521	0.00986	0.01507
124	**CAS 182926**	*Natrix natrix*	male	531	0.00548	0.00422	0.0097
125	**CAS 185291**	*Natrix natrix*	male	637	0.01213	0.01507	0.0272
126	**CAS 182694**	*Natrix natrix*	male	509	0.00988	0.00513	0.01501
127	**CAS 182662**	*Natrix natrix*	female	674	0.01610	0.00984	0.02594
128	**CAS 182666**	*Natrix natrix*	female	499	0.0083	0.00509	0.01339
129	**CAS 185286**	*Natrix natrix*	female	802	0.00548	0.00422	0.0097
130	**CAS 182691**	*Natrix natrix*	female	410	0.00538	0.00254	0.00792
131	**CAS 183055**	*Natrix natrix*	female	824	0.03133	0.03261	0.06394
132	**USNM 524232**	*Natrix natrix*	female	609	0.01021	0.01349	0.0237

Table 2.2. Continued.

	Catalogue #	Species	Sex	SVL (mm)	L. Adrenal Mass (g)	R. Adrenal Mass (g)	Total Adrenal Mass
133	**USNM 196480**	*Natrix natrix*	female	910	0.06642	0.06655	0.13297
134	**USNM 154495**	*Natrix natrix*	female	630	0.00604	0.01705	0.02309
135	**USNM 56016**	*Natrix natrix*	female	653	0.01156	0.01762	0.02918
136	**FNMH 62454**	*Natrix natrix*	female	803	0.04260	0.06230	0.1049
137	**CAS 183103**	*Natrix tessellata*	male	542	0.00720	0.00846	0.01566
138	**CAS 183111**	*Natrix tessellata*	male	586	0.01070	0.00455	0.01525
139	**CAS 183104**	*Natrix tessellata*	male	476	0.00679	0.00811	0.0149
140	**CAS 185300**	*Natrix tessellata*	male	614	0.01750	0.01420	0.0317
141	**CAS 185306**	*Natrix tessellata*	male	622	0.01013	0.00805	0.01818
142	**USNM 124723**	*Natrix tessellata*	male	529	0.01423	0.01818	0.03241
143	**FMNH 19622**	*Natrix tessellata*	male	482	0.01250	0.00620	0.0187
144	**FMNH 19594**	*Natrix tessellata*	male	630	0.01430	0.01720	0.0315
145	**FMNH 19558**	*Natrix tessellata*	male	419	0.00280	0.00580	0.0086
146	**FMNH 19559**	*Natrix tessellata*	male	416	0.00460	0.00250	0.0071
147	**USNM 124722**	*Natrix tessellata*	female	551	0.01155	0.01504	0.02659
148	**FMNH 171241**	*Natrix tessellata*	female	865	0.03690	0.03280	0.0697
149	**FMNH 171223**	*Natrix tessellata*	female	830	0.03740	0.04570	0.0831
150	**FMNH 19607**	*Natrix tessellata*	female	710	0.02960	0.02750	0.0571
151	**FMNH 171245**	*Natrix tessellata*	female	765	0.02890	0.01490	0.0438
152	**CAS 183107**	*Natrix tessellata*	female	750	0.02958	0.0339	0.06348
153	**CAS 183110**	*Natrix tessellata*	female	798	0.02762	0.01552	0.04314
154	**CAS 185303**	*Natrix tessellata*	female	786	0.03930	0.03428	0.07358
155	**CAS 185301**	*Natrix tessellata*	female	710	0.01280	0.02650	0.0393
156	**CAS 217587**	*Natrix tessellata*	female	578	0.01504	0.00791	0.02295

Data Analysis

To analyze the relationship between adrenal mass, diet, and sex, I performed analyses of covariance (ANCOVA) using snout-vent length as the covariate. This allowed me to account for variability in adrenal mass related to differences in body size.

Skewness in the data was normalized using $\log_e$ transformation. Two outlier individuals, USNM 516619 and AMNH 2009-032, were removed from the data set under the subsequent suspicion that they were juveniles.

Statistical analyses were conducted using PASW Statistics for Mac, ver. 17.0.2. Graphs were generated using the same program.

Results

In an initial ANCOVA I compared the adrenal masses of bufophagous and nonbufophagous snakes as groups, with males and females pooled (Figure 2.3). Despite the taxonomic and sexual heterogeneity of the samples, the bufophagous snakes generally cluster on the basis of higher adrenal mass, and the interaction of adrenal mass and diet is highly significant ($F = 15.476$, $p < 0.001$, $df = 1$). However, there also is a significant interaction with SVL ($F = 7.577$, $p < 0.001$, $df = 1$), indicating that adrenal mass scales differently with SVL in bufophagous snakes and nonbufophagous snakes. Therefore, adrenal glands cannot be compared simply as a proportion of body mass across species. Adrenal mass increases with SVL at a slower rate in bufophagous snakes than in nonbufophagous species, and the lower slope and higher intercept for the bufophagous species indicates that this is due to relatively larger glands among smaller individuals as compared to the nonbufophagous group. Figure 2.3 reveals a cluster of bufophagous individuals with adrenal masses that overlap those of nonbufophagous species. Examination of the data by species (Figure 2.4) reveals that all of these individuals are *Natrix natrix*. Furthermore, the slope of the line for that species does not resemble that of

other bufophagous species, indicating that adrenal glands of this species do not follow the same pattern as those of the other three bufophagous species examined.

One caveat in the comparison of adrenal masses should be noted: the lack of small bufophagous snakes in the data set. However, there are no bufophagous species known which possess adult SVLs similar to that of *Hypsiglena torquata*, the smallest nonbufophagous snake included in this study.

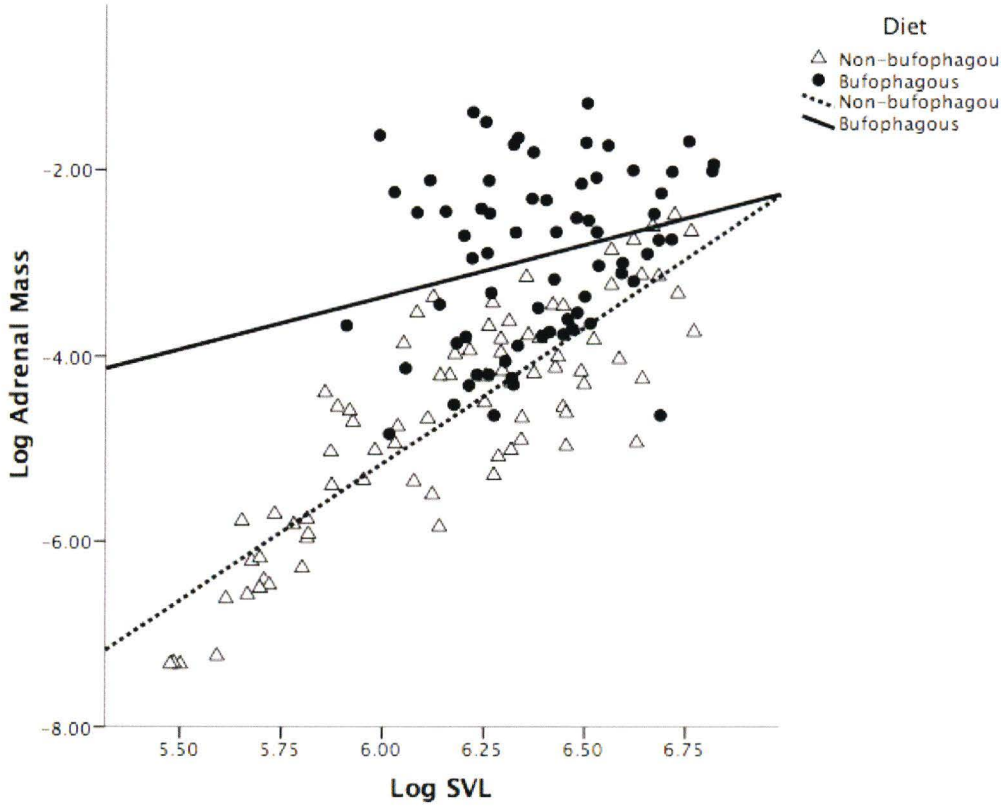

Figure 2.3. ANCOVA graph showing significant differences in log adrenal mass vs. log SVL between bufophagous and nonbufophagous snakes.

Because sexual dimorphism in adrenal mass was previously identified in *Heterodon platirhinos* (Spaur and Smith, 1971), I investigated whether other bufophagous species also display this pattern. However, the more general question of whether sexual dimorphism in adrenal gland size occurs in most species of snakes, independent of diet, has not been addressed previously. Therefore, I first compared male and female nonbufophagous snakes (Figure 2.5). The results of the ANCOVA indicate that there is no significant interaction between adrenal mass and sex ($F = 1.908$, $p = 0.172$, $df = 1$) in nonbufophagous species. I then compared male and female bufophagous snakes (Figure 2.6) and, although greater separation of points is detectable visually -- with males generally exhibiting higher adrenal mass -- the results were not significant ($F = 1.908$, $p = 0.172$, $df = 1$). This is most likely attributed to a cluster of male *Natrix natrix* whose values overlap with those of the females of all four species. I decided to test this idea by removing *N. natrix* from the bufophagous species dataset, and as expected the ANCOVA results revealed sexual dimorphism (Figure 2.7; $F = 4.471$, $p = 0.040$, $df = 1$). Combined with the previous analysis, we see that *N. natrix* diverges from the pattern seen in the other three bufophagous species.

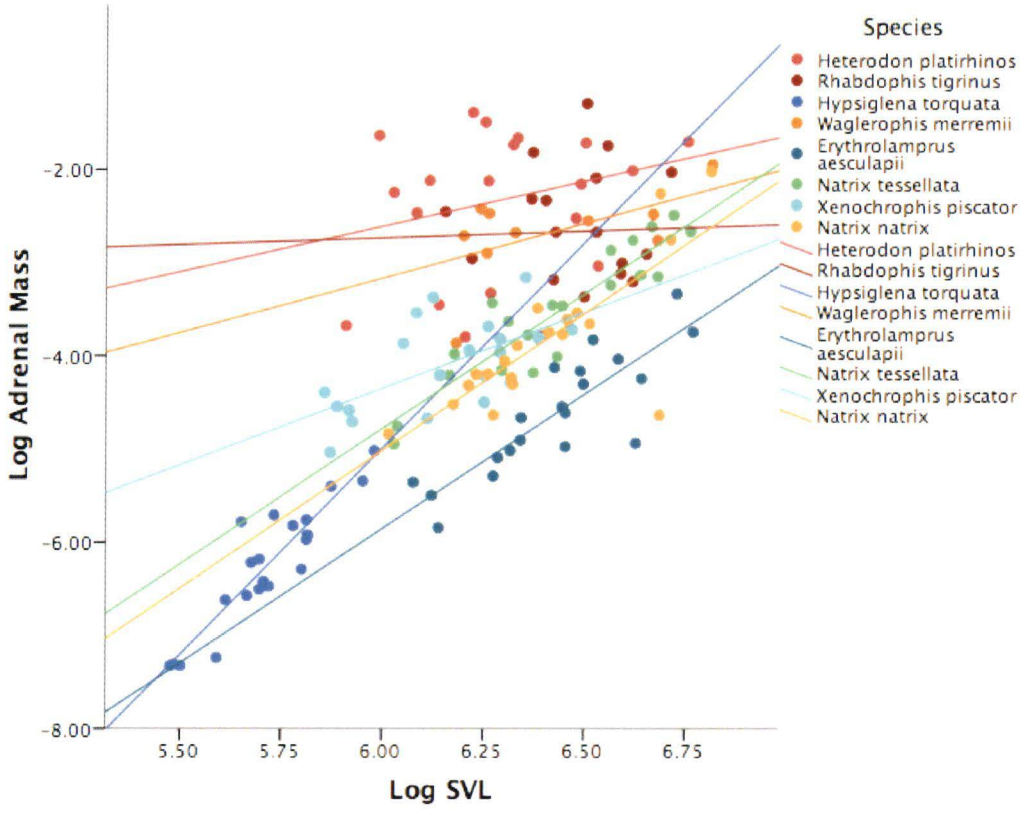

Figure 2.4. ANCOVA showing a comparison of log adrenal mass vs. log SVL between all species (bufophagous and nonbufophagous).

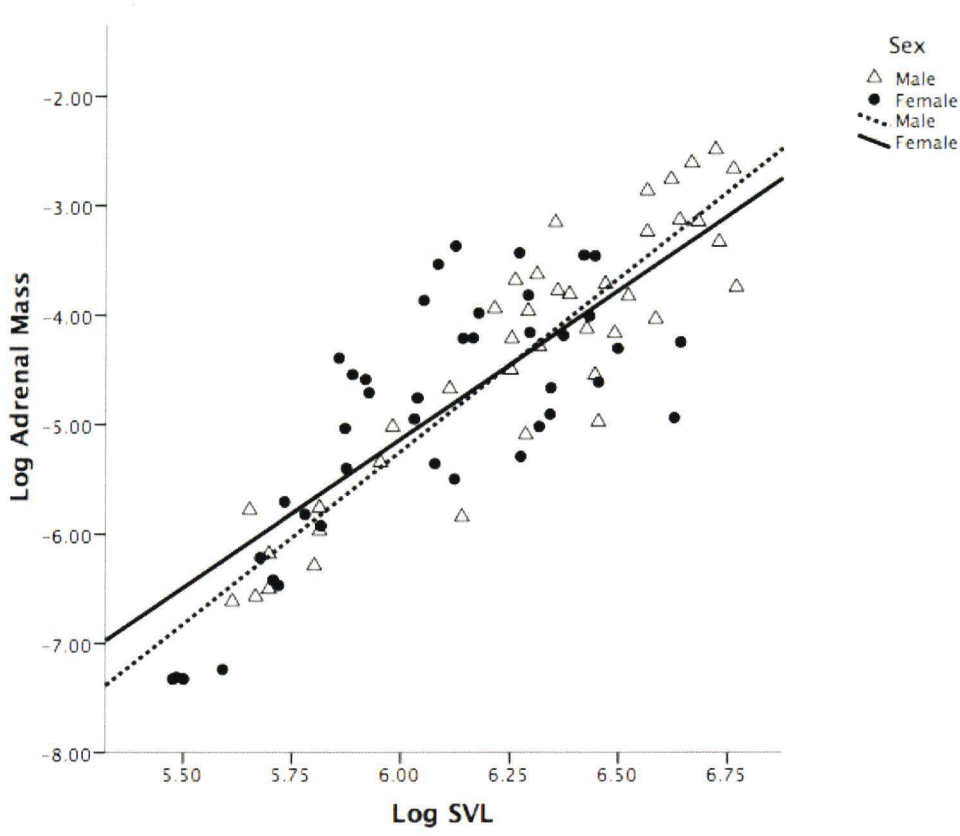

Figure 2.5. ANCOVA showing comparison of male and female adrenal mass for all nonbufophagous species pooled. The two groups completely overlap, showing no sexual dimorphism in adrenal mass.

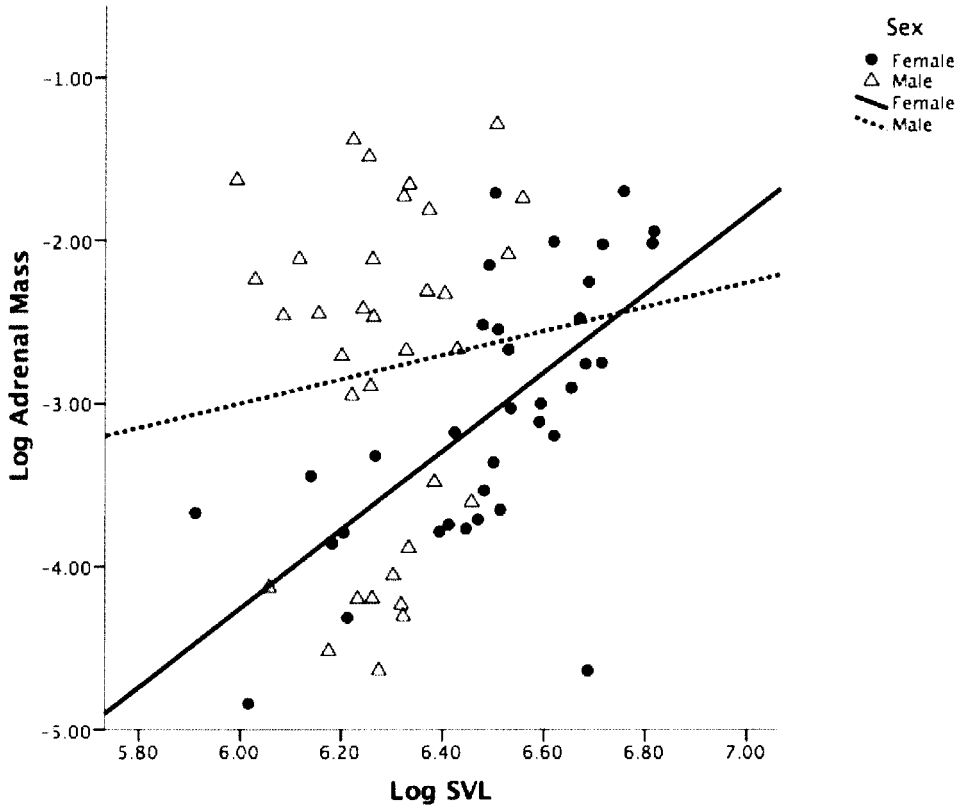

Figure 2.6. ANCOVA showing comparison of male and female adrenal mass in all bufophagous species pooled. The result of the ANCOVA is not significant. The cluster of male data points overlapping with females consists of specimens of *Natrix natrix*.

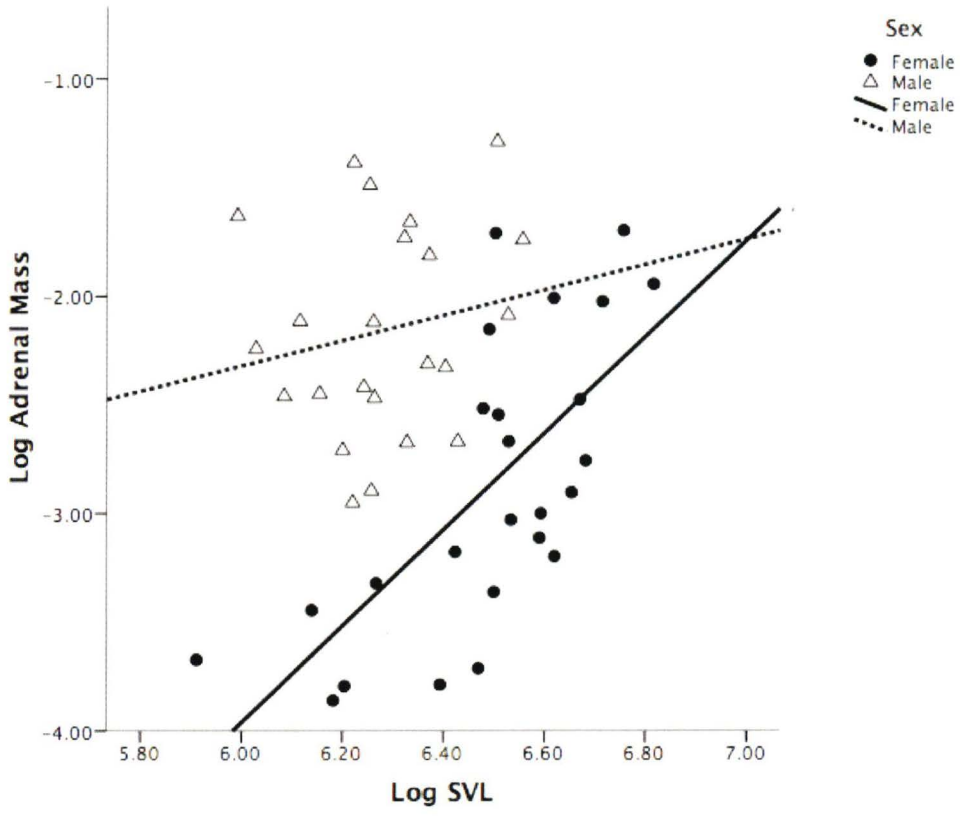

Figure 2.7. ANCOVA showing comparison of male and female adrenal mass in bufophagous species without *Natrix natrix*. The result of the ANCOVA is significant.

Discussion

The results of this study support the hypothesis that bufophagy is associated with adrenal enlargement, although it also raises new questions regarding this phenomenon. My data reveal that adrenal enlargement has evolved repeatedly in bufophagous snakes belonging to three phylogenetically independent lineages. Curiously, the data also show that *Natrix natrix* differs from the other three bufophagous species in having adrenal glands that are similar in size, relative to SVL, to those of the nonbufophagous snakes.

My data also demonstrate, for the first time, that adrenal mass is not sexually dimorphic in nonbufophagous snakes, and so the adrenal enlargement previously reported for *Heterodon platirhinos* (Spaur and Smith, 1971) does not simply represent a proportional amplification of a more widespread sexual size dimorphism, resulting in proportionately larger glands in males. Indeed, the same sexual dimorphism reported for *H platirhinos* also occurs in both *Waglerophis merremii* and *Rhabdophis tigrinus*. Curiously, however, the same pattern does not occur in *Natrix natrix*, a strongly bufophagous species that does not differ in this respect from its sister taxon *N. tessellata* (i.e., neither species exhibits sexual dimorphism in adrenal size). The lack of significant sexual dimorphism when all bufophagous species were pooled was a reflection of the impact of *N. natrix* on the analysis, as revealed when the ANCOVA was run without including that species.

Although there is no direct experimental evidence that adrenal glands function in bufophagous snakes' resistance to the toxic effects of bufadienolides, I have established that there is a strong, if not universal, pattern of adrenal enlargement in at least three bufophagous species belonging to independent lineages of colubroid snakes. There are two possible explanations for these findings. The first is that the enlarged adrenal glands are a functional requirement for bufadienolide resistance, perhaps producing an increased supply of hormones that play pivotal roles in maintaining NKA, and thereby counteracting the adverse effects of bufonid toxins. The second explanation is that adrenal enlargement occurs as a secondary consequence of bufophagy, either as a response to stress invoked by bufadienolide metabolism (see Chapter 1) or perhaps as a developmental response to the presence of the steroidal toxins of toads. Although the

finding of Spaur and Smith (1971) that naïve (unfed) hatchling Heterodon platirhinos exhibit both adrenal hypertrophy and sexual dimorphism of the glands would initially suggest that those conditions could not result from individual exposure to the toxins, the finding of Hutchinson, et al. (2007, 2008) that embryonic Rhabdophis can be provisioned with bufadienolides prior to oviposition indicates that embryonic exposure to the toxins must also be considered. In any event, further work is required to distinguish which, if either, of these alternative hypotheses is correct.

Additional uncertainty surrounds the uniqueness of *Natrix natrix* in comparison to the other bufophagous species, in regard to both adrenal mass and sexual dimorphism of the glands. Inasmuch as both *N. natrix* and *Rhabdophis tigrinus* are members of the colubroid family Natricidae, phylogenetic constraint is unlikely to explain this difference. Furthermore, several comprehensive ecological studies (Gregory and Isaac, 2004; Luiselli and Capula, 1997) leave little doubt that these snakes consume toads as a major portion of their diet. There is also no reason to believe that the species of toad that *N. natrix* consumes, *Bufo bufo*, contains lower unusually low concentrations of bufadienolides. At this time I can offer no explanation for the lack of adrenal modification in *N. natrix*.

NKA isoforms may also play a role in bufadienolide resistance because some isoforms have been shown to have lower affinity for cardiotonic steroids (see Chapter 1). This idea is reinforced by More et al. (2009), who examined the evolution of bufadienolide resistance in toads by sequencing the NKA gene of several amphibian species. They found distinct mutations in toads, suggesting that the cardiotonic steroid

binding sites have been altered. A similar study by Feldman et al. (2009) on tetrodotoxin resistant garter snakes (*Thamnophis*) found mutations in the genes coding for sodium channels ($NA_v1.4$), which are the target ligand of TTX.

CHAPTER III

A COMPARISON OF TISSUE DISTRIBUTION IN THE ADRENAL GLANDS OF BUFOPHAGOUS AND NONBUFOPHAGOUS SNAKES

Introduction

In snakes, as in other amniotes, the adrenal glands are composed of catecholamine-producing chromaffin cells and corticosteroid-producing interrenal cells (Hebard and Charipper, 1955; Norris, 1997). These classes of hormones play important roles in the up-regulation of sodium-potassium ATPase (NKA; see Chapter I) in other vertebrates, and presumably also in snakes.

Aldosterone and corticosterone are corticosteroid hormones produced by the interrenal tissue. Aldosterone controls sodium and potassium levels by binding to a specific mineralocorticoid site on the α-subunit of NKA, the same binding site that has an affinity for cardiotonic steroids (Ikeda et al., 1991). Aldosterone also influences gene expression of NKA (Therien and Blostein, 2000). The effects of corticosterone are similar to those of aldosterone, having direct impacts on the activity levels of NKA (Ikeda et al., 1991). Adrenergic agents produced in the chromaffin tissue of the adrenal glands have also been found to stimulate activity of the sodium-potassium pump through various pathways. They can signal the synthesis of corticosteroids through stimulation of ACTH (Axelrod and Resine, 1984), a pathway that exerts an indirect effect on NKA, whereas other pathways have been shown to stimulate NKA directly (Therien and Blostein, 2000).

Because I have shown that gross adrenal enlargement occurs in three phylogenetically independent species of bufophagous snakes, I hypothesize that the adrenal glands are in some way related to these snakes' ability to resist bufadienolide toxins. If this resistance is mediated by a particular adrenal hormone, then it can reasonably be expected that the hormone is produced in relatively higher quantities in bufophagous snakes. Moreover, it is likely that the tissue producing that hormone exists at a higher proportion in the adrenal glands of those snakes and contributes differentially to adrenal enlargement. To test this, I quantified the proportions of chromaffin and interrenal tissues and blood vessels in two bufophagous and two non-bufophagous species, controlling as much as possible for phylogeny. I present evidence here that, unexpectedly, these two groups of species do not differ in the relative proportions of these tissues.

Methods

Sample Collection

Twelve individuals from four of the species used for comparison of adrenal mass (see Chapter 2) were selected for this analysis (Table 3.1). The left and right adrenal glands were removed from each preserved individual and stored in 70% ethanol.

Histology

For larger snakes, the ends of the glands were cut off and the middle was processed for routine paraffin embedding (Presnell and Schreibman, 1997), embedded in Paraplast Plus, serially sectioned using a Leitz 1512 rotary microtome, and mounted on

subbed slides (Presnell and Schreibman, 1997). For those snakes in which the adrenal glands were very short, the entire gland was embedded.

The sections were differentially stained for chromaffin tissue using the ferric ferricyanide method (Lillie, 1965). This method stains chromaffin cells dark green while leaving interrenal cells light green. Blood cells also stained dark green but were distinguishable by their level of color saturation and shape. The sections were observed under a compound microscope to assess the staining quality. Many of the sections did not stain well, presumably because all of the specimens were acquired from museum collections and thus represented a wide range of preservation histories and tissue conditions. For this reason the ultimate sample size for this analysis was much smaller than anticipated. In fact, the original sample included a third pair of species, *Waglerophis merremii* and *Erythrolamprus aesculapii*. However, I was not able to obtain any adequately stained sections from individuals of these snakes. Nonetheless, I was able to obtain an adequate number of well-stained sections from the four species described here to make a general comparison between the two diet groups.

Image Analysis

Sections were photographed using a Nikon Coolpix 990 attached to a Nikon Optiphot compound microscope. For each slide, every tenth section was photographed until 20 photographs were recorded per individual, 10 of the right adrenal gland and 10 of the left. However, the right adrenal sections of one individual *Hypsiglena torquata*, USNM 244649, were too degraded for analysis, seemingly due to the histology process, for analysis, so only ten photographs of the left adrenal sections were collected. In total,

240 photographs were available for analysis. The photographs were analyzed in ImageJ ver. 1.41o (Rasband, 2008). Tissues were manually outlined in ImageJ, and the cross-sectional areas of chromaffin tissue, interrenal tissue, blood vessels and total adrenal gland section were calculated for each photo. The mean of the combined left and right adrenal measurements for a given tissue type was calculated, resulting in three final measurements for each individual. These final values were used for the statistical analysis (Table 3.1).

Table 3.1. List of specimens used in tissue proportion comparison with museum catalogue numbers and associated data.

	Catalogue #	Species	Sex	Avg. Section Area (mm^2)	Avg. Blood Vessel Area (mm^2)	Avg. Chromaffin Area (mm^2)	Avg. Interrenal Area (mm^2)
1	**USNM 561161**	*Heterodon platirhinos*	male	1.728	0.2200383	0.0490370	1.4593135
2	**USNM 516610**	*Heterodon platirhinos*	male	1.226	0.0750621	0.0339749	1.1173091
3	**USNM 516620**	*Heterodon platirhinos*	female	0.237	0.0461758	0.0133885	0.1772856
4	**USNM 132762**	*Hypsiglena torquata*	male	0.123	0.0129534	0.0257538	0.0841002
5	**USNM 244649**	*Hypsiglena torquata*	female	0.1624	0.0387955	0.0179629	0.1058249
6	**USNM 240874**	*Hypsiglena torquata*	female	0.148	0.0255843	0.0178017	0.104364
7	**FMNH 26814**	*Natrix natrix*	male	0.526	0.1842148	0.1100945	0.2318907
8	**FMNH 234288**	*Natrix natrix*	male	0.192	0.0384248	0.0200260	0.1330992
9	**USNM 196480**	*Natrix natrix*	female	0.977	0.1728856	0.0942427	0.7103217
10	**FMNH 19594**	*Natrix tessellata*	male	0.395	0.0495558	0.0365267	0.3092675
11	**USNM 124723**	*Natrix tessellata*	male	0.298	0.1132697	0.0393746	0.1458057
12	**FMNH 19607**	*Natrix tessellata*	female	0.303	0.0718933	0.0154360	0.215776

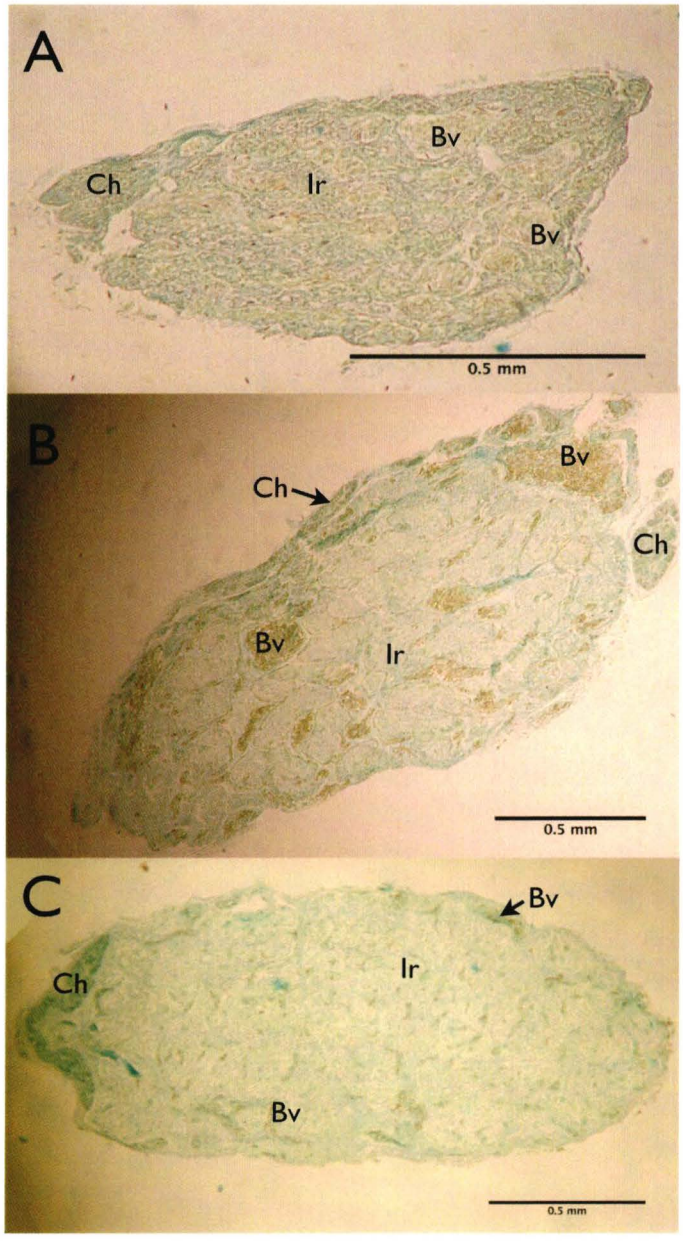

Figure 3.1. Adrenal sections stained with ferric ferryicyanide. (A); *Natrix tessellata* (USNM 124723); (B) *Natrix natrix* (USNM 196480); (C) *Heterodon platirhinos* (USNM 561161) The dark green tissue with a slightly blue tint is chromaffin (Ch), the light green tissue is interrenal (Ir), and the dark green areas with a slightly yellow tint are erythrocytes within blood vessels (Bv).

Data Analysis

To compare the proportions of the two fundamental adrenal tissues between diets and sexes, I conducted model I, two-factor analyses of variance (ANOVAs) for chromaffin tissue, interrenal tissue, and blood vessels. Due to the reduced sample size, and the fact that differences attributable to snake species were not of primary interest, species was treated as a random effect (block). The assumptions for normality were checked using the Shapiro-Wilk test and those for homogeneity of variance were checked using the Levene test. To meet these assumptions, the data were arcsine transformed. Statistical analyses were conducted using PASW Statistics for Mac, ver. 17.0.2. Graphs were generated using the same program.

Results

The mean proportions of the two adrenal tissues and blood vessels are shown in Fig. 3.2. All three ANOVAs yielded non-significant results. The first ANOVA compared the proportions of chromaffin tissue between bufophagous and non-bufophagous snakes (Fig. 3.2; Table 3.2; $F = 1.163$, $P = 0.312$, $df = 1$) and between males and females ($F = 0.644$, $P = 0.445$, $df = 1$), as revealed by their cross-sectional areas in sections. The second ANOVA compared the proportions of interrenal tissue between the same groups (Fig. 3.2; Table 3.2; $F = 0.347$, $P = 0.572$, $df = 1$; $F = 0.318$, $P = 0.250$ $df = 1$), whereas the third compared the proportions of blood vessels (Fig. 3.2; Table 3.2 ; $F < 0.001$, $P = 0.984$, $df = 1$; $F = 1.535$, $P = 0.250$, $df = 1$).

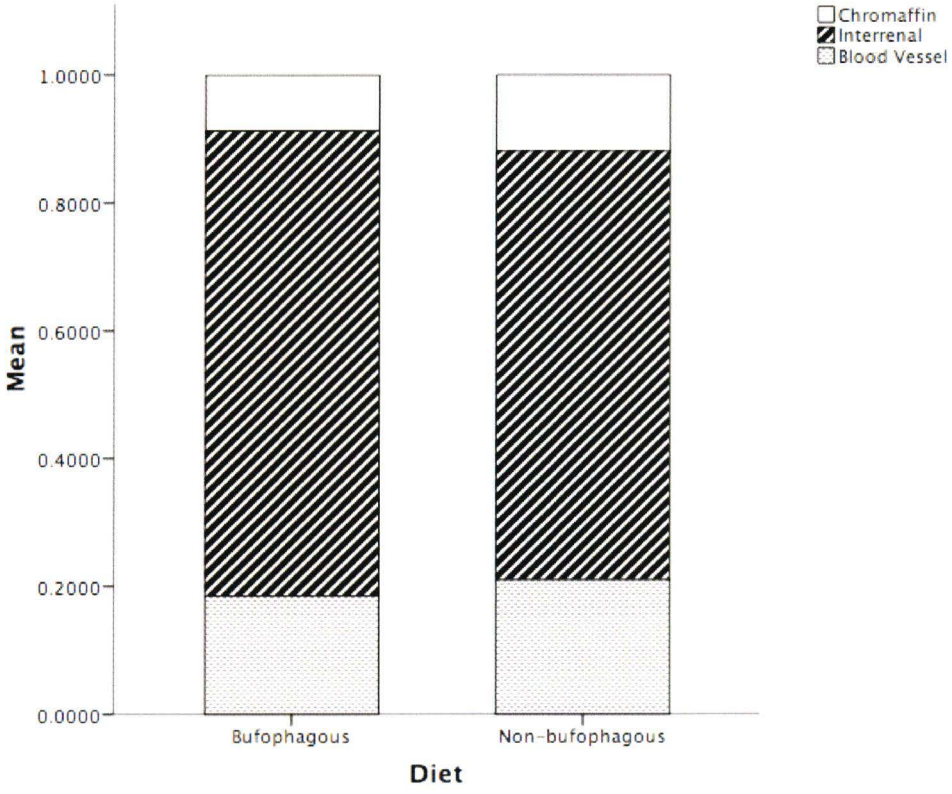

Figure 3.2. Mean chromaffin, interrenal and blood vessel proportions in bufophagous and non-bufophagous snakes. The results of the ANOVA were not significant in chromaffin, interrenal and blood vessel proportion comparisons ($F = 1.163$, $P = 0.312$, $df = 1$; $F = 0.347$, $P = 0.572$, $df = 1$; $F < 0.001$, $P = 0.984$, $df = 1$).

Table 3.2. ANOVA tables for tissue proportion comparisons between bufophagous and nonbufophagous snakes.

Chromaffin	df	MS	F	P
Diet	1	0.005	1.163	0.312
Sex	1	0.003	0.664	0.445
Diet * Sex	1	0.001	0.15	0.708
Error	8	0.004		
Total	11			

Interrenal	df	MS	F	P
Diet	1	0.015	0.347	0.572
Sex	1	0.013	0.318	0.588
Diet * Sex	1	0.009	0.203	0.664
Error	8	0.042		
Total	11			

Blood Vessels	df	MS	F	P
Diet	1	3.86E-06	< 0.001	0.984
Sex	1	0.014	1.535	0.25
Diet * Sex	1	0.014	1.524	0.252
Error	8	0.009		
Total	11			

Additionally, I compared the data between male and female snakes of the two dietary groups (Figs. 3.3 & 3.4; Tables 3.3 & 3.4). However, no significant differences were observed in chromaffin tissue, interrenal tissue, or blood vessel proportions in bufophagous snakes, perhaps due to small sample sizes (Fig. 3.3; Table 3.3; $F = 0.063$, $P = 0.814$, $df = 1$; $F = 0.004$, $P = 0.953$, $df = 1$; $F < 0.001$, $P = 0.998$, $df = 1$) or non-

bufophagous snakes (Fig. 3.4; Table 3.4; F = 1.105, P = 0.353, df = 1; F = 1.399, P = 0.302, df = 1; F = 4.492, P = 0.101, df = 1).

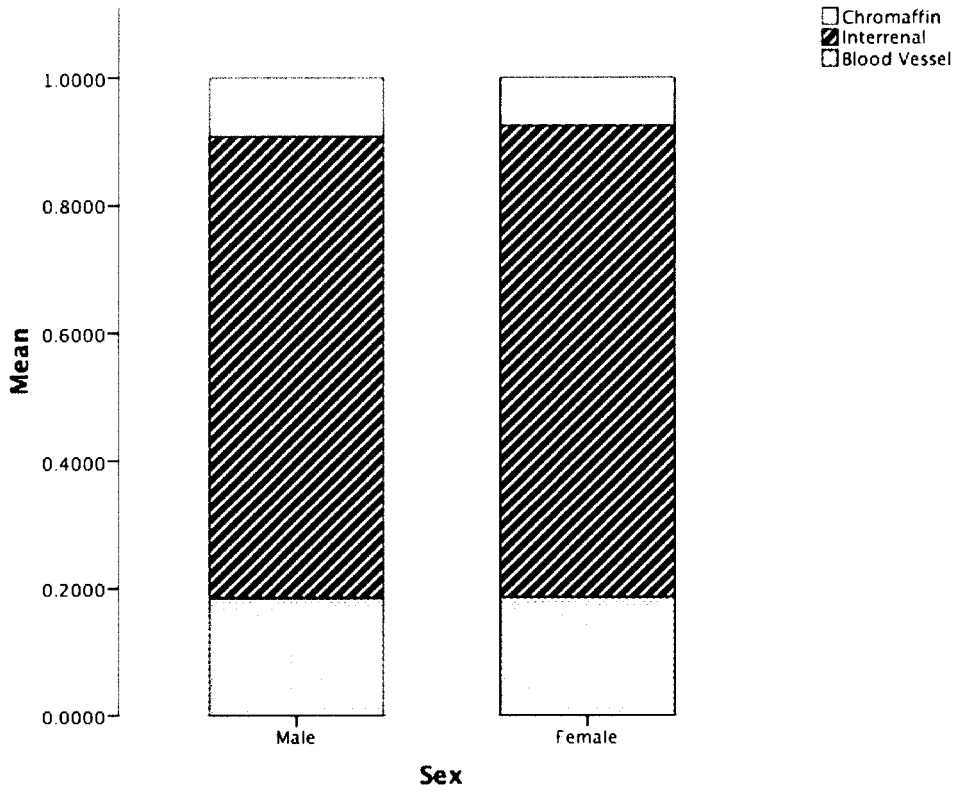

Figure 3.3. Mean chromaffin, interrenal and blood vessel proportions between bufophagous male and female snakes. The results of the ANOVA were not significant in chromaffin, interrenal and blood vessel proportion comparisons (F = 0.063, P = 0.814, df = 1; F = 0.004, P = 0.953, df = 1; F < 0.001, P = 0.998, df = 1).

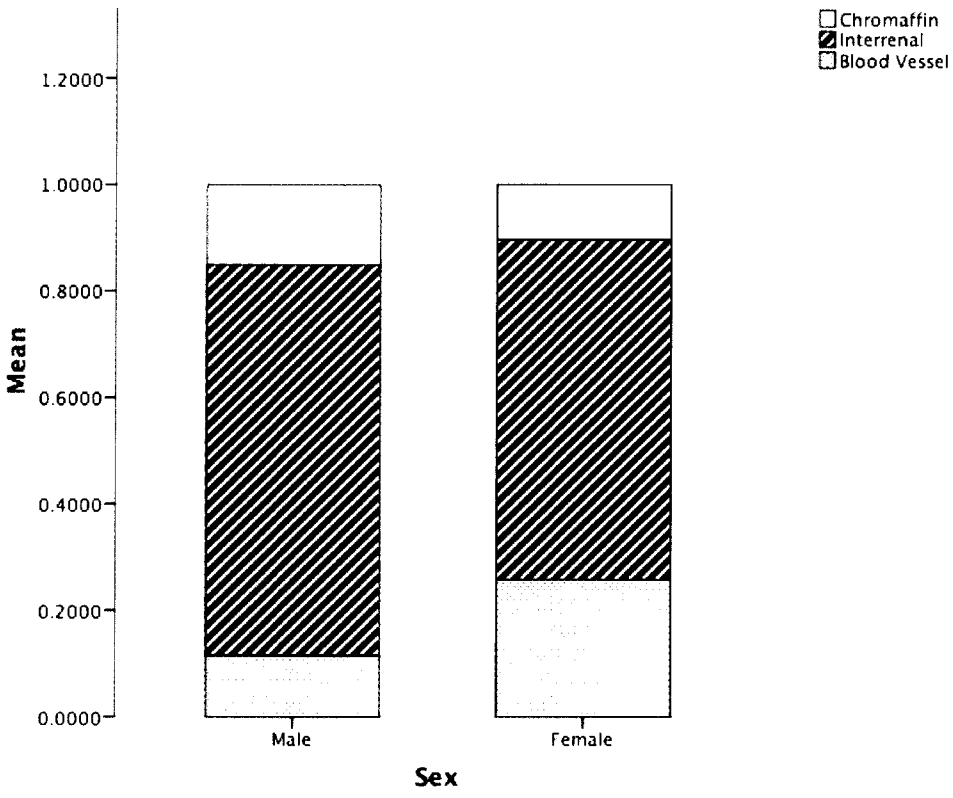

Figure 3.4. Mean chromaffin, interrenal and blood vessel proportions in non-bufophagous male and female snakes. The results of the ANOVA were not significant in chromaffin, interrenal and blood vessel proportion comparisons ($F = 1.105$, $P = 0.353$, $df = 1$; $F = 1.399$, $P = 0.302$, $df = 1$; $F = 4.492$, $P = 0.101$, $df = 1$).

Table 3.3. ANOVA tables for tissue proportion comparisons between male and female bufophagous snakes.

Chromaffin	df	MS	F	P
Sex	1	< 0.001	0.063	0.814
Error	4	0.006		
Total	5			

Interrenal	df	MS	F	P
Sex	1	< 0.001	0.004	0.953
Error	4	0.069		
Total	5			

Blood Vessels	df	MS	F	P
Sex	1	9.70E-08	< 0.001	0.998
Error	4	0.012		
Total	5			

Table 3.4. ANOVA tables for tissue proportion comparisons between male and female non-bufophagous snakes.

Chromaffin	df	MS	F	P
Sex	1	0.003	1.105	0.353
Error	4	0.003		
Total	5			

Interrenal	df	MS	F	P
Sex	1	0.022	1.399	0.302
Error	4	0.015		
Total	5			

Blood Vessels	df	MS	F	P
Sex	1	0.028	4.492	0.101
Error	4	0.006		
Total	5			

I compiled an additional graph to examine the distribution of tissues among species (Fig. 3.5). This comparison revealed a disproportionately large quantity of interrenal tissue in *Heterodon platirhinos* and a relatively large vascular cross-section in both species of *Natrix*. The constraints imposed by low sample sizes prevented me from comparing these groups statistically, but these observations may be relevant and should be pursued further.

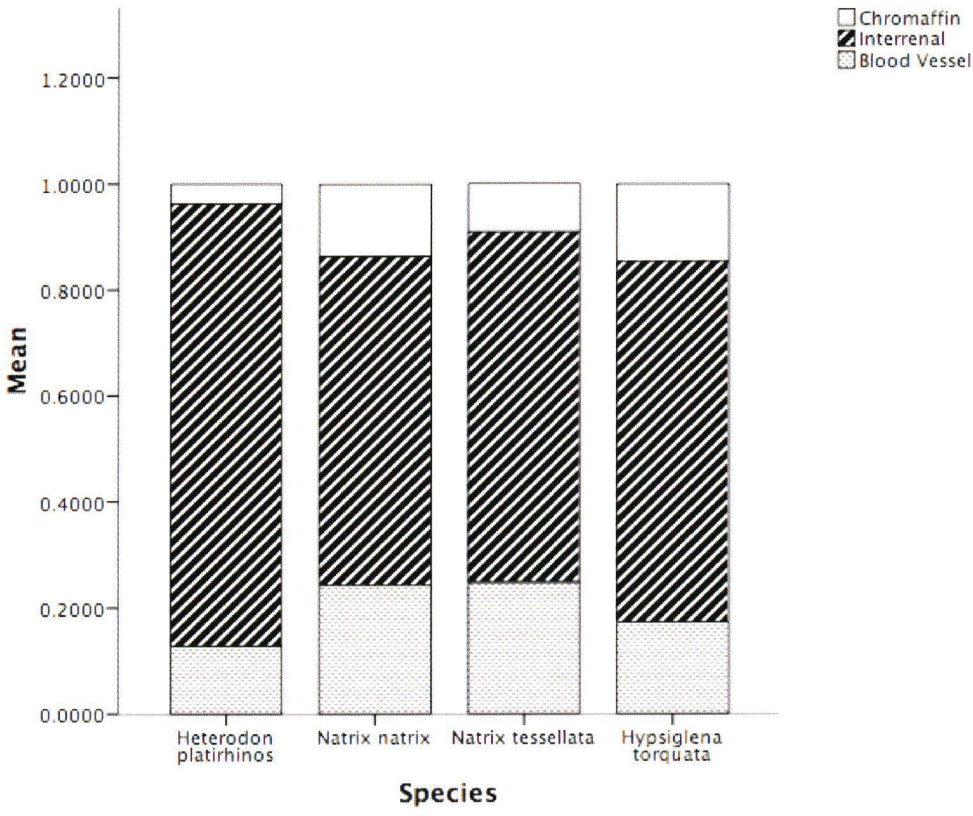

Figure 3.5. Mean chromaffin, interrenal and blood vessel proportions between species.

An important observation concerning the gross anatomy of the adrenal gland in *Hypsiglena torquata* was made during the course of this study. Adrenal glands harvested from all specimens of *H. torquata* for the comparison of adrenal mass (all from the USNM) exhibited an unusual mass of tissue associated with the gland (Fig. 3.6). These unusual adrenal masses were found in both sexes and in both left and right adrenal glands of individuals. The specimens came from various populations in Mexico and the United States, so the feature does not appear to represent a localized phenomenon. I left these

adrenal glands intact throughout the preparation of histological sections, in an attempt to identify the unusual tissue, as well as the analysis of tissue proportions. However, although the ferric ferricyanide stain revealed a reasonable amount of detail for these cells (Fig. 3.7), I was unable to identify their histological nature. Each cell in these masses measured approximately 0.18 μm^2 in area and 19 μm in diameter, with the nucleus measuring approximately 8 μm in diameter. The cells were very densely packed, and the tissue was not well vascularized.

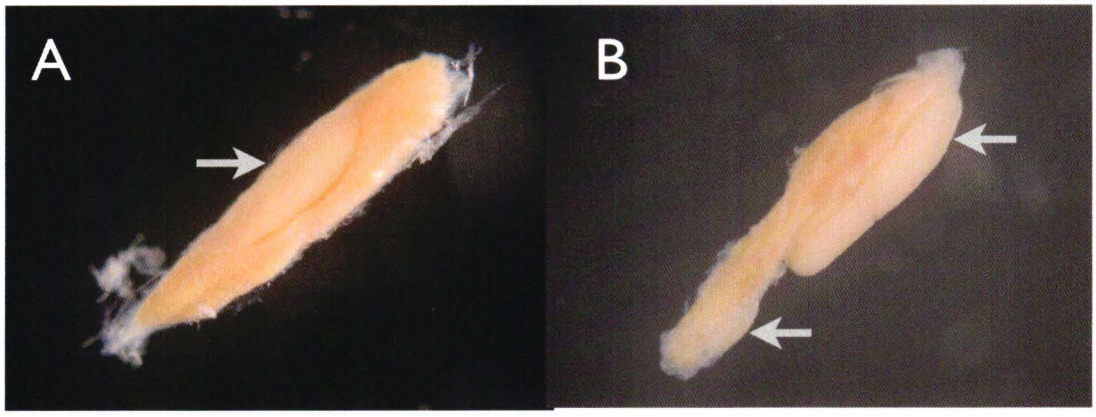

Figure 3.6. Images of whole adrenal glands removed from *Hypsiglena torquata* museum specimens showing unidentified tissue masses (arrows) on the adrenal glands. In some specimens (A) these masses appear to be closely integrated with the adrenal glands. In other specimens (B) they are not as closely associated. A: USNM 247755, B: USNM 240874.

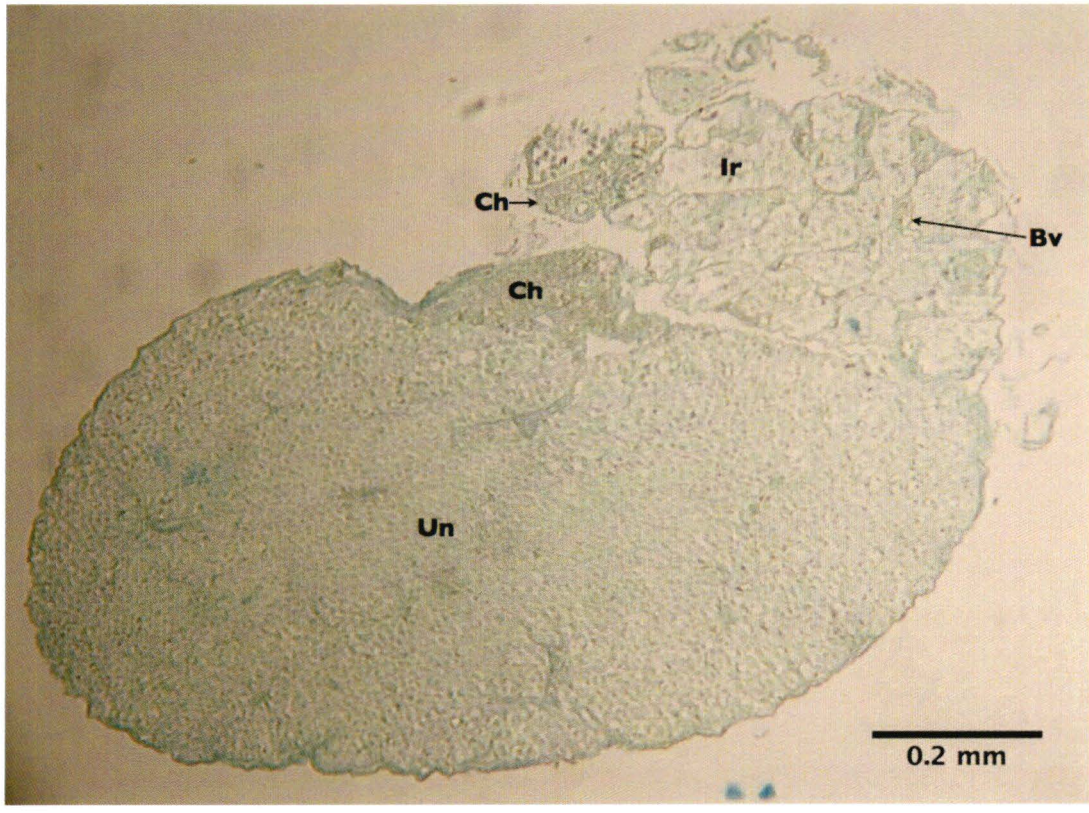

Figure 3.7. Adrenal section of *Hypsiglena torquata* (USNM 240874) showing the unidentified tissue cells (Un) along with the adrenal gland chromaffin tissue (Ch), interrenal tissue (Ir) and blood vessels (Bv).

Discussion

I have demonstrated that adrenal mass scales differently with body size in bufophagous snakes compared with non-bufophagous species (Chapter 2), with the former having larger glands. The results of this analysis, however, indicate that, within the limitations of my samples sizes, the differences in adrenal size cannot be explained by the differential enlargement of any one adrenal tissue. However, given the distinction in relative adrenal mass between *Natrix natrix* and other bufophagous species (Chapter 2)

and the observed differences in tissue proportions between this species and *Heterodon platirhinos* (Fig. 3.5), it would be helpful to extend these analyses to larger sample sizes and more taxa to obtain a more accurate measure of tissue proportions between the two dietary groups. I therefore consider the results of this study to be preliminary.

Although the sample sizes for this analysis are small, visual inspection of the data (Figs. 3.3 & 3.4) do not reveal any obvious differences related to diet or sex. It is possible that all adrenal tissues function in the resistance of bufadienolides, resulting in isometric scaling of those components. Alternatively, it may be that the adrenal hormones do not play a major role in bufadienolide resistance, in which case adrenal enlargement may simply be a response to stresses induced by ingestion of bufadienolides (see discussion in Chapter 2).

The tissue masses associated with the adrenal glands of *Hypsiglena torquata* (Figs. 3.6 & 3.7) remain unidentified. Because the adrenal glands in snakes are closely associated with the gonads and the masses superficially resembled ovaries, the initial assumption was that they were gonadal tissue. However, that interpretation was abandoned when the masses were found in both sexes. Likewise, the possibility that they might be neoplasms was also eliminated because the nuclei of the cells are not irregularly shaped and it would be unlikely that neoplasms would occur in so many individuals from different populations.

Finally, it was noted that the adrenal glands in both species of *Natrix* are highly vascularized compared to the other species examined (though they do not differ statistically, perhaps due to small sample sizes). If this proves to be a valid phylogenetic

character, it may offer an alternative mechanism for the resistance of *Natrix natrix* to bufadienolide toxins, perhaps underlying the difference between the latter species and other bufophagous species in regard to adrenal scaling (Chapter 2). Increased vasculature presumably would serve to transport adrenal hormones more quickly, if such secretions do in fact play a role in bufadienolide resistance.

CHAPTER IV

CONCLUSIONS

The preceding two chapters provide support for the hypothesis that the adrenal glands of bufophagous snakes are in some way related to the ability of such snakes to resist bufadienolide toxins. For the first time it has been established that adrenal enlargement occurs in several phylogenetically independent lineages of bufophagous species. Unexpectedly, however, the bufophagous species *Natrix natrix* does not exhibit the adrenal enlargement observed in other bufophagous snakes, despite the species ability to resist bufadienolides, suggesting that *Natrix natrix* employs a different mechanism to resist bufadienolides than do other bufophagous species. Further analysis revealed that the adrenal glands in bufophagous species with adrenal enlargement also exhibit sexual dimorphism in adrenal mass, with males possessing significantly larger glands. This trait, however, also does not occur in *N. natrix*.

Preliminary investigation of the histology of the adrenal glands did not demonstrate that the adrenal enlargement observed in bufophagous snakes reflects differential hypetrophy or hyperplasia of either interrenal or chromaffin tissue. Although not statistically significant, perhaps owing to small sample sizes, histological comparisons among species suggest that *Heterodon platirhinos* exhibits an unusually large proportion of interrenal tissue whereas *Natrix natrix and N. tesselata* exhibit extensively vascularized adrenal glands. The latter observation may be related to the lack of adrenal enlargement in *N. natrix*.

This study constitutes only an initial investigation into the evolution of toxin resistance in bufophagous snakes, and the results have generated more questions than answers. Several avenues of future investigation should be pursued. First, the analysis should be expanded to include more of the known species of bufophagous snakes and related non-bufophagous sister taxa. It would be interesting to see whether the relatively small but highly vascularized adrenal glands of *Natrix natrix* occur in any other bufophagous species. The analysis of adrenal tissue proportions would benefit not only from much larger sample sizes, but also from the use of fresh material (for more definitive histological differentiation of tissue types) and from the inclusion of additional taxa. Furthermore, immunohistochemical techniques could be employed to improve different staining, and controlled feeding experiments could be used to investigate whether changes in the gross morphology and/or histology of the adrenal glands occur as a result of consuming bufadienolide toxins.

It also would be interesting to compare the protein sequences for sodium-potassium ATPase (NKA) in bufophagous and non-bufophagous snakes, to test the hypothesis that bufophagous snakes have evolved isoforms of NKA with different affinities for bufadienolides. Feldman et al. (2009) compared gene sequences that code for sodium channel proteins in garter snakes with differing resistance to tetrodotoxin and found that several different mutations evolved independently among several resistant populations. Similarly, Moore et al. (2009) compared NKA sequences among anurans and determined that mutations in NKA associated with bufadienolide resistance are present among lineages ancestral to, and within, the Bufonidae. Conversely, however,

preliminary data from *Rhabdophis tigrinus* (Alexei Bagrov and Olga Federova, pers. comm.) indicate that cardiac muscle of unfed snakes is, in fact, more sensitive to bufadienolides than that of a non-bufophagous species, suggesting that such initial sensitivity may lead to the upregulation of more highly resistant isoforms of NKA. These are only a few examples of the different approaches that could be considered for future research. Hopefully such studies would help to answer the question of how toad-eating snakes resist bufadienolide toxicity.

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Publications

Manuscripts In Preparation

McDiarmid, R. W., J. A. Campbell, and **S. Mohammadi**. Snake species of the world volumes II and III. MS being edited and expanded.

Hill, J. G., D. S. McLeod, K. Miller Hesed, **S. Mohammadi**, T. Artchawakom. Herpetofaunal Diversity at Sakaerat Environmental Research Station, Northeast Thailand: Revisiting a Historically Important Site. Final MS being reviewed by co-authors.

Hill, J. G., T. Tamashiro, D. Bagsby, **S. Mohammadi**, Y. Amano, D. Karns, K. Thirakupt, H. K. Voris. Ecology and Thermal Biology of the Big Eyed Pit Viper (*Cryptelytrops macrops*): a tropical arboreal pitviper, in Northeast Thailand. MS being reviewed by co-authors.

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