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Molecular and Physiological Mechanisms of Toxin Resistance in Toad-Eating Snakes

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MOLECULAR AND PHYSIOLOGICAL MECHANISMS OF TOXIN RESISTANCE IN TOAD-EATING SNAKES

by

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ABSTRACT

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by

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Many plants and animals are defended by toxic compounds, and circumvention of those defenses often has involved the evolution of elaborate mechanisms for tolerance or resistance of the toxins. Toads synthesize potent cardiotonic steroids known as bufadienolides (BDs) from cholesterol and store those toxins in high concentrations in their cutaneous glands. Those toxins protect toads from the majority of predators, including most snakes that readily consume other species of frogs. BDs exert their effect by inhibiting ion transport by the Na+/K+-ATPase (NKA). This ubiquitous transmembrane enzyme consists of a catalytic alpha-subunit, which carries out the enzyme's functions, and a glycoprotein beta-subunit, which provides structural stability. Inhibition of the NKA causes highly elevated intracellular Ca^{2+} levels and results in often lethal increased cardiac contraction strength. Molecular resistance to bufadienolides in snakes is conferred by mutations in the alpha-subunit of the Na^{+}/K^{+}-ATPase. I have found that these mutations are more prevalent in snakes than previously suggested, and that many genetically resistant species do not feed on toads. This suggests that possession

of the mutations alone does not carry substantial negative consequences, and that feeding on toads may have been an ancestral habit in some groups of snakes. I have further found evidence of tissue-specific variation in resistance to bufadienolides, and gene expression investigations revealed that the bufadienolide resistance-conferring mutations are not expressed equally among different organs. Variation in resistance among different tissues indicates that possession of the mutations does not protect all cells equally. Finally, by testing the physiological responses of resistant snakes to exposure to cardiotonic steroid, I have found that feeding on toads incurs negative consequences and that toad-specialized resistant snakes respond differently from nontoad-specialized resistant snakes. The presence of physiological consequences of toxin exposure may explain why feeding on toads has been lost in some lineages of snakes that retain resistance-conferring mutations. In summary, these findings indicate that genetic resistance of the Na+/K+-ATPase is necessary in order for snakes to survive acute toxicity of bufadienolides, but it is not sufficient to explain fully the physiological mechanisms involved in dealing with chronic exposure to the toxins.

(186 pages)

PUBLIC ABSTRACT

Molecular and Physiological Mechanisms of Toxin Resistance in Toad-Eating Snakes

Shabnam Mohammadi

Many plants and animals are defended by toxic compounds, and the evasion of those defenses by predators often has involved the evolution of elaborate mechanisms for resistance to those toxins. Toads are chemically defended by cardiotonic steroids known as bufadienolides, which exert their lethal effects by binding to, and inhibiting, Na^+/K^+-ATPases on cell membranes. Select lineages of snakes from several geographic regions have evolved resistance to bufadienolides through mutations in the gene encoding their Na^+/K^+-ATPases and are able to feed on toads without suffering the normally lethal effects. I have found that mutations for resistance of Na^+/K^+-ATPases are more prevalent in snakes than previously suggested, and that many genetically resistant species do not feed on toads. This suggests that possession of the mutations alone does not carry substantial negative consequences, and a diet of toads may have been an ancestral trait in some groups of snakes. I have further found that certain organs of resistant snakes are better protected from the toxins than others. Variation in resistance among different organs indicates that the physiology involved in tolerance of the toxins is complex and that possession of resistance mutations does not protect all cells equally. Further investigations revealed that the mutations are not expressed equally among different organs. Finally, by testing the physiological responses of resistant snakes to cardiotonic

steroid exposure, I have found that the exposure incurs negative physiological consequences and that the physiological responses differ between resistant snakes that are toad-specialized and those that are not. The presence of physiological consequences of toxin exposure may explain why feeding on toads has been lost in some lineages of snakes that retain resistance-conferring mutations. In summary, these findings indicate that genetic resistance of the Na+/K+-ATPase is necessary in order for snakes to survive acute toxicity of bufadienolides, but it is not sufficient to explain fully the physiological mechanisms involved in dealing with chronic exposure to the toxins.

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CHAPTER I

INTRODUCTION

Cardiotonic Steroids and their Modes of Action

Many plants and animals are defended by toxic compounds, and circumvention of those defenses often has involved the evolution of elaborate mechanisms for tolerance or resistance of the toxins (Geffeney et al. 2002; Feldman et al. 2009; Agrawal et al. 2012; Ujvari et al. 2015; Mohammadi et al. 2016a). Toads synthesize potent cardiotonic steroids known as bufadienolides (BDs) from cholesterol and store those toxins in high concentrations in their cutaneous glands (Krenn and Kopp 1998). Those toxins protect toads from the majority of predators, including most snakes that readily consume other species of frogs (Phillips and Shine 2004; Phillips and Shine 2006; Mohammadi et al. 2016b). Nonetheless, toad-eating (bufophagy) has evolved in at least 17 genera of snakes (Mohammadi et al. 2016a).

Bufadienolides are one of two classes of cardiotonic steroids, cardenolides being the other (Krenn and Kopp 1998; Dvela et al. 2007). All cardiotonic steroids share the general structure of a steroidal body with a lactone ring on carbon 17 (Dvela et al. 2007). These compounds exert their effect by inhibiting ion transport by the Na+/K+-ATPase (NKA; Schoner 2002). This ubiquitous transmembrane enzyme consists of a catalytic alpha-subunit, which carries out the enzyme's functions, and a glycoprotein beta-subunit, which provides structural stability (Köksoy 2002; Aperia 2007). Inhibition of the NKA causes highly elevated intracellular Ca^{2+} levels and results in often-lethal increased strenfth of cardiac contraction (Barry and Bridges 1993; Schoner 2002). Molecular

resistance to BDs in snakes is conferred by mutations in the alpha-subunit of the NKA (encoded by the gene *ATP1a3*; Ujvari et al. 2015; Mohammadi et al. 2016a). These mutations significantly lower the toxins' affinity to the enzyme (Ujvari et al. 2015). Such a system of chemical defense and resistance between predator and prey exemplify the co-evolutionary arms races that may drive genetic and phenotypic changes in the participating organisms. The ability to feed on toxic prey is an important innovation in snakes. However, our knowledge of the mechanisms underlying this innovation remains superficial.

Molecular Resistance to Bufadienolides in Snakes

The molecular mechanisms of resistance to BDs in bufophagous snakes remained unknown until recently published work revealed amino acid substitutions in the BD binding pocket of one of three NKA gene paralogs (*ATP1a3*; Ujvari et al. 2015; Ujvari et al. 2016; Mohammadi et al. 2016a; the other two paralogs remain uninvestigated). Site-directed mutagenesis has revealed that these mutations confer significantly increased resistance to BDs at the cellular level (Ujvari et al. 2015), and dose-response experiments have revealed a high level of resistance to acute exposure at the whole-body level in snakes that possess the mutations (Mohammadi et al. 2016b). Evidence from parallel systems of toxin adaptation suggests that the presence of amino acid substitutions in the NKA is not the only adaptation that confers resistance to steroidal toxins. Cardenolide-resistant insects, for example, use a variety of mechanisms to deal with the toxins, in addition to target-site insensitivity of the NKA (Scudder and Meredith 1982; Despres 2007; Petschenka et al. 2013; Bramer et al. 2015). To understand the origin and retention

of a trait, it is essential to determine the physiological mechanisms associated with possession of the trait. Therefore, it is necessary to combine genetic screens with physiological and functional assays.

Physiological Mechanisms of Bufadienolide Resistance in Snakes

Adrenal hormones are likely candidates for promoting a resistant snake's assimilation and digestion of BDs. Previous work has revealed a strong association between adrenal gland enlargement and bufophagy in snakes (Mohammadi et al. 2013). These endocrine glands produce the steroidal hormones aldosterone and corticosterone (Norris 2007). Aldosterone is a mineralocorticoid hormone known to signal increased expression of NKA, especially in the heart (Oguchi et al. 1993; Ikeda et al. 1991; Weber 2001; Slayer et al. 2013). This suggests a possible compensatory role in counteracting the NKA-blocking effects of ingested cardiotonic steroids. It is therefore possible that the adrenal enlargement previously observed in bufophagous snakes is due in part to an increased number of aldosterone-producing cells. Corticosterone is a glucocorticoid hormone that is often used as a measure of physiological stress (Rich and Romero 2005; French et al. 2007; Hews and Baniki 2013). Previous studies have shown that an organism under sustained physiological stress will respond with enlargement of the adrenal glands (Fickess 1963; Ulrich-Lai et al. 2006). It is possible that physiological stress is a consequence of chronic exposure to bufadienolides through a diet rich in toads. Therefore the adrenal enlargement observed in bufophagous snakes might be due in part to stress resulting from consumption of the toxins. It is also possible that a combination of increased corticosterone and aldosterone contributes to that enlargement.

Physiological Consequences of Bufophagy in Snakes

Beyond the scope of the endocrine system, there are strong effects of cardiotonic steroids on cardiac contractility (Barry and Bridges 1993; Prassas and Diamandis 2008). These effects have been studied extensively in mammalian systems, as these steroids have a long history of use in the treatment of congestive heart failure (Barry and Bridges 1993; Ruch et al. 2003; Nesher et al. 2007; Schoner and Scheiner-Bobis 2007; Prassas and Diamandis 2008; Wansapura et al. 2016). The increased intracellular Ca^{2+} caused by inhibition of the NKA in myocardial cells results in an overload of Ca^{2+} in the sarcoplasmic reticulum (Ruch et al. 2003). To compensate for this overload the sarcoplasmic reticulum delivers Ca^{2+} at higher concentrations and faster rates to muscle fibers, thereby causing stronger and faster cardiac contractions (Barry and Bridges 1993). One previous study investigated the effects of crude toad secretions on cardiac contractility in both resistant and non-resistant snakes, and found that the compounds generally caused tachycardia (Licht and Low 1968). However, other studies have shown that individual compounds can have differing effects on cardiac contractility (reviewed by Dvela et al. 2007).

Dissertation Overview

The chapters that follow constitute an investigation of the molecular and physiological mechanisms of bufadienolide resistance in snakes. Chapter II provides evidence that molecular resistance to bufadienolides is widespread in snakes and not tightly coupled with bufophagy. Chapter III shows that tissue-level resistance to bufadienolides is not uniform among different organs in the body of resistant snakes.

Furthermore, Chapter IV shows that gene expression levels of *ATP1a3* are also not equal among different organs. Finally, Chapters V and VI provide evidence for the presence of physiological consequences of bufophagy.

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CHAPTER 2

TOXIN-RESISTANT ISOFORMS OF NA^+/K^+-ATPASE IN SNAKES DO NOT CLOSELY TRACK DIETARY SPECIALIZATION ON TOADS

Toads are chemically defended by bufadienolides, a class of cardiotonic steroids that exert toxic effects by binding to and disabling the Na^+/K^+-ATPases of cell membranes. Some predators, including a number of snakes, have evolved resistance to the toxic effects of bufadienolides and prey regularly on toads. Resistance in snakes to the acute effects of these toxins is conferred by at least two amino acid substitutions in the cardiotonic steroid binding pocket of the Na^+/K^+-ATPase. We surveyed 100 species of snakes from a broad phylogenetic range for the presence or absence of resistance-conferring mutations. We found that such mutations occur in a much wider range of taxa than previously believed. Although all sequenced species known to consume toads exhibited the resistance mutations, many of the species possessing the mutations do not feed on toads, much less specialize on that food source. This suggests that either there is little performance cost associated with these mutations or they provide an unknown benefit. Furthermore, the distribution of the mutation among major clades of advanced snakes suggests that the origin of the mutation reflects evolutionary retention more than dietary constraint.

1. Introduction

Many plants and animals are defended by toxic compounds, and circumvention of those defenses often has involved the evolution of elaborate mechanisms for resistance to

the toxins [1–5]. Among animals, some defensive compounds are synthesized from nontoxic precursors, whereas others are acquired and stored (sequestered) from environmental sources, primarily diet, and redeployed defensively [3,6–8]. Toads (Bufonidae) synthesize potent cardiotonic steroids known as bufadienolides (BDs) from cholesterol and store those toxins in high concentrations in their cutaneous glands [6]. Those toxins protect toads from the majority of predators, including many snakes that readily consume other species of frogs. Nonetheless, specialized bufophagy (the consumption of toads as a major (> 80%) dietary component) or the sequestration of dietary BDs for defense occur in at least three genera of snakes on different continents (Table 2-1) [9–11]. Such systems of chemical defense and resistance between prey and predators exemplify the co-evolutionary arms races that can drive molecular and physiological changes in the participating organisms [12].

Studies involving independently evolved systems of chemical defense and their circumvention by predators have shown that resistance to toxic dietary items can be accomplished by similar molecular changes that confer target-site insensitivity [11–15]. Furthermore, there is evidence that tight coupling between resistance of predators and toxicity of prey in a co-evolutionary arms race can result when the predator incurs a performance cost from resistance [12]. When a toxin targets a critical cellular function, phenotypes resistant to that toxin sometimes evolve through parallel or convergent mutations of the same gene across multiple species or populations [11–13,16–18].

BDs and other cardiotonic steroids, including the plant-derived cardenolides (CDs) [19,20], target the ubiquitous cell-membrane enzyme Na^+/K^+-ATPase, inhibiting the transport of sodium ions out of the cell and potassium ions in. The Na^+/K^+-ATPase

molecule consists of a catalytic α subunit, with 10 transmembrane segments, which carries out the functional activities of the enzyme, and a glycosylated β subunit, with one transmembrane segment, which provides structural stability (Figure 2-1A) [22–25]. The H1-H2 extracellular loop of the α subunit comprises a significant portion of the binding pocket for cardiotonic steroids (Figure 2-1B). However, some sites on the H3-H4 and the H5-H6 extracellular loops also contribute to the binding pocket to a lesser extent [13], and recent evidence suggests that mutations near the C-terminal end also confer resistance [26].

Several studies have found that two amino acid substitutions, one at position 111 and the other at or near position 122 of the H1-H2 loop, confer lowered affinity for cardiotonic steroids in resistant insects, lizards, and snakes [11,13–15,27–30]. Dobler et al. [13] identified various amino acid replacements encoded by the gene for Na^+/K^+-ATPase, including replacements at two positions in the H1-H2 extracellular loop, of several independent lineages of CD-resistant insects. Ujvari et al. [15] identified two similar amino acid substitutions in the H1-H2 extracellular loop of BD-resistant species of varanid lizards: a leucine (L) replaces a glutamine (Q) at position 111 (Q111L) and an arginine (R) replaces a glycine (G) at position 120 (G120R). Together, the Q111L and G120R substitutions were shown to confer significantly reduced affinity of Na^+/K^+-ATPase for BDs when transfected into human kidney cells [15]. These two substitutions were subsequently found to occur in several species of snakes that are resistant to BDs [11]. Mohammadi et al. [29] investigated the extent of resistance conferred at the whole-body level in a species of snake known to possess the Q111L and G120R mutations but

known to feed only rarely on toads. Those snakes were able to withstand concentrations of intraperitoneally injected BDs 200-fold higher than the mouse LD_{50}.

Site-directed mutagenesis of the gene that encodes for Na^+/K^+-ATPase in *Drosophila melanogaster* has revealed that the Q111L substitution alone, in CD-resistant insects, contributes slightly to resistance, whereas the mutation at position 122 (N122H, with histidine replacing asparagine) contributes significantly [13]. The N122H substitution results in a substantial biochemical change, replacement of a neutral amino acid by a positive one [13], so it is no surprise that this mutation significantly alters the sensitivity of insect Na^+/K^+-ATPases to CDs. Furthermore, since the G120R mutation in BD-resistant reptiles results in the same biochemical change two amino acid positions away from the N122H mutation of insects [11,15,29], it is safe to assume that the G120R mutation is likely the major contributor to lowered BD affinity in mutant reptile Na^+/K^+-ATPases [15].

Adaptation to similar dietary toxins through the evolution of virtually identical amino acid substitutions across a range of taxa, spanning invertebrates and vertebrates, represents a striking example of convergent evolution. Previous studies have typically concluded that the resistance-conferring mutations co-occur with dietary specialization [11–14], suggesting that resistance to dietary toxins occurs only under strong selective pressure.

A recent survey of genetic resistance to cardiotonic steroids across the Metazoa [11] noted a strong correspondence in snakes between Q111L and G120R mutations and predation on toads. Ujvari et al. [11] recovered three origins of resistance in their sample of 27 snakes and associated each origin with the assumption of toad-eating habits. Here

we expand the sample to 100 species of snakes, finding many more species that possess the resistance-conferring Q111L and G120R substitutions. We reconstruct the ancestral states for these taxa and find, in contrast to the more limited taxon sampling of Ujvari et al. [11], that the mutations for resistance occur more widely among snakes than does either specialized or opportunistic bufophagy. Indeed, the mutations occur in some species with diets restricted to prey that lack cardiotonic steroids. This lack of close correspondence between the mutation and toad consumption suggests that, in contrast to similar studies of predators on amphibians defended by tetrodotoxin (which blocks sodium channels) [12], resistance to BDs does not impose a significant performance cost or there is some other, unknown benefit. Furthermore, our results suggest that target-site insensitivity is necessary, but may not be sufficient, to permit dietary specialization on toads or the sequestration of dietary BDs.

2. Materials and Methods

(a) Selection and classification of study taxa

Because our goal was to test whether a mutated Na^+/K^+-ATPase alone could explain the evolution of specialized bufophagous habits, we began by sequencing part of the *ATP1a3* gene in previously unsequenced toad-eating species. Our criteria for toad-eating habits is restrictive and does not include diet data from general and secondary sources, which classify some species based upon anecdotal dietary records or assumptions of diet based on the predator's habitat. We based our dietary classification (Table 2-1) almost exclusively on dietary studies and other published reports in the primary literature. However, we also scored as (potentially) bufophagous those species

that possess the resistance mutations *and* have a broad diet of amphibians, even in the absence of specific reports of toads in their diet. This conservative assumption maximizes the perceived co-occurrence of the mutations and bufophagy.

We also broadened our genetic sampling to include nontoad-eating sister taxa of the specialized and facultative toad-eaters, to determine whether the distribution of mutations for BD resistance closely correspond to toad-eating habits. Combined with other sequence data available in GenBank, we included data for 100 species of snakes (see supporting online materials). Although not shown in the figures, our phylogenetic analysis included 21 lizards (18 of them in the genus *Varanus*, plus one dactyloid, one agamid, and one scincid), as well as *Sphenodon*, *Alligator*, *Gallus*, and *Xenopus*, as outgroups.

(b) Sequence data

Ethanol-preserved tissue samples were obtained from the Louisiana State University Museum of Zoology. Other tissue samples were acquired from snakes collected in the field in Louisiana (permit no. LNHP-12-064), Utah (permit no. 1COLL9134), and Nebraska (permit no. 477) or from previously frozen and ethanol-preserved specimens in our laboratories at Utah State University and Kyoto University. DNA was isolated from tissues using either the DNeasy Blood and Tissue Kit (Qiagen™) or the Phire Direct PCR Kit (Thermo Fisher™). The H1-H2 extracellular loop of the *ATP1a3* gene was amplified using the primers (Integrated DNA Technologies ®) ATP1a3Fwd (CGAGATGGCCCCAATGCTCTCA) and ATP1a3Rvs (TGGTAGTAGGAGAAGCAGCCGGT), which were modified from Moore et al. [31].

Amplified DNA product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega™). DNA was sequenced on an ABI 3730xo Genetic Analyzer using BigDye Terminator Kit V.3.1. Sequences were aligned and analyzed using Geneious v. 6.1.8 (Biomatters Ltd.). Sequences were translated to amino acids, and the hydrophobicity and isoelectric points of each residue were calculated using Geneious v. 6.1.8 (Biomatters Ltd.). Some sequences were obtained from GenBank (accession numbers KP238131-KP23176, XM_007437572, and AZIM01015831). DNA sequence data generated in this study have been submitted to GenBank with the accession numbers KU738063-KU738139, KU933521, and KU950321.

(c) Ancestral state reconstruction

Stochastic character mapping [32] was used to obtain probabilistic reconstructions of ancestral states for nonsynonymous substitutions at amino acid positions 111 (2nd codon position, A vs. T) and 120 (1st codon position A vs. G or C) (see Results). Character mapping was based on the time-calibrated squamate reptile phylogeny from Zheng and Weins [33], which we pruned to retain only the 121 species for which we had *ATP1a3* sequences. Sequences were available for seven species that were not included in Zheng and Wiens' phylogeny, although congeners were included in that tree. Therefore, we considered the sequenced species to be equivalent to their congeners for the purposes of the analysis, placing them at the same point on the phylogeny. The included species were *Conophis vittatus* (replacing *C. lineatus*), *Rhabdophis leonardi* (replacing *R. nuchalis*), *Cyclophiops semicarinatus* (replacing *C. major*), *Dendrelaphis subocularis*

(replacing *D. tristis*), *D. punctulatus* (replacing *D. bifrenalis*), *Causus maculatus* (replacing *C. rhombeatus*), and *Vipera aspis* (replacing *V. ammodytes*).

We first obtained the joint posterior distribution of ancestral character states and the transition matrix for each nucleotide position. We allowed all transition rates to differ and assumed a flat prior on the root states. Posteriors were inferred using Markov chain Monte Carlo (MCMC) with a 10,000 iteration burn-in, 100,000 sampling iterations, and a thinning interval of 50 iterations. Two chains were run to evaluate convergence of the MCMC algorithm to the stationary distribution, and results were combined for inference. Stochastic character maps were simulated over the posterior distribution of the transition matrix and thus account for uncertainty in transition rates. This analysis was conducted using the *make.simmap* function in the R package *phytools* [34].

In contrast to CD-resistant insects, in which position 122 of Na^+/K^+-ATPase has a positive amino acid, we always observed a shift from a neutral to positive amino acid at position 120 in BD-resistant snakes. Therefore, we asked whether such a pattern could be explained by chance under the null model that both changes would have an equivalent effect and are equally likely. Our calculations were made over the posterior distribution of the character state reconstructions, thus accounting for uncertainty in the number of changes.

We next compared models of correlated versus independent evolution for the two amino acids. We fit continuous-time Markov models for trait evolution, either with separate transition matrixes for each amino acid (independent evolution model) or with a single transition matrix for the pair of amino acids (dependent, or correlated, evolution model) [34]. We obtained maximum likelihood parameter estimates using BayesTraitsV2

(25 starts were used for parameter estimation). Models were then compared using a likelihood ratio-test as described by Pagel [35].

3. Results

(a) Amino acid substitutions

Our analysis of Na^+/K^+-ATPase α3 subunit sequences for the H1-H2 extracellular loop revealed substitutions at several residues. In our survey of 100 species of snakes, we found two consistent amino acid substitutions in all taxa known to consume toads. At position 111 we found the Q111L substitution and at position 120 the G120R substitution, a result that is consistent with previously published findings (Figure 2-1C) [11]. These two substitutions always occurred together and almost always involved the same nucleotide changes. Notably, in addition to the occurrence of the Q111L and G120R substitutions in all bufophagous species included in our analysis, we found many instances of these same two substitutions (hereinafter, "resistance mutations") across the phylogeny of snakes, including many species that rarely or never feed on toads. In addition to the Q111L and G120R substitutions, several other substitutions occurred at various positions among different groups. However, these changes are not known to alter the biochemical properties of the binding pocket to any significant extent, and thus are believed to be of no major functional consequence [11].

(b) Ancestral state reconstruction

Stochastic character mapping supported multiple gains and losses of the resistance mutations across the tree (Figure 2-2). We found evidence of 11 (95% credible intervals

[CIs] 2-18) transitions from *A* to *T* (with 8 reversals, 95% CIs 2-19) at position 2 of amino acid 111 and 11 (95% CIs 1-33) transitions from *A* to *G* (with 10 reversals, 95% CIs 1-32) at position 1 of amino acid 120. Given the total number of transitions and the paucity of taxa with only one of the resistance mutations, we can confidently reject the null model that changes at amino acid 111 and 120 are equivalent (binomial probability: median across MCMC replicates, $p = 0.0005$; mean across MCMC replicates = 0.0511). Moreover, a model of correlated or dependent evolution for amino acid positions 111 and 120 was preferred relative to a null model of independent evolution (likelihood ratio = 88.85, chi-sq with 4 d.f., $p < 0.001$).

4. Tables and Figures

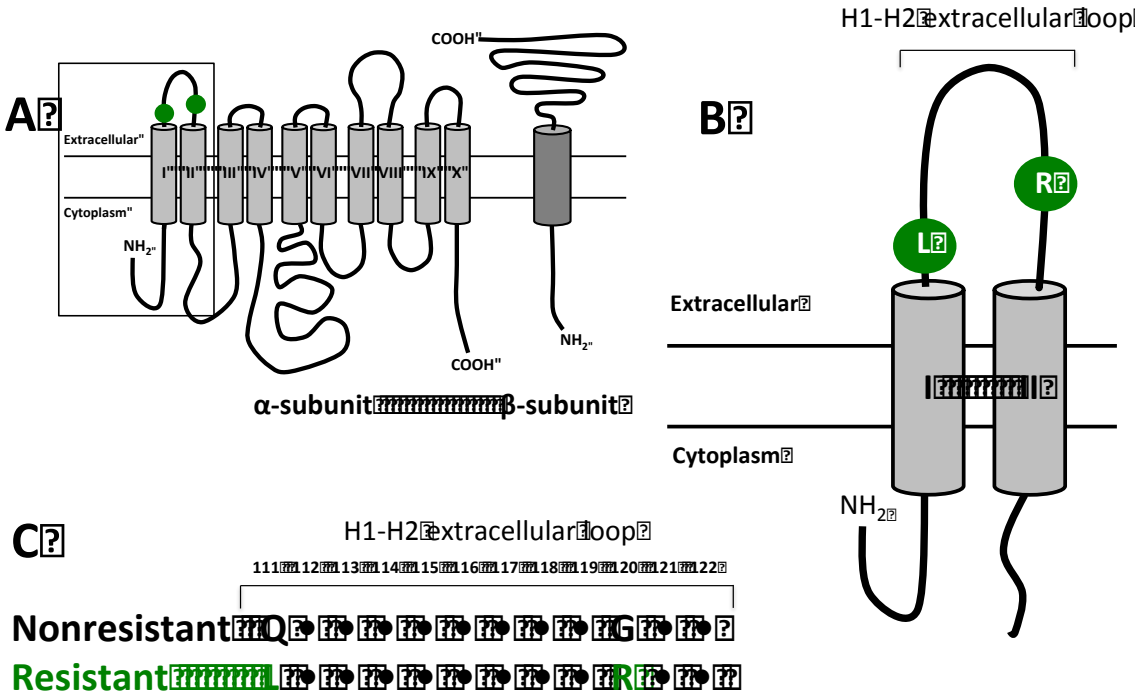

Figure 2-1. (A) Schematic diagram of an unfolded Na^+/K^+-ATPase molecule, showing the location of the Q111L and G120R substitutions on the H1-H2 extracellular loop. Box shows area enlarged in B. (B) Enlargement of the H1-H2 extracellular loop and the first two transmembrane domains. (C) The amino acid sequence of the first extracellular loop, indicating the substitutions at positions 111 and 120 that confer resistance to bufadienolides in snakes. Redrawn from Köksoy [21]. For a more detailed structural reconstruction of the interactions of the Na^+/K^+-ATPase and a BD, see Laursen et al. [25].

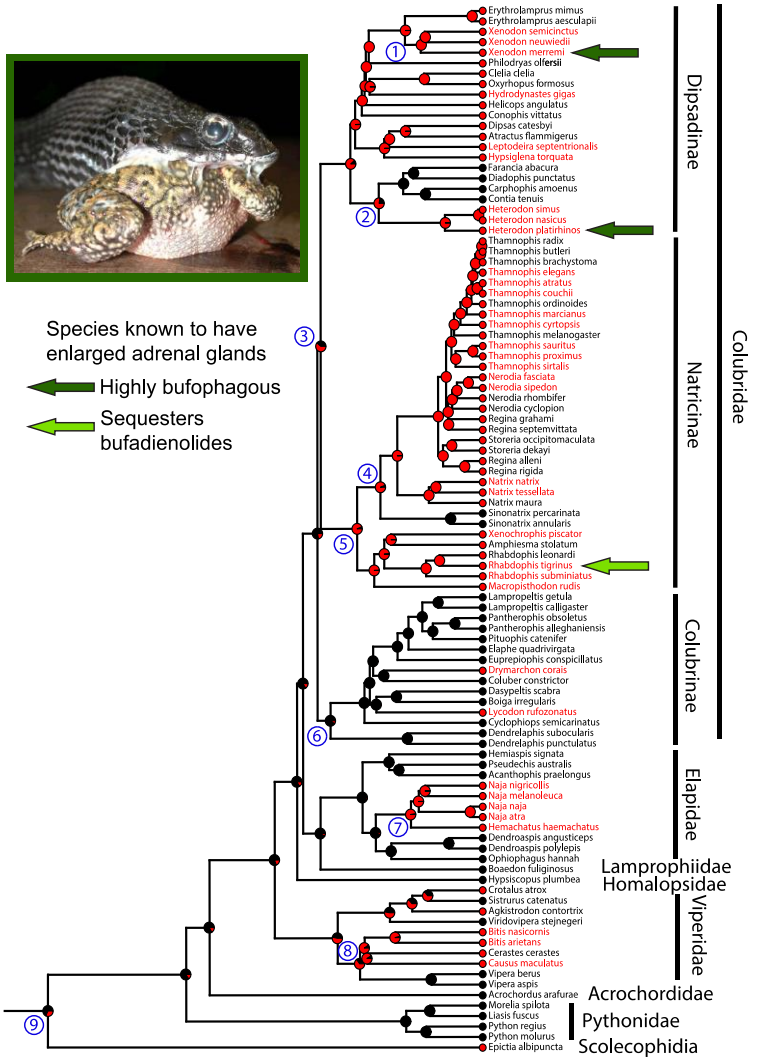

Figure 2-2. Ancestral state reconstruction of the mutations to Na^+/K^+-ATPase that confer resistance to bufadienolides in snakes. Colored circles beside terminal taxa indicate the amino acid composition of Na^+/K^+-ATPase at the H1-H2 extracellular loop (red = resistant L111 and R120 and black = nonresistant Q111 and G120). The names of bufophagous species are shown in red, and nonbufophagous species are shown in black (Table 2-1) [10, 35–36]. Inferred ancestral states are denoted by pie charts that indicate the posterior probability of having (red) versus not having (black) the resistance mutations. Selected higher taxa are identified, and circles numbers identify nodes that are discussed in the text. Note that the resistance mutations to Na^+/K^+-ATPase are not limited to taxa that prey on toads (as shown by the number of black species names beside red circles). Two highly bufophagous species (*Heterodon platirhinos* and *Xenodon merremii*) and one that sequesters BDs (*Rhabdophis tigrinus*), indicated by green arrows, possess enlarged adrenal glands that may function in countering the effects of chronic exposure to bufadienolides. Inset: *Heterodon platirhinos* feeding on the toad *Anaxyrus terrestris*. Note the viscous skin secretion of the toad, which is rich in bufadienolides.

Table 2-1. Snake species included in this study, indicating the presence or absence of the resistance mutations of Na^+/K^+-ATPase and the presence or absence of toads in the diet. The dietary data are compiled almost exclusively from primary literature, a large portion of which includes comprehensive dietary studies rather than anecdotal observations. We considered most secondary literature to be unreliable for accurate dietary data. Due to the imperfect nature of dietary studies, we have taken a conservative approach and consider a species bufophagous if it is a generalist predator and there exists a record of at least one individual consuming a toad (i.e., marked "Yes" under "Toad in Diet"). Furthermore, we considered a bufadienolide-resistant snake bufophagous if it is a generalist predator with a high frequency of amphibians in the diet, even though there are no published records of toads. Bufadienolide-resistant species that are not considered bufophagous include primarily species with dietary specializations that do not include amphibians. Asterisks indicate species with diets including >80% toads or those that sequester dietary bufadienolides.

Species	Resistance mutations	Toad in diet	References
Erythrolamprus mimus	**Yes**	**No**	[1–2]
Erythrolamprus aesculapii	**Yes**	**No**	[1,3–8]
Xenodon semicinctus	**Yes**	**Yes**	[4]
Xenodon neuwiedii	**Yes**	**Yes**	[9–12]
Xenodon merremi	**Yes**	**Yes***	[9–10,13–12]
Philodryas olfersii	**Yes**	**No**	[7,14–18]
Clelia clelia	**Yes**	**No**	[19]
Oxyrhopus formosus	**Yes**	**No**	[17–18]
Hydrodynastes gigas	**Yes**	**Yes**	[20]
Helicops angulatus	**Yes**	**No**	[7,14,21–24]
Conophis vittatus	**Yes**	**No**	[25]
Dipsas catesbyi	**Yes**	**No**	[23,26–27]
Atractus flammigerus	**Yes**	**No**	[15]
Leptodeira septentrionalis	**Yes**	**Yes**	[28–30]
Hypsiglena torquata	**Yes**	**Yes**	[31]
Farancia abacura	**No**	**No**	[32–33]
Diadophis punctatus	**No**	**No**	[34–35]
Carphophis amoenus	**No**	**No**	[34]
Contia tenuis	**No**	**No**	[36]
Heterodon simus	**Yes**	**Yes**	[37–38]
Heterodon nasicus	**Yes**	**Yes**	[37–39]
Heterodon platirhinos	**Yes**	**Yes***	[37,40–41]
Thamnophis radix	**Yes**	**No**	[42–45]

(Table 2-1 continued.)

Thamnophis butleri	**Yes**	**No**	[46–47]
Thamnophis brachystoma	**Yes**	**No**	[48–49]
Thamnophis elegans	**Yes**	**Yes**	[12,50–51]
Thamnophis atratus	**Yes**	**Yes**	[52–54]
Thamnophis couchii	**Yes**	**Yes**	[55–56]
Thamnophis ordinoides	**Yes**	**No**	[49,56]
Thamnophis marcianus	**Yes**	**Yes**	[57]
Thamnophis cyrtopsis	**Yes**	**Yes**	[58]
Thamnophis melanogaster	**Yes**	**No**	[59–63]
Thamnophis sauritus	**Yes**	**Yes**	[64]
Thamnophis proximus	**Yes**	**Yes**	[57,65–67]
Thamnophis sirtalis	**Yes**	**Yes**	[12,51,68–70]
Nerodia fasciata	**Yes**	**Yes**	[71–73]
Nerodia sipedon	**Yes**	**Yes**	[34–35,74]
Nerodia rhombifer	**Yes**	**Yes**	[71,74–76]
Nerodia cyclopion	**Yes**	**No**	[74–75]
Regina grahami	**Yes**	**No**	[71,74]
Regina septemvittata	**Yes**	**No**	[74,77–78]
Storeria occipitomaculata	**Yes**	**No**	[79–80]
Storeria dekayi	**Yes**	**No**	[79,81]
Regina alleni	**Yes**	**No**	[74,82–84]
Regina rigida	**Yes**	**No**	[74–75]
Natrix natrix	**Yes**	**Yes**	[85–88]
Natrix tessellata	**Yes**	**Yes**	[86,89]
Natrix maura	**Yes**	**No**	[90–91]
Sinonatrix percarinata	**No**	**No**	[92]
Sinonatrix annularis	**No**	**No**	[92]
Xenochrophis piscator	**Yes**	**Yes**	[93]
Amphiesma stolatum	**Yes**	**No**	[94]
Rhabdophis leonardi	**Yes**	**No**	[95]
Rhabdophis tigrinus	**Yes**	**Yes***	[96–98]
Rhabdophis subminiatus	**Yes**	**Yes**	[99]
Macropisthodon rudis	**Yes**	**Yes**	[100]
Lampropeltis getula	**No**	**No**	[66]
Lampropeltis calligaster	**No**	**No**	[35,66]

(Table 2-1 continued.)

Pantherophis obsoletus	**No**	**No**	[35,66,101]
Pantherophis alleghaniensis	**No**	**No**	[102]
Pituophis catenifer	**No**	**No**	[103]
Elaphe quadrivirgata	**No**	**No**	[96–97,104]
Euprepiophis conspicillatus	**No**	**No**	[105]
Drymarchon corais	**Yes**	**Yes**	[106–107]
Coluber constrictor	**No**	**No**	[35,66,108–109]
Dasypeltis scabra	**No**	**No**	[110–111]
Boiga irregularis	**No**	**No**	[112]
Lycodon rufozonatus	**Yes**	**Yes**	[113]
Cyclophiops semicarinatus	**No**	**No**	[105]
Dendrelaphis subocularis	**No**	**No**	[114–115]
Dendrelaphis punctulatus	**No**	**No**	[116–118]
Hemiaspis signata	**No**	**No**	[119–121]
Pseudechis australis	**No**	**No**	[121]
Acanthophis praelongus	**No**	**No**	[122]
Naja nigricollis	**Yes**	**Yes**	[123]
Naja melanoleuca	**Yes**	**Yes**	[123–126]
Naja naja	**Yes**	**No**	[127–128]
Naja atra	**Yes**	**No**	[127–129]
Hemachatus haemachatus	**Yes**	**Yes**	[126]
Dendroaspis angusticeps	**No**	**No**	[130–131]
Dendroaspis polylepis	**No**	**No**	[131]
Ophiophagus hannah	**No**	**No**	[132]
Boaedon fuliginosus	**No**	**No**	[133]
Hypsiscopus plumbea	**No**	**No**	[134]
Crotalus atrox	**Yes**	**No**	[135–137]
Sistrurus catenatus	**No**	**No**	[138–140]
Agkistrodon contortrix	**Yes**	**No**	[66,141–143]
Viridovipera stejnegeri	**No**	**No**	[127,144]
Bitis nasicornis	**Yes**	**Yes**	[124,145]
Bitis arietans	**Yes**	**Yes**	[146]
Cerastes cerastes	**Yes**	**No**	[147–148]
Causus maculatus	**Yes**	**Yes**	[149]

(Table 2-1 continued.)

Vipera berus	**No**	**No**	[150–151]
Vipera aspis	**No**	**No**	[152–154]
Acrochordus arafurae	**No**	**No**	[155–156]
Morelia spilota	**No**	**No**	[157–158]
Liasis fuscus	**No**	**No**	[159–161]
Python regius	**No**	**No**	[162]
Python molurus	**No**	**No**	[163–165]
Epictia albipuncta	**Yes**	**No**	[166–167]

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5. Discussion

Our results demonstrate that the mutations at positions 111 and 120 in the H1-H2 extracellular loop of the Na^+/K^+-ATPase α3 subunit, which confer resistance to toad toxins in snakes [11,15,29], occur in a much wider range of species than previously believed, including many species that do not regularly, if ever, prey on toads (Table 2-1). The first extracellular loop (amino acid positions 111-122) comprises a major part of the cardiotonic steroid binding pocket, and the nonresistant gene codes for the amino acids glutamine at position 111 and glycine at position 120. The observed substitution of leucine at position 111 results in a biochemical residue change from polar-neutral to nonpolar-neutral, which increases the hydrophobicity of the residue but does not change the isoelectric point. The substitution of arginine at position 120 results in a biochemical residue change from nonpolar-neutral to polar-positive, which increases the isoelectric point and decreases the hydrophobicity of the residue. Although the physiological effect of the G120R substitution has not been specifically tested, it is biochemically similar to the residue change caused by the N122H substitution in milkweed insects (change from a neutral to positively charged residue), which has been shown to confer significantly lowered binding affinity to CDs [13]. Furthermore, the combined substitutions of Q111L and G120R have been shown to confer a much lower affinity to BDs than the wild-type

gene in vertebrate cells [15]. Although the Q111L substitution does contribute to resistance in cardenolide-resistant invertebrates [13], evidence from insect phylogenies suggests that it is a necessary intermediate step that leads to the later appearance of other substitutions in the H1-H2 extracellular loop [4]. A similar relationship presumably explains the consistent co-occurrence of substitution Q111L with G120R in BD-resistant snakes.

The distribution of the resistance mutations Q111L and G120R among colubroid snakes, including their widespread appearance in many species that do not prey on toads, raises a number of important issues that were obscured by the limited taxon sampling of previous studies. The occurrence of such mutations among nontoad-eating species suggests that possession of these mutations does not carry a high performance cost. Paradoxically, however, investigations of the ion transport activities of some resistant Na^+/K^+-ATPases have shown that the mutations do in fact lower the enzyme's function [36-37]. It appears that in insects, the deleterious effects of the lowered enzyme function are circumvented by the presence of a secondary Na^+/K^+-ATPase isoform [37]. It is thus likely that in snakes Na^+/K^+-ATPase isoforms α1 and α2 might be contributing similarly. This contrasts sharply with the limited distribution of mutations for resistance in snakes that consume amphibians defended by tetrodotoxin (TTX) [12]. TTX targets voltage-gated sodium channels, and the mutations that confer resistance to TTX also impart a substantial countervailing cost, as measured by locomotor performance [38–39]. Several point mutations have been documented among TTX-resistant snakes [12], and their occurrence is tightly linked to the consumption of TTX-defended amphibians. In the best-documented case, involving the natricine snake *Thamnophis sirtalis* and the newt *Taricha*

granulosa, geographic differences in the level of TTX in the newt are mirrored by differences in the level of innate resistance of the sodium channels in the snake [40]. Populations of snakes that consume low levels of TTX exhibit low levels of resistance.

In contrast to such close correspondence between prey toxicity and sodium channel resistance to TTX, our data document the occurrence of BD-resistance mutations in Na^+/K^+-ATPase among many species that are not known to consume toads at all, including specialized predators on mollusks (*Dipsas catesbyi*, *Storeria occipitomaculata*), crayfishes (*Regina* spp.), fishes (*Nerodia rhombifer*, *Helicops angulatus*), and mammals (*Crotalus atrox*, *Agkistrodon contortrix*) (Table 2-1). It is possible that the same mutation protects a few of these taxa against other toxins that exert yet unknown pharmacological effects (as in some mollusks [41] and other invertebrates [42-46]). However, most nontoad-eating species that possess the resistance mutations appear not to derive any benefit nor incur a substantial cost from the mutated form of Na^+/K^+-ATPase. Future studies should address that inference directly.

Our study includes relatively broad taxon sampling of the three largest subfamilies of colubrid snakes. The sister lineages Natricinae and Dipsadinae (Figure 2-2) both include large numbers of taxa that possess the resistance mutations. The species in both lineages consume a wide variety of prey, and both subfamilies include species that are highly bufophagous or sequester BDs. Our results indicate a 0.61 probability that resistance to BDs originated in the common ancestor of both of these groups (Figure 2-2, node 3) and was independently lost in two extant lineages. Interestingly, both of these subfamilies include early branches that are highly bufophagous or sequester BDs, suggesting the possibility that consumption of toads characterized early members of both

radiations, and possibly their common ancestor. Arguably the most highly bufophagous genus of snakes, *Heterodon* of North America, lies near the base of the Dipsadinae, although members of the sister lineage of *Heterodon* (which includes *Farancia*, *Diadophis*, *Carphophis*, and *Contia*) lack the resistance mutations, most likely through secondary loss of those residues (Figure 2-2, node 2). The other highly bufophagous lineage of dipsadines, the genus *Xenodon* (*sensu lato,* including the nominal genera *Waglerophis* and *Lystrophis* [47]), is nested more deeply within the Dipsadinae, as a member of a diverse South American radiation (Figure 2-2, node 1).

Among the Natricinae, the overwhelming majority of species possesses the resistance mutations. A basal split (Figure 2-2, node 5) separates an exclusively Asian lineage from one that includes a few Asian species, as well as a number in Europe and the large North American tribe Thamnophiini. The exclusively Asian lineage includes the genus *Rhabdophis*, most members of which possess defensive structures known as nuchal or nucho-dorsal glands [48]. Some species accumulate high concentrations of BDs from toads consumed as prey and release the toxins to deter the snakes' predators [3,49]. Furthermore, female *Rhabdophis tigrinus* can transfer prey toxins to their embryos before oviposition [50], and a recent field study demonstrated that females preferentially forage for toads during the reproductive season, presumably increasing the likelihood of provisioning their embryos with BDs [51]. Therefore, although toads comprise a numerically small element of the diet in this species [52], the snakes often retain high levels of BDs in their tissues. The sampled species of Natricinae exhibit only a single example of likely secondary loss of the resistance mutations, the East Asian genus *Sinonatrix* (Figure 2-2, node 4). That genus of highly aquatic fish-eaters (Table 2-1), is

sister to the West Asian, European, and North American natricines, all of which possess the resistance mutations and one of which, *Natrix natrix* of Europe, frequently consumes toads.

The third broadly sampled colubrid subfamily, the Colubrinae, displays a different evolutionary picture. Of 15 species sampled, only two distantly related species possess the resistance mutation (and are known to feed on toads). Our analysis suggests a 91% probability that the ancestor of the Colubrinae (Figure 2-2, node 6) lacked the mutation. Thus, although all species in our sample that are known to prey on toads possess the resistance mutations, the recovered pattern is one in which only a few members of the ancestrally nonresistant Colubrinae evolved resistance, whereas many members of the ancestrally resistant Natricinae and Dipsadinae retained the mutations despite consuming prey other than toads. These results argue that historical contingency plays a strong role in the origin of genetic resistance to BDs and suggest that the performance cost of retaining the mutations is low, even when it confers no evident advantage.

Two other large and widespread families, Viperidae and Elapidae, possess bufophagous genera, both of which occur in Africa. Viperids exhibit greater variation in the distribution of the resistance mutations than other major lineages. However, the bufophagous viper *Causus* is basal to a radiation of African Viperinae (Figure 2-2, node 8), of which all sequenced taxa retain those mutations. The Eurasian genus *Vipera* diverged before the African radiation and lacks the mutations. The other major lineage of vipers, the Crotalinae, exhibits a diversity of Na^+/K^+-ATPase sequences. However, our sampling of Viperidae is limited, and further sequencing is needed to resolve the origins of resistance in this family with confidence. Among the Elapidae, the bufophagous

African genus *Hemachatus* is sister to the diverse radiation of African and Asian *Naja* (Figure 2-2, node 7), which includes a number of facultative toad-eaters. Unfortunately, our sampling includes only a single species of Lamprophiidae, a large and ecologically diverse African colubroid radiation that is sister to the Elapidae. The only sequenced lamprophiid, *Boaedon fuliginosus*, lacks the resistance mutations, but further sampling of this family is desirable, especially considering that one species of the lamprophiid genus *Crotaphopeltis* feeds largely on toads [9].

It is possible that some generally nontoad-eating taxa may rarely encounter and consume bufonids, either as adults or as juveniles. (Information on the diets of young snakes are especially limited.) However, it is doubtful that rare instances of toad consumption would have driven the evolution of the resistance mutations, especially in the varied dietary specialists discussed above. It is more likely that species belonging to ancestrally resistant lineages may occasionally consume bufonids when encountered, despite a diet consisting primarily of other prey. Such a scenario is not inconsistent with our conclusions. Conversely, however, we predict that nonresistant snakes would rarely, if ever, survive such a rare predatory event.

Recent time-calibrated phylogenetic studies of toads and snakes support the hypothesis that toads arrived on all occupied continents prior to, or coincident with, the evolution of specialized bufophagous snakes. The Bufonidae are believed to have arisen in South America, after the breakup of Gondwana approximately 88 Ma, and dispersed north and then west across the Bering Strait, establishing lineages in Eurasia and Africa before returning to the Americas across a North Atlantic land connection about 43 Ma [53]. The presence of BDs in the most basal genus of bufonids, *Melanophryniscus*, is

uncertain, although its species are chemically defended by the indolealylamine bufotenine and by alkaloids sequestered from arthropod prey [54]. Skin extracts from *Melanophryniscus* appear to inhibit Na^+/K^+-ATPase [55]. BDs are present in *Atelopus* [55], which is sister to all remaining bufonids in both the New and Old Worlds. Furthermore, a more derived clade of Neotropical bufonids (*Rhinella*) diverged from a North American ancestor about 41 Ma, so the presence of BD-defended toads in South America clearly predates the origin of the bufophagous South American genus *Xenodon* about 18 Ma. Similarly, the origin of the notably bufophagous genera *Heterodon* (North America, 30 Ma), *Rhabdophis* (East Asia, 29 Ma), *Natrix natrix* (Europe, 13 Ma), *Hemachatus* (Africa, 23 Ma), and *Causus* (Africa, 30 Ma) all postdate the completion of the bufonid diaspora [33].

Importantly, dates vary for the divergence of the related colubrid subfamilies Natricinae and Dipsadinae, both of which include relatively basal bufophagous genera and large numbers of genera that possess the resistance mutations. Indeed, our analysis indicated a fairly high probability that the ancestor of Natricinae + Dipsadinae (Figure 2-2, node 3) possessed those mutations. A detailed analysis of the Natricinae suggested that they originated in eastern Asia approximately 35 Ma (Figure 2-2, node 5), later establishing lineages in Africa, Europe, and North America [56]. Consistent with that scenario, the most basal dipsadines, *Thermophis* and *Stichophanes*, occur in Asia, whereas all other members of the subfamily are limited to the New World, including the highly bufophagous *Heterodon* and *Xenodon*. More recently, Zheng and Wiens [33] dated the divergence of Natricinae and Dipsadinae to about 48 Ma. These two divergence estimates bracket the date for the re-entry of toads into the New World from Eurasia

(about 43 Ma; 38) and are reasonably consistent with an origin of the mutations in the ancestor of Natricinae + Dipsadinae in Asia while in sympatry with bufonids.

The earliest lineage of snakes in which we found the resistance mutations is *Epictia albipuncta* (Leptotyphlopidae; Figure 2-2, node 9), our only sample from the large but poorly understood radiation of Scolecophidia. That possibly paraphyletic group of five families and about 400 species [33,57] underwent an initial split into three major lineages about 123-125 Ma [57], long before the origin of toads. Given the presence of the resistance mutations in some varanid lizards [11,15], it is possible that the mutations may have been present in the common ancestor of all snakes. Alternatively, resistance may have evolved independently in scolecophidians, nearly all of which specialize on a diet of social insects, either ants or termites [8]. Ants are defended by a diverse array of alkaloids [42–43], and some termites possess terpene toxins [43–46], although the diversity of toxins in the early life history stages on which scolecophidians largely feed is not well known. The pharmacological effects of most of ant and termite compounds also are poorly understood, and thus it is uncertain whether the same mutations to Na^+/K^+-ATPase that confer resistance to BDs would protect against those prey. However, evidence of resistance to both BDs and alkaloids has been demonstrated in *Heterodon platirhinos*, a highly bufophagous species that also is resistant to TTX in newts, although the mechanism for its resistance to TTX remains unknown [58]. Clearly, additional sequencing of diverse scolecophidian species is needed, together with a better understanding of the physiological effects of their prey's defensive toxins.

Although our sampling efforts were biased toward certain lineages, our data are sufficient to demonstrate that the presence of BD resistance mutations across the

phylogeny of snakes does not correspond closely to the consumption of toads. Nonetheless, it is true that all bufophagous snakes in our sample possess the resistance mutations. Comparison of the physiological effects of a controlled dose of the BD gamabufotalin on a species with the resistance mutations, *Nerodia rhombifer*, and one that lacks the mutations, *Pituophis catenifer*, revealed sharply different responses [29]. *N. rhombifer*, a specialized predator on fishes, exhibited no signs of distress when subjected to mass-specific doses 20 times greater than those that proved lethal to *P. catenifer*. This suggests that the resistance mutations are indeed protective against the acute effects of BD consumption. Furthermore, the widespread occurrence of the resistance mutations among nontoad-eating snakes suggests that possession of the resistance mutations does not impose a large performance cost. However, the inclusion of toads as an occasional prey item among the genetically resistant species, together with the relative rarity of species that either specialize on a diet of toads or sequester dietary BDs, suggests that the cost of chronic exposure to those toxins poses a greater physiological challenge and may require additional mechanisms of resistance.

In parallel invertebrate systems, a variety of mechanisms confer resistance to CDs, including detoxification by enzymes, excretion, and exclusion from sensitive tissues, in addition to target-site insensitivity [1–2,4–5]. As such, target-site insensitivity may not always be required as a precursor for tolerance to toxic prey. Furthermore, several resistance mechanisms against environmental toxins can exist within a single species. For example, dichlorodiphenyltrichloroethane (DDT) resistance in insects can be achieved by amino acid substitutions in the voltage-gated sodium channel [17,59] and also through increased expression of detoxifying enzymes [17,60]. This further suggests

that, although the resistance mutations of Na^+/K^+-ATPase may be sufficient to protect a snake from the acute effects of BD ingestion, additional physiological adaptations may be required to protect highly bufophagous taxa and those that sequester BDs against the chronic effects of toxin exposure. Some CD-resistant insects show tissue-specific increases of mutant Na^+/K^+-ATPase expression that reflect the relative exposure of different tissues to CDs [14]. A similar mechanism in bufophagous snakes may involve a specific endocrine response to BDs. Mohammadi et al. [10] demonstrated that three independently evolved snakes that specialize on toads possess highly enlarged adrenal glands in comparison with their nontoad-eating sister taxa (Figure 2-2). Adrenal corticosteroid hormones exert diverse physiological effects, and aldosterone in particular has been linked to increased expression of Na^+/K^+-ATPase in cardiac cells [61].

Although target-site insensitivity clearly plays an essential role in the survival of toad-eating snakes, it is not tightly coupled to bufophagy. The occurrence of the resistance mutations among species with varied diets suggests that the mutations impose little performance cost. To understand the evolution of highly specialized bufophagous snakes, it will be necessary to move beyond target-site insensitivity and investigate the complex, interconnected physiological pathways that confer resistance to chronic BD exposure.

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CHAPTER 3

TOAD TOXIN-RESISTANT SNAKE (*THAMNOPHIS ELEGANS*) EXPRESSES HIGH LEVELS OF MUTANT NA^+/K^+-ATPASE mRNA IN CARDIAC MUSCLE

Toads are chemically defended by bufadienolides, which are lethal to most predators. These toxins exert their lethal effects by binding to and disabling the Na^+/K^+-ATPases of cell membranes. Many species of snakes exhibit resistance to the effects of bufadienolides due to target-site insensitivity of the Na^+/K^+-ATPase. Mutations that confer resistance have previously been identified in *ATP1a3*, the gene that codes for the Na^+/K^+-ATPase α-3 paralog. We have found that this mutant gene is expressed at a significantly elevated level in heart tissue compared to gut, kidney, and liver of the bufadienolide-resistant snake, *Thamnophis elegans*. Furthermore, we found that exposure to bufadienolides elicits a significant increase in the expression levels of *ATP1a3* in the heart, but not in the kidneys, liver, or gut one hour after exposure. We suggest that upregulation of *ATP1a3* in the heart plays an important role in the physiological processes involved in tolerance of bufadienolides among genetically resistant snakes.

1. Introduction

Toads are chemically defended by potent cardiotonic steroids known as bufadienolides (BDs; Chen and Kovaříková, 1967; Garraffo and Gros, 1986; Krenn and Kopp, 1998), which render them lethal to most predators (Phillips and Shine, 2006; Ujvari et al., 2013; Mohammadi et al., 2016a). BDs are one of two classes of cardiotonic steroids (together with cardenolides), which exert their lethal effects by binding to and

disabling Na^+/K^+-ATPases (NKAs) of cell membranes (Barry and Bridge, 1993; Rose and Valdes, 1994; Barrueto et al., 2006; Dvela et al., 2007). Inhibition of this transmembrane enzyme triggers a compensatory reaction by the Na^+/Ca^{2+} exchanger, which causes highly elevated intracellular Ca^{2+} levels and results in increased and prolonged cardiac muscle contraction and decreased heart rate (Rose and Valdes, 1994; Krylov, 2000). BDs occur in toads (Bufonidae), as well as fireflies (Lampyridae; Krenn and Kopp, 1998; Dvela et al., 2007), and are distinguished from cardenolides by a six-membered, as opposed to five-membered, lactone group at the C-17 position. Two plant genera, *Helleborus* and *Kalanchoe*, also produce BDs (Wissner and Kating, 1971; Muhr et al., 1995; Krenn and Kopp, 1998). Among the cardenolides are pharmacologically significant compounds such as digoxin and ouabain, which have been used by humans in the treatment of heart failure for over 200 years (Schoner, 2002).

Several lineages of snakes have evolved resistance to BDs, through mutations that lower the affinity of the toxins to NKAs, and can prey readily on toads (Ujvari et al., 2015; Mohammadi et al., 2016b), whereas snakes without the mutations die from attempted predation on toads (Shine, 2010; Mohammadi et al., 2016a). Ujvari et al. (2015) demonstrated that taxa across the Metazoa have independently evolved mutations of NKA that confer resistance to cardiotonic steroids. A phylogenetic analysis concluded that absence of a mutation that confers resistance is ancestral to the family Colubridae, although the presence of the mutated form appears to be ancestral to the subfamily Natricinae, to which *T. elegans* belongs (Mohammadi et al., 2016b).

The α-subunit of NKA contains the BD binding pocket (Morth et al., 2007; Ogawa et al., 2009; Laursen et al., 2015), and snakes possess three paralogs of this

subunit (frequently referred to as isoforms in the literature) encoded by the genes *ATP1a1*, *ATP1a2*, and *ATP1a3* (Babonis et al., 2011; Castoe et al., 2013; Vonk et al., 2013). Differences in their function are poorly understood. Mutations that confer resistance to BDs in snakes are known to occur in *ATP1a3* (Ujvari et al., 2015; Mohammadi et al., 2016b). It is possible that *ATP1a1* and *ATP1a2* also possess mutations that confer resistance, but the amino acid sequences of those paralogs have not yet been studied in BD resistant snakes.

To date, there have been no published studies investigating the abundance of the mRNAs for the genes encoding the three α-paralogs in snakes. Previous studies that have investigated the mechanisms of BD resistance in reptiles have focused almost exclusively on target-site insensitivity of the NKA (*ATP1a3*; Ujvari et al., 2013; Ujvari et al., 2015; Mohammadi et al., 2016a; Mohammadi et al., 2016b). However, in order to understand better the physiology of BD resistance, it is imperative to move beyond target-site insensitivity and investigate the physiological pathways that may also contribute to resistance. A logical step is to determine the tissue-specific expression levels of the mutant NKA α-3 gene, *ATP1a3*. We predict higher levels of *ATP1a3* mRNA in tissues that receive significant exposure to BDs and those that rely heavily on high numbers of functioning NKAs, such as the kidneys (Katz, 1982; Ferrandi et al., 1996). Increased expression of *ATP1a3* mRNA in such tissues would differentially protect them from NKA inhibition.

We tested this prediction by measuring the expression levels of *ATP1a3* in tissues that would likely be affected or come in contact with ingested BDs of a species known to possess the resistant mutations of *ATP1a3* (Mohammadi et al., 2016b), *Thamnophis*

elegans. We measured *ATP1a3* expression levels in the absence of BDs and following exposure to an injected BD. We limited our study to the paralog *ATP1a3* because it has been the focus of all previous studies of NKA resistance in reptiles. Furthermore, DNA sequences of paralogs *ATP1a1* and *ATP1a2* are not available for BD-resistant snakes.

2. Materials and methods

2.1 Experimental subjects

Eleven adult snakes (*T. elegans*; Table 3-1) were collected in Utah (Cache County; permit no. 1COLL9134), transported to Utah State University (Logan, Utah, USA), housed individually in 37.8 L glass terraria with newspaper substrate, provided with water ad libitum and a hide shelter, and fed with thawed, commercially purchased mice (RodentPro.com, LLC) once per week. Snakes were cared for and handled in compliance with Utah State University Institutional Animal Care and Use Committee (IACUC) regulations (protocol #2078).

2.2 Experimental treatments

The snakes were randomly assigned to one of two treatments: control or BD. Five percent dimethyl sulfoxide (DMSO; Fisher Scientific, Inc.) served as the solvent for the BD doses, and was thus used as the control treatment. Six snakes were given a mass-adjusted volume dose (equivalent to BD dose) of 5% DMSO as a control, and the other five were given a mass-adjusted dose (0.149 M/g) of the BD gamabufotalin (PiChemicals ®, Lot # PI201406082068 and # PI1201206031583) dissolved in DMSO (Table 3-1). The BD dose was selected based on previously published experiments and represents 100

times the intravenous LD_{50} of mice and approximately 10 times the intraperitoneal lethal dose for a non-resistant snake (Yoshida et al., 1976; Mohammadi et al., 2016a). Doses were administered by intraperitoneal injection. At exactly one hour post-injection, snakes were sacrificed by decapitation and immediately dissected for tissue samples of heart, liver, kidney, gut (small intestine), and skeletal muscle. Tissue samples were preserved in RNAlater® (Ambion™) and stored at -80°C until RNA extraction.

2.3 Extraction of mRNA and cDNA synthesis

RNA was extracted from tissues using the RNeasy Mini Kit (Qiagen©), quantified and checked for purity using a micro-volume spectrophotometer (NanoDrop 2000, Thermo Scientific™). We were unable to extract sufficient RNA from the skeletal muscle samples, and thus that tissue was omitted from the analysis. For cDNA synthesis we used the Superscript III reverse-transcription kit (Invitrogen™) including both Oligo-dT_{20} and random hexamer primers. A final concentration of 50 ng/µl in 20 µl volume was reverse transcribed for each sample, and homogeneity across samples was confirmed visually using a 2% agarose gel. All cDNA was stored at -20°C until use in the qPCR analysis.

2.4 Primer design and qPCR analysis

Quantitative PCR primers were designed to amplify the target gene, *ATP1a3* (FWD 5'-GTTTTGTCGCCAGCTCTTCG-3' RVS 5'-ACCCAGGTACAGGTTGTCTC-3'; Primer3 software Untergasser et al., 2012), and the ribosomal protein reference genes, *L8* (FWD 5'-AACTGTTCATTGCAGCGGAGG-3' RVS 5'-

TGAGCTGAGCTTTCTTGCCAV-3'; Babonis et al., 2011) and *18S* (FWD 5'-CCGATGCTCTTAACTGAGTGTCT-3' RVS 5'-GGTCCAAGAATTTCACCTCTAGC-3'; Primer3 software Untergasser et al., 2012) in *T. elegans*. *L8* and *18S* were chosen as reference genes due to their consistent expression across tissue types (Filby and Tyler, 2007; Babonis et al., 2011). We checked primer specificity for *ATP1a3* against *ATP1a1* and *ATP1a2* with a nucleotide BLAST against the *Python bivittatus* genome on GenBank (NCBI). The BLAST search returned with a 100% match on *ATP1a3*, a 77% match on *ATP1a1*, and an 85% match on *ATP1a2*. All qPCR primers amplified ~100-215 bp amplicons. Primers were designed to cross exon-exon borders, to ensure there was no amplification of genomic DNA. Primer specificities were first tested using standard PCR amplification and visualized on a 2% agarose gel. Specificity was further ensured by visualization of the amplicons' melting curves (StepOne™ Real-Time PCR System; Applied Biosystems™).

Primer efficiencies, which represent measurements of amplification efficiencies in qPCR reactions, were measured using a five-point 1:10 dilution series of cDNA combined from all tissues. A linear regression of the dilution curves was performed for each gene, and primer efficiencies were calculated using the following formula: PCR-efficiency = $10^{(-1/\text{slope})}$ (Pfaffl, 2001). Primer efficiencies were as follows: *ATP1a3* 87.21%, *L8* 82.94% and *18S* 92.27%.

We measured mRNA abundance in the tissues from which we were able to extract sufficient mRNA (n = 26) using qPCR. We loaded 10 µl duplicates of reaction mixtures (Maxima SYBR Green/ROX qPCR Master Mix, Thermo Scientific™) into 48-well optical plates (MicroAmp®, Applied Biosystems™). In addition to the standard qPCR

master mix, each reaction contained 10 ng of reverse transcribed cDNA. Reactions were run in duplicate on a StepOne™ Real-Time PCR System (Applied Biosystems™), using the following cycling protocol: step 1: 98°C for 10 mins (initial denature); step 2: 98°C for 15s, 60°C for 20s, 72°C for 30s (repeated for a total of 45 cycles); and step 3: melting curve analysis (to ensure amplification of only a single product). Inter-plate variation was 5.88% for *18S* amplification, 0.87% of *L8* amplification, and 3.6% for *ATP1a3* amplification.

2.5 Statistical analysis

Fold-increase was calculated from cycle threshold (Ct) values for each sample using the method of Pfaffl (2001), according to the following formula: ratio = $(E_{target})^{\Delta CP_{target}\,(control - sample)} / (E_{ref})^{\Delta CP_{ref}\,(control - sample)}$. When comparing fold-increase between different tissue types, calculations for each sample were normalized to the mean Ct values of all tissues for the target gene and the reference genes. When comparing fold-increase within tissue types, calculations for each sample were normalized to the controls of that tissue. The data did not meet the assumption of homogeneity of variance, and thus we used nonparametric analyses. Both untransformed and log-transformed data were analyzed, with identical statistical results, so log-transformed data are presented here to improve their graphic presentation. To test for differences in expression between tissues, we used the Kruskal-Wallis multiple comparison approach with Bonferroni corrections. To test for differences between control and BD-treated samples, we used independent Welch's t-tests (unequal variance two-sample t-tests) for each tissue type. Statistical analyses were performed using JMP 12.2.0 (SAS Institute Inc., 2015). Graphs were

generated using GraphPad Prism 6.0h (GraphPad software, 2015). The significance level was set at $\alpha < 0.05$ for all statistical comparisons unless when corrected for multiple comparisons with Bonferroni corrections, in which case alpha level was set at $\alpha < 0.0083$.

3. Results

To quantify differences in *ATP1a3* expression between heart, liver, gut (small intestine), and kidney tissues, we analyzed the expression levels relative to the average Ct values for all tissues combined, for the target and reference genes (Figure 3-1; 3-1). The Kruskal-Wallis test with Bonferroni corrections revealed significant differences between heart and gut (adjusted $p = 0.0486$), kidney (adjusted $p = 0.0258$), and liver (adjusted $p = 0.0348$), with heart expressing higher levels in all three comparisons.

To determine whether exposure to BDs causes upregulation of *ATP1a3* we compared expression between control and BD-treated samples for each tissue type (Figure 3-1). The Welch's t-tests revealed significant differences in the heart ($t(2) = 3.2085$, $p = 0.0495$) (Figure 3-1). There were no statistically significant changes in the levels of expression following injection of BD among gut ($t(2) = 0.9165$, $p = 0.4275$), kidney ($t(2) = 1.1635$, $p = 0.3382$), or liver ($t(3) = 0.2783$, $p = 0.7947$) (Figure 3-1).

4. Tables and Figures

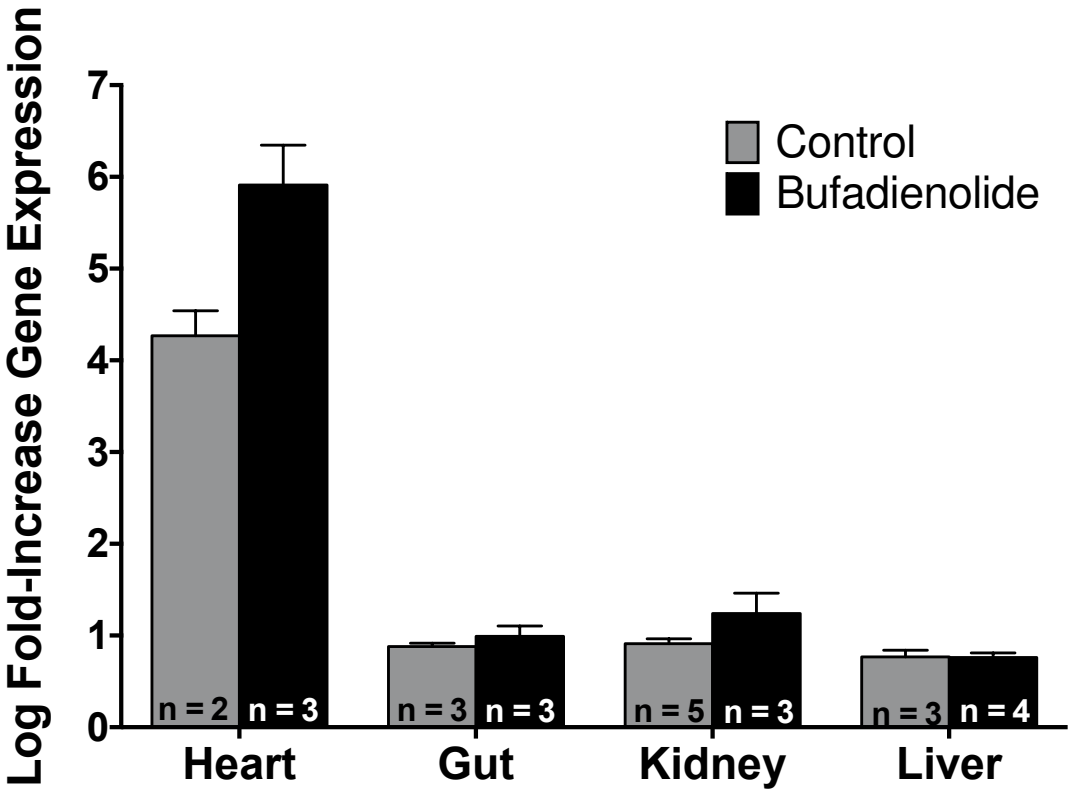

Figure 3-1. Change in *ATP1a3* expression in tissues of BD-treated snakes (DMSO + BD) compared to those of control snakes (DMSO only). Data for each tissue are normalized to the mean Ct value of all tissues combined. Heart exhibited significantly higher expression of *ATP1a3* compared to kidney (adjusted $p = 0.0258$), gut (adjusted $p = 0.0486$), and liver (adjusted $p = 0.0348$). The heart also exhibited significant upregulation in response to BD exposure of the snake ($t(2) = 3.2085$, $p = 0.0495$). Changes in gene expression were measured one hour after injection. Data are represented as mean ± SEM.

Table 3-1. List of snakes used in this study, with accompanying locality and morphometric data. GB denotes gamabufotalin.

ID	Species	Locality	Sex	Mass (g)	SVL (cm)	TL (cm)	Treatment Administered	Dose Time	Mass (GB) Administered (mg)	Dissection Time	Volume Injected (mL)
KM18	*Thamnophis elegans*	Utah - Cache Valley	M	37.8	54	69	5% DMSO	12:42	-	13:42	0.630
KM13	*Thamnophis elegans*	Utah - Cache Valley	M	20.7	47	62	5% DMSO	12:43	-	13:43	0.345
KM27	*Thamnophis elegans*	Utah - Cache Valley	M	25.7	50	61	5% DMSO	12:45	-	14:45	0.428
KM26	*Thamnophis elegans*	Utah - Cache Valley	M	23.8	48	59	5% DMSO	12:46	-	13:46	0.397
KM38	*Thamnophis elegans*	Utah - Cache Valley	M	29.8	51	66	5% DMSO	12:47	-	13:47	0.497
KM21	*Thamnophis elegans*	Utah - Cache Valley	M	22.4	45	59	5% DMSO	12:48	-	13:48	0.373
KM33	*Thamnophis elegans*	Utah - Cache Valley	M	13.5	41	51	0.149 M/g GB	12:50	1.350	13:50	0.225
KM31	*Thamnophis elegans*	Utah - Cache Valley	M	15.4	39	54	0.149 M/g GB	12:54	1.540	13:54	0.257
KM34	*Thamnophis elegans*	Utah - Cache Valley	M	16.8	45	58	0.149 M/g GB	12:55	1.680	13:55	0.280
KM35	*Thamnophis elegans*	Utah - Cache Valley	M	34.2	51	61	0.149 M/g GB	12:56	3.420	13:56	0.570
KM36	*Thamnophis elegans*	Utah - Cache Valley	M	29.9	56	70	0.149 M/g GB	12:58	2.990	13:58	0.498

5. Discussion

The results of this study provide preliminary insights into the regulatory patterns of *ATP1a3* in a BD-resistant snake. Specifically, we have found that the mutant *ATP1a3* gene is expressed at significantly higher levels in heart tissue than in kidney, gut, or liver tissues in the genetically BD-resistant snake, *T. elegans*. Furthermore, we have shown that BD exposure causes significant upregulation of *ATP1a3* mRNA in the heart. These results suggest that the heart is more strongly protected against BDs than the other tissues tested from *T. elegans*. Given the known effect of cardiotonic steroids on cardiac muscle, this is not a surprising result. Unfortunately, it is not known whether the other two NKA α–paralogs, *ATP1a1* and *ATP1a2*, also possess resistance-conferring mutations or how their levels of expression vary among tissues. We predict that other snakes that achieve BD resistance through target-site insensitivity of the NKA may share a similar tissue-specific pattern of *ATP1a3* gene expression as *T. elegans*. A recent upsurge in studies of the evolution of mutant NKAs in a wide range of organisms (Dobler et al., 2012; Zhen et al., 2012; Ujvari et al., 2013; Ujvari et al., 2015; Petschenka et al., 2013; Aardema and Andolfatto, 2016; Mohammadi et al., 2016b) has made this enzyme a model for the study of parallel and convergent evolution (Storz, 2016). However, in contrast to that strong focus on the molecular basis for cardiotonic steroid resistance (i.e., target-site insensitivity), there is a paucity of data on whether additional physiological mechanisms are involved in the resistance among vertebrates.

Although cardiotonic steroids target the NKAs of all cells, research on the effects of these compounds in mammalian systems has focused on their impacts on the heart (Licht and Low, 1968; Katz, 1969; Hirai et al., 1992; Fridman et al., 2002; Schoner and

Scheiner-Bobis, 2007). Furthermore, the discovery of endogenous cardiotonic steroids in mammals has shifted the view of these compounds from toxins to hormones that function in maintaining blood pressure and cardiac contractile homeostasis (Kelly and Smith, 1994; Schoner, 2002; Schoner et al., 2003; Schoner and Scheiner-Bobis, 2005; Schoner and Scheiner-Bobis, 2007). Accordingly, their toxic effects are often viewed now as a hormonal effect at levels 10-fold or higher than the physiological level. Evidence suggests that endogenous cardiotonic steroids in mammals are produced in the adrenal glands (Dimitrieva et al., 2000; Schoner and Scheiner-Bobis, 2007), and thus it is possible that they are transported throughout the body in a manner similar to that of other adrenal hormones. Binding globulins transport non-polar steroid hormones through the bloodstream to their target cells, where in some cases interactions with docking proteins cause them to release the steroids, which then interact with other membrane receptors (Rosner, 1990; Antolovic et al., 1998; Rosner et al., 1999; Schoner, 2002; Willnow and Nykjaer, 2010). Previous studies have shown that mammals possess a cardiotonic steroid-specific binding globulin, which binds to the steroids with high affinity and inhibits their function (Antolovic et al., 1998; Antolovic et al., 2000; Schoner et al., 2003). These binding globulins are produced at high concentrations in the kidneys, where they likely protect the NKAs of those tissues (Antolovic et al., 2003). We do not yet know whether snakes, like mammals, possess BD-specific binding globulins or endogenous cardiotonic steroids, much less the role they might play in the metabolism of BDs in snakes.

Beyond heart tissue, it is interesting that *ATP1a3* mRNA was not relatively abundant in the kidneys (Figure 3-1), where overall expression of the NKA is known to be greatest (Katz, 1982). The NKA plays a vital role in maintaining sodium homeostasis,

and thereby water balance, in all vertebrates (Ferrandi et al., 1996). We anticipate that *ATP1a1* and/or *ATP1a2*, which code for alternative paralogs of the NKA α-subunit, will be shown to exhibit relatively higher levels of expression in those tissues. Mammalian kidneys and liver primarily express *ATP1a1* (Orlowski and Lingrel, 1988), and recent evidence from insect NKAs suggests that mutations of the NKA may compromise the enzyme's functionality as an ion pump (Dalla and Dobler, 2016). It is possible that the mutations of *ATP1a3* in resistant snakes might also compromise the enzyme's ion pumping function, and thus may explain the low level of mutant *ATP1a3* mRNA in the kidneys of *T. elegans*, where functioning NKAs are vital. However, this hypothesis has yet to be tested in snakes.

Although our results did reveal a significant change in *ATP1a3* expression in the heart in response to BD exposure in snakes, it is likely that one hour following exposure was insufficient to elicit a full response. Ikeda et al. (1991) showed that the adrenal hormone aldosterone causes an increase in NKA gene expression that peaks at six hours after exposure in an in vitro preparation of rat heart tissue. Oguchi et al. (1993) found that expression in rat heart peaks 24 hours after aldosterone exposure in vitro. Although we do not know whether aldosterone stimulates expression of NKA in snakes, Mohammadi et al. 2017 showed that the Japanese snake *Rhabdophis tigrinus*, which sequesters BDs from its prey, responds to BD exposure with increased levels of circulating aldosterone. Furthermore, Mohammadi et al. (2013) showed that several highly specialized toad-eating snakes, including *R. tigrinus*, possess greatly enlarged adrenal glands. It is therefore possible that BD exposure in genetically resistant snakes elicits increased NKA expression in response to higher circulating levels of aldosterone. However, in order to

determine whether BDs play any role in mediating NKA regulation, it would be necessary to measure simultaneously the changes in NKA expression and circulating aldosterone for at least 48 hours after BD exposure.

The results of this experiment provide preliminary insights into the role of *ATP1a3* gene expression in BD resistance of a resistant snake and suggest that further exploration of such expression would be worthwhile. We have documented the relative abundance of the resistance-conferring *ATP1a3* mRNA in heart, liver, gut, and kidney tissues of a genetically BD-resistant snake and have compared the relative change in expression among tissues following exposure to a BD. Future studies should examine the expression of *ATP1a1* and *ATP1a2* under the same conditions, to determine the levels of expression of the other two α-paralogs, and should compare those values among a number of phylogenetically independent BD-resistant species.

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CHAPTER 4

TISSUE-SPECIFIC VARIATION IN CARDIOTONIC STEROID SENSITIVITY OF NA^+/K^+-ATPASE IN SNAKES

Cardiotonic steroids comprise a diverse group of compounds found in both animals and plants. Some species of plants, insects, and amphibians chemically defend themselves from predators with high concentrations of these compounds. Resistance to cardiotonic steroids in snakes that prey on chemically defended amphibians is conferred by amino acid substitutions in at least one of three Na^+/K^+-ATPase paralogs, the molecular target of the compounds. These substitutions lower the enzyme's binding affinity to cardiotonic steroids. Despite recent advances in our understanding of this adaptation, little is known about the physiological mechanisms that contribute to resistance in snakes. We have used an *in vitro* assay to determine the tissue-specific levels of sensitivity to cardiotonic steroids in resistant snakes. We measured sensitivities in brain, heart, liver, gut, and kidney, and we compared these sensitivities between resistant and non-resistant species. We found that the heart and kidney are the least sensitive tissues in resistant snakes, whereas brain, gut, and liver are sensitive to cardiotonic steroids. All tissues are sensitive in non-resistant species.

1. Introduction

Cardiotonic steroids comprise a diverse group of plant and animal-derived compounds that exert their effects by binding to and disabling the Na^+/K^+-ATPases (NKAs) of cell membranes (Krenn and Kopp, 1998; Steyn and van Heerden, 1998;

Barrueto et al., 2006; Laursen et al., 2015). The shared structural core of all cardiotonic steroids includes a steroidal skeleton with a lactone ring at C3 (Krenn and Kopp, 1998; Laursen et al., 2015; Tang et al., 2016). Bufadienolides and cardenolides comprise the two main classes, and they differ with respect to the number of carbons in the lactone ring (Krenn and Kopp, 1998; Tang et al., 2016). All bufadienolides possess a 6-membered lactone ring, whereas cardenolides exhibit a 5-membered ring (Krenn and Kopp, 1998; Laursen et al., 2015). Animal-derived compounds are typically nonglycosylated, whereas plant-derived compounds often are glycosylated (Laursen et al., 2015; Tang et al., 2016). These properties can have specific influences on the binding properties of NKA (Laursen et al., 2015). However, all forms of cardiotonic steroids dock similarly to a specific extracellular binding pocket formed by the αM1-6 transmembrane domains and extracellular loops of the NKA α-subunit (Laursen et al., 2015; Tang et al., 2016).

The NKA is a member of the P-type ATPase family, and it consists of a catalytic α subunit, which carries out the functional activities of the enzyme, and a regulatory β subunit, which provides structural stability (Bagrov et al., 2009). The roles of the NKA in physiological homeostasis are vast (reviewed by Bagrov et al., 2009). The enzyme continuously transports three Na^+ ions out of the cell through phosphorylation of ATP and two K^+ ions into the cell through dephosphorylation (Bagrov et al., 2009). This ion transport plays a vital role in the regulation of smooth and cardiac muscle contractions through the indirect maintenance of intracellular Ca^{2+} levels (Barry and Bridges, 1993; Dvela et al., 2007; Lichtstein et al., 2012). The presence of a cardiotonic steroid-specific binding pocket on the extracellular surface of the enzyme and the production of

endogenous cardiotonic steroids in mammalian adrenal glands indicate that these compounds have a physiological function in mammals (Dvela et al., 2007; Lichtstein et al., 2012).

The adaptations that allow select organisms, including many snakes, to tolerate normally lethal concentrations of cardiotonic steroids have been investigated widely in recent studies (Dobler et al., 2012; Zhen et al., 2012; Petschenka et al., 2012; Petschenka et al., 2013a; Mohammadi et al., 2013; Ujvari et al., 2013; Ujvari et al., 2015; Bramer et al., 2015; Mohammadi et al., 2016a; Mohammadi et al., 2017a; Mohammadi et al., 2017b). Genetic resistance to cardiotonic steroids in snakes has been attributed to the presence of two amino acid substitutions in the αM1-2 extracellular loop of NKA, which lower the binding affinity of the enzyme to the steroids (Ujvari et al., 2015; Mohammadi et al., 2016a). Snakes possess three NKA α-paralogs (Babonis et al., 2011; Castoe et al., 2011; Vonk et al., 2013). The resistance-conferring mutations have thus far only been identified in α-3 (*ATP1a3*) of the NKA (Ujvari et al., 2015; Mohammadi et al., 2016a). In a recent study, Mohammadi et al. (2017a) showed that the mutant *ATP1a3* paralog was expressed at significantly higher levels in the heart of a resistant species, *Thamnophis elegans*, as compared to gut, liver, and kidney. The presence of amino acid substitutions beyond the αM1-2 extracellular loop or in α-1 and α-2 remain to be investigated.

Although much attention has been focused on the molecular source of resistance, the physiological adaptations of resistance can involve diverse mechanisms beyond target-site insensitivity (Scudder and Meredith, 1982; Despres et al., 2007; Petschenka et al., 2013b; Mohammadi et al., 2017a; Mohammadi et al. 2017b). High expression levels of *ATP1a3* in the heart of resistant snakes suggest that cardiac tissue plays an important

role in mediating the toxic effects of cardiotonic steroids (Mohammadi et al., 2017b). Furthermore, the low levels of gene expression in the gut, liver, and kidneys suggest that alternate paralogs of the NKA might also possess resistance-conferring mutations or that other mechanisms may contribute to resistance. However, these possibilities have yet to be investigated.

Here we compare tissue-specific NKA sensitivities of brain, heart, liver, gut, and kidney to the cardenolide ouabain in three species of snakes, in order to gain a better understanding of the potential significance of each tissue in the physiological resistance of snakes. *Rhabdophis tigrinus* and *Thamnophis elegans* represent resistant species that possess target-site insensitivity (Ujvari et al., 2015; Mohammadi et al., 2016a). *R. tigrinus* has further evolved the ability to sequester and store dietary toxins in specialized structures known as nuchal glands (Hutchinson et al., 2007; Mori et al., 2012) and is thus compared separately. *Elaphe quadrivirgata* does not possess resistance-conferring mutations and is susceptible to the lethal effects of cardiotonic steroids (Mohammadi et al., 2017b). We hypothesized that tissue sensitivity to ouabain was dependent on the possession of resistance-conferring mutations in *ATP1a3* and on the distribution and concentration of the mutant NKA throughout various tissues. We predicted that (1) the tissues of *R. tigrinus* and *T. sirtalis* would be less sensitive to ouabain, whereas those of *E. quadrivirgata* would be highly sensitive; and (2) that the heart would be the least sensitive tissue in the resistant species.

2. Materials and methods

2.1. Study animals

Live snakes were collected from the field in Utah (Cache and Tooele Counties; permit no. 1COLL9134), Nebraska (Garden County; permit no. 477), and Japan (Kyoto, Okayama, Shizuoka, Shiga, Shimane, and Wakayama Prefectures; no permit required). All snakes were housed and used in accordance with Utah State University Institutional Animal Care and Use Committee (IACUC) regulations (protocol #2078), and with Kyoto University Graduate School of Science (protocol #H2605).

2.2. Tissue preparation

Tissue samples (brain, heart, liver, gut, and kidney) were dissected from snakes and immediately stored at -80°C. NKAs were extracted from tissue samples following the protocol described by Petschenka et al. (2012). In brief, tissue samples were thawed and homogenized on ice in a 1 mL all-glass grinder (Wheaton) with 500 µl distilled H2O. Homogenates were subjected to sonication for 15 minutes in a chilled bath. Thereafter, samples were centrifuged at 10,000 x g for 10 minutes to precipitate debris. The supernatants were ultracentrifuged at 84,000 x g for 30 minutes at 4°C to sediment the membranes (centrifuge: Beckman Coulter; Optima TLX; rotor: TLA-100.3). The supernatants were discarded and the microsomal pellets were washed three times in distilled H2O, and then resuspended in 500 µl of distilled H2O. Samples were sonicated in a chilled bath for 30 minutes to fully re-suspend the microsomal pellets. The extracts were stored at -20°C until assayed.

2.3. Na^+/K^+-ATPase assay

Tissue extracts were assayed following the protocol described by Petschenka et al. (2013a). In brief, each sample was incubated with exponentially increasing concentrations of the cardenolide ouabain (Acrōs Organics, Lot # A0343825). Ouabain is used as a surrogate for a bufadienolide in this assay due to its high polarity, a characteristic that is required for this assay and not shared by any commercially available bufadienolide. The activity of Na^+/K^+-ATPases following ouabain incubation was determined by quantification of inorganic phosphate released from enzymatically hydrolyzed ATP (Sigma Aldrich, lot # SLBB8329V). Reactions were performed in 96-well microplates (Fisherbrand), and absorbance at 700 nm was measured with a plate reader (BioRad Xmark spectrophotometer).

2.4. Statistical analysis

Absorbance values were converted to percentage non-inhibited activity based on control measurements (Petschenka et al., 2013a). These data were plotted and IC_{50} values were obtained from nonlinear fitting, using a four-parameter logistic curve (Equation 1), with the bottom asymptote set to zero.

$$\frac{Y = Bottom + (Top - Bottom)}{1 + 10^{(LogIC50 - X) * HillSlope}}$$

Equation 1. Four-parameter logistic curve equation, where Top and Bottom represent the plateaus in the units of the Y axis, the IC_{50} is the concentration of agonist that gives a response halfway between Top and Bottom, and HillSlope describes the steepness of the family of curves.

IC_{50} values were log transformed to better meet the assumptions of normality and homogeneity of variance and compared statistically using pairwise t tests. First, the IC_{50} differences between species were compared by tissue types (Table 2), then the IC_{50} differences between tissue types were compared by species (Table 3). P-values of multiple comparisons were adjusted using the Holm-Bonferroni method (Holm, 1979). All data were analyzed statistically with JMP software (Version 12.2.0, SAS Institute Inc.). Statistical significance was set at $\alpha = 0.05$. Results are represented as means ± S.E.

3. Results

Mean IC_{50} values for each independent group are listed in Table 4-1. Comparisons of tissue sensitivities to ouabain between species revealed that the resistant species, *R. tigrinus* and *T. elegans*, exhibited significantly higher IC_{50} values than the non-resistant *E. quadrivirgata* for heart and kidney tissues (Table 4-2; Fig. 4-1). There were no significant differences between the IC_{50} values of *T. elegans* and *R. tigrinus* in any tissues (Table 4-2).

Comparisons of tissue sensitivities to ouabain between tissues within species revealed that in *R. tigrinus*, the heart exhibited significantly higher IC_{50} values than the brain, gut, or liver, and the kidney displayed significantly higher IC_{50} values than the brain (Table 4-3; Fig. 4-2). *T. elegans* showed no significant differences between its tissues, and *E. quadrivirgata* exhibited significant differences between liver and kidney (Table 4-3; Fig. 4-2).

4. Tables and Figures

Table 4-1. Mean IC_{50} values for each species by tissue, and the corresponding number of replicates used in this study.

Tissue	Species	IC50 [M] ± SE	*n*
Kidney	*R. tigrinus*	$1.71 \times 10^{-2} \pm 8.24 \times 10^{-3}$	6
Kidney	*T. elegans*	$2.05 \times 10^{-2} \pm 3.0 \times 10^{-3}$	5
Kidney	*E. quadrivirgata*	$9.73 \times 10^{-6} \pm 8.31 \times 10^{-6}$	4
Heart	*R. tigrinus*	$1.66 \times 10^{-1} \pm 1.49 \times 10^{-1}$	8
Heart	*T. elegans*	$1.3 \times 10^{-2} \pm 4.36 \times 10^{-3}$	5
Heart	*E. quadrivirgata*	$5.6 \times 10^{-5} \pm 2.18 \times 10^{-5}$	4
Brain	*R. tigrinus*	$3.54 \times 10^{-4} \pm 1.6 \times 10^{-4}$	7
Brain	*T. elegans*	$3.38 \times 10^{-3} \pm 2.5 \times 10^{-3}$	6
Brain	*E. quadrivirgata*	$4.62 \times 10^{-4} \pm 4.2 \times 10^{-4}$	6
Gut	*R. tigrinus*	$4.69 \times 10^{-3} \pm 3.9 \times 10^{-3}$	7
Gut	*T. elegans*	$4.48 \times 10^{-3} \pm 2.15 \times 10^{-3}$	6
Gut	*E. quadrivirgata*	$1.95 \times 10^{-4} \pm 1.4 \times 10^{-4}$	5
Liver	*R. tigrinus*	$7.42 \times 10^{-3} \pm 4.84 \times 10^{-3}$	7
Liver	*T. elegans*	$1.03 \times 10^{-2} \pm 1.03 \times 10^{-2}$	2
Liver	*E. quadrivirgata*	$7.37 \times 10^{-4} \pm 4.6 \times 10^{-4}$	5

Table 4-2. Results of log IC_{50} t-test comparisons between species by tissues. P-values are adjusted with the Holm-Bonferroni correction. Comparisons that yielded significant differences are bolded. *R. tigrinus* and *T. elegans* represent species with cardiotonic steroid resistance-conferring mutations in *ATP1a3*, whereas *E. quadrivirgata* does not possess these mutations and is susceptible to cardiotonic steroids. These comparisons are illustrated in Fig. 4-1.

Tissue	Comparison	t value	df	Adjusted p-value
Kidney	***R. tigrinus* vs. *E. quadrivirgata***	**6.2632**	**12**	**0.0003**
Kidney	*R. tigrinus* vs. *T. elegans*	0.5384	12	0.6001
Kidney	***T. elegans* vs. *E. quadrivirgata***	**6.5128**	**12**	**0.0003**
Heart	***R. tigrinus* vs. *E. quadrivirgata***	**6.8333**	**14**	**0.0003**
Heart	*R. tigrinus* vs. *T. elegans*	-1.1129	14	0.2845
Heart	***T. elegans* vs. *E. quadrivirgata***	**5.292**	**14**	**0.0003**
Gut	*R. tigrinus* vs. *E. quadrivirgata*	2.0439	15	0.1178
Gut	*R. tigrinus* vs. *T. elegans*	0.6208	15	0.5440
Gut	*T. elegans* vs. *E. quadrivirgata*	2.5468	15	0.0669
Brain	*R. tigrinus* vs. *E. quadrivirgata*	1.2965	16	0.4246
Brain	*R. tigrinus* vs. *T. elegans*	0.9332	16	0.4246
Brain	*T. elegans* vs. *E. quadrivirgata*	2.1487	16	0.1419
Liver	*R. tigrinus* vs. *E. quadrivirgata*	0.3073	11	0.7643
Liver	*R. tigrinus* vs. *T. elegans*	-1.3462	11	0.6159
Liver	*T. elegans* vs. *E. quadrivirgata*	-1.0749	11	0.6159

Table 4-3. Results of log IC_{50} comparisons between tissues by species. P-values are adjusted with the Holm-Bonferroni correction. Comparisons that yielded significant differences are bolded. *R. tigrinus* and *T. elegans* represent species with cardiotonic steroid resistance-conferring mutations in *ATP1a3*, whereas *E. quadrivirgata* does not possess these mutations and is susceptible to cardiotonic steroids. These comparisons are illustrated in Fig. 4-2.

Species	Comparison	t value	df	Adjusted p-value
E. quadrivirgata	**liver vs. kidney**	**2.7588**	**19**	**0.1250**
E. quadrivirgata	heart vs. kidney	-1.8851	19	0.5984
E. quadrivirgata	liver vs. gut	1.9529	19	0.5913
E. quadrivirgata	brain vs. kidney	-1.7729	19	0.6461
E. quadrivirgata	heart vs. gut	1.0694	19	1.0000
E. quadrivirgata	liver vs. brain	1.1662	19	1.0000
E. quadrivirgata	gut vs. kidney	-0.9175	19	1.0000
E. quadrivirgata	brain vs. gut	-0.8734	19	1.0000
E. quadrivirgata	liver vs. heart	0.7717	19	1.0000
E. quadrivirgata	heart vs. brain	0.292	19	1.0000
R. tigrinus	**heart vs. brain**	**4.3002**	**30**	**0.0020**
R. tigrinus	**heart vs. gut**	**3.7845**	**30**	**0.0063**
R. tigrinus	**kidney vs. brain**	**3.2176**	**30**	**0.0248**
R. tigrinus	**heart vs. liver**	**-3.0116**	**30**	**0.0364**
R. tigrinus	kidney vs. gut	2.7379	30	0.0618
R. tigrinus	kidney vs. liver	-2.0189	30	0.2625
R. tigrinus	liver vs. brain	1.2476	30	0.8872
R. tigrinus	heart vs. kidney	-0.8062	30	1.0000
R. tigrinus	liver vs. gut	0.7483	30	1.0000
R. tigrinus	gut vs. brain	0.4993	30	1.0000
T. elegans	kidney vs. liver	-2.9554	19	0.0810
T. elegans	heart vs. liver	-2.6725	19	0.1359
T. elegans	gut vs. liver	-1.8779	19	0.6064
T. elegans	brain vs. liver	-1.6671	19	0.6714
T. elegans	kidney vs. brain	1.8355	19	0.6064
T. elegans	kidney vs. gut	1.5513	19	0.6865
T. elegans	heart vs. brain	1.4446	19	0.6865
T. elegans	heart vs. gut	1.1603	19	0.7809
T. elegans	kidney vs. heart	0.3474	19	1.0000
T. elegans	gut vs. brain	0.2981	19	1.0000

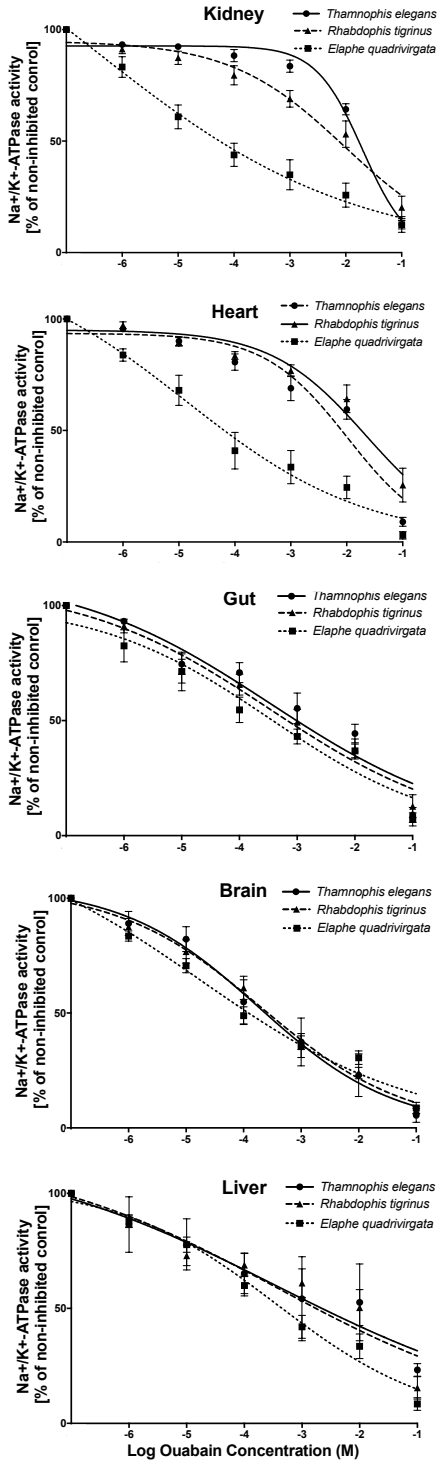

Fig. 4-1. Ouabain inhibition curves for the five tissues compared in this study. Each graph represents a comparison of tissue sensitivity for all species. Data points represent mean IC_{50} values ± S.E. See Table 4-2 for results of statistical comparisons of these data.

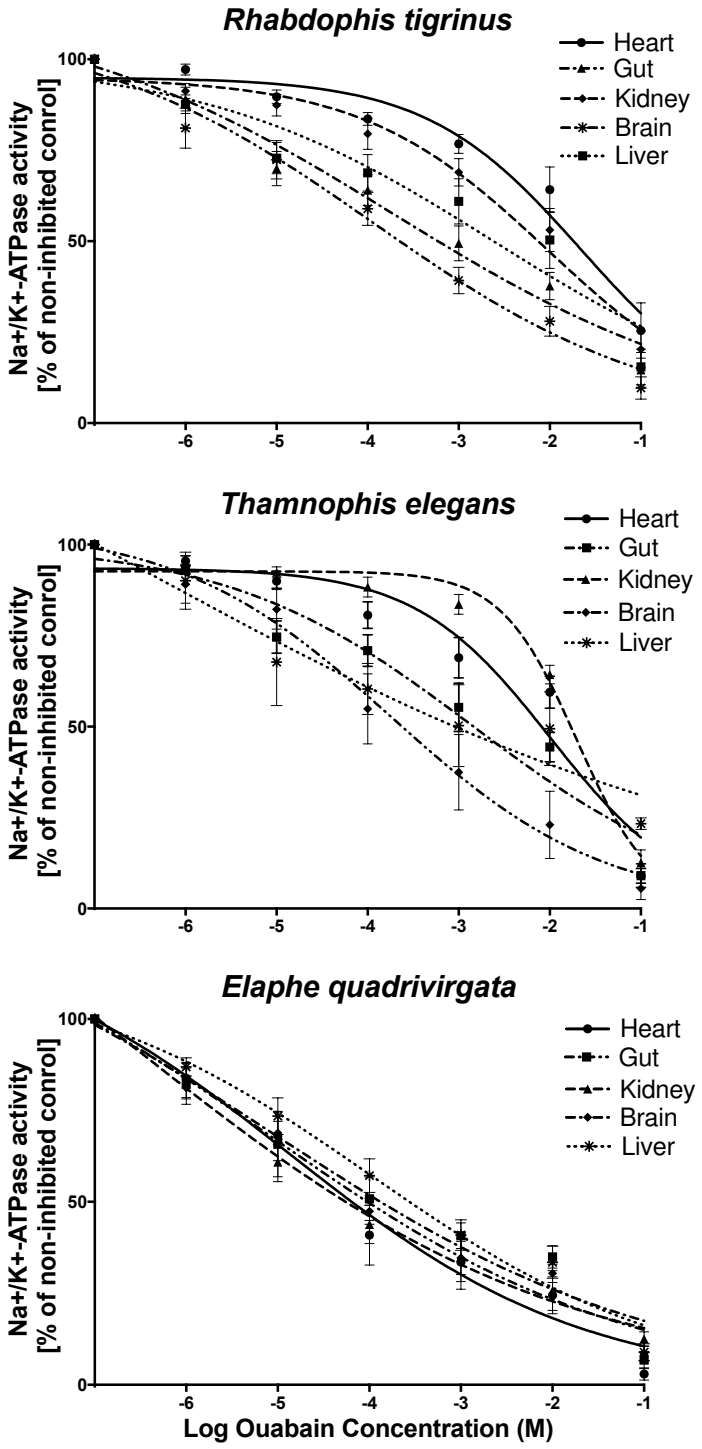

Fig. 4-2. Ouabain inhibition curves of the three species used in this study. Each graph represents a comparison of the different tissue sensitivities within a species. Data points represent mean IC_{50} values ± S.E. See Table 4-3 for results of statistical comparisons of these data.

5. Discussion

The results of this study provide a tissue-level physiological measurement of resistance in snakes that are and are not adapted to withstand lethal concentrations of cardiotonic steroids. As predicted we have found that in the non-resistant species, *E. quadrivirgata,* all tissues are sensitive to ouabain (Fig. 4-1 and 4-2). We also found that in the genetically resistant species, *R. tigrinus* and *T. sirtalis*, both the heart and the kidney are highly insensitive to ouabain (Table 4-1; Fig. 4-1). Since *T. elegans* is known to express significantly higher levels of the resistant NKA paralog, *ATP1a3,* in the heart compared to liver, gut, or kidney (Mohammadi et al., 2017a), it is not surprising that we observed low sensitivity in cardiac tissue. However, despite the comparatively low expression levels of *ATP1a3* in the kidney, we observed equally low sensitivity of kidneys to ouabain, compared to heart tissue, in the resistant species. However, the kidneys' importance in maintaining sodium balance in the body relies upon the activity of NKA (Ferrandi et al., 1996), which may require the tissue to maintain resistance against ingested cardiotonic steroids in snakes that feed on toads. Several hypotheses could explain the observed insensitivity of the kidney in spite of its low level of *ATP1a3* expression. The first is that an alternate paralog of NKA also possesses resistance-conferring mutations, and that this alternate paralog is expressed at high levels in kidneys. Another possibility is that the kidneys simply express very high levels of non-resistant NKA to compensate for those that are disabled by cardiotonic steroids. A recent investigation of the effects of resistance-conferring mutations on the ion transport activity of NKA in cardenolide-resistant insects showed that the same mutations that protect them from cardiotonic steroids ingested through their diet also compromise the enzyme's

efficiency (Dalla and Dobler, 2016). These insects compensate for the loss of function in their resistant NKA by co-expressing a non-mutated paralog.

Because the kidneys play an important role in the maintenance of sodium and potassium balance in the body, compromising the efficiency of NKA in that tissue can have negative physiological consequences (Ferrandi et al., 1996; Tipsmark and Madsen, 2003). The kidneys are also theoretically more susceptible to toxicity than most organs, due to the high rate of blood flow through the kidneys, even in a resting state, resulting in a higher rate of toxin delivery than to many other tissues (Hura and Stein, 2011). Furthermore, kidney secretion and diffusion processes can allow toxins to concentrate locally within the organs, further exacerbating the effects of toxins (Dantzler, 1992). Thus, they are likely one of the first organs to evolve insensitivity or resistance via natural selection.

High sensitivity of brain tissue in both non-resistant and resistant species is not surprising, due to the presence of a blood-brain barrier in reptiles (Shivers, 1979). That barrier protects brain tissue from exposure to circulating toxins, including ingested cardiotonic steroids (Shivers, 1979; Abbott et al., 2010). Thus, there would be no selective pressure to protect the NKA in those tissues from such toxins.

Perhaps the most interesting result of this study was the relatively high sensitivities of the gut and liver compared to the heart and kidney in resistant species (Tables 4-1 and 4-3; Fig. 4-2). The variation in sensitivity between different tissues within a resistant snake indicates the presence of complex physiological mechanisms involved in the uptake, transport, and metabolism of dietary cardiotonic steroids. One explanation could be related to how cardiotonic steroids are transported in the body. We

know that cardiotonic steroids are produced from cholesterol in the adrenal glands of mammals (Dmitrieva et al., 2000). As such, it is likely that these compounds are transported throughout the circulatory system in a manner similar to the transport of other adrenal steroid hormones.

Corticosteroid binding globulins transport steroid hormones through the bloodstream (Dunn et al., 1981; Rosner, 1990; Antolovic et al., 1998; Rosner et al., 1999; Schoner, 2002). Mammals possess a cardiotonic steroid-specific binding globulin that binds to the steroids with high affinity, inhibiting their function (Antolovic et al., 1998; Antolovic et al., 2000; Schoner et al., 2003). Interestingly, these binding globulins are produced at high concentrations in the kidneys of mammals, where they likely protect the NKAs of those tissues from endogenous cardiotonic steroids (Antolovic et al., 2000). We have no knowledge of any analogous mechanisms in reptiles, and further work is needed to determine whether protection of tissues via sequestration of bufadienolides occurs in snakes.

One final relevant outcome of these results involves the biphasic character of sensitivity curves. All species exhibited evidence of a biphasic response to ouabain in the heart, gut, and liver as indicated by the discontinuous decline in NKA activity of the data points (Fig. 4-1 and 4-2). The discontinuous decline indicates the co-existence of at least two forms of NKA with different ouabain sensitivities in these tissues (Blanco and Mercer, 1998). However, further work is necessary to confirm whether multiple forms of NKA do indeed occur in these tissues.

Although the results of this study provide important information on the physiological mechanisms of cardiotonic steroid resistance in snakes, they present only a

superficial view of what increasingly appears to be a complex adaptive system. Future work should aim to determine the routes of uptake, metabolism, and excretion of ingested bufadienolides in order to better understand the physiology of this dietary adaptation in snakes.

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CHAPTER 5

CORTICOSTEROID RESPONSES OF SNAKES TO TOXINS FROM TOADS (BOFADIENOLIDES) AND PLANTS (CARDENOLIDES) REFLECT DIFFERENCES IN DIETARY SPECIALIZATIONS

Toads are chemically defended by cardiotonic steroids known as bufadienolides. Resistance to the acute effects of bufadienolides in snakes that prey on toads is conferred by target-site insensitivity of the toxin's target enzyme, the Na^+/K^+-ATPase. Previous studies have focused largely on the molecular mechanisms of resistance but have not investigated the physiological mechanisms or consequences of exposure to the toxins. Adrenal enlargement in snakes often is associated with specialization on a diet of toads. These endocrine glands are partly composed of interrenal tissue, which produces the corticosteroids corticosterone and aldosterone. Corticosterone is the main hormone released in response to stress in reptiles, and aldosterone plays an important role in maintaining ion balance through upregulation of Na^+/K^+-ATPase. We tested the endocrine response of select species of snakes to acute cardiotonic steroid exposure by measuring circulating aldosterone and corticosterone concentrations. We found that *Rhabdophis tigrinus*, which specializes on a diet of toads, responds with lower corticosterone and higher aldosterone compared to other species that exhibit target-site resistance to the toxins but do not specialize on toads. We also found differences between sexes in *R. tigrinus*, with males generally responding with higher corticosterone and aldosterone than females. This study provides evidence of physiological adaptations, beyond target-site resistance, associated with tolerance of bufadienolides in a specialized toad-eating snake.

1. Introduction

Toads (Bufonidae) are defended by potent cardiotonic steroid toxins known as bufadienolides (BDs), which are synthesized from cholesterol in their cutaneous glands and stored there in high concentrations (Krenn and Kopp, 1998). Those toxins protect toads from most predators, including many snakes that feed readily on other groups of frogs. Nonetheless, bufophagy (toad-eating) has evolved in several phylogenetically distant lineages of snakes (Mohammadi et al., 2016a; Ujvari et al., 2015; Mohammadi et al., 2013), which have evolved the ability to tolerate the normally lethal BDs of toads (Mohammadi et al., 2016b). At least one of those lineages has also evolved the ability to sequester, or store, those toxins for use in its own defense (Mori, 2012; Hutchinson et al., 2012).

Cardenolides (CDs) are another class of cardiotonic steroid and are primarily produced from sterols in plants (Agrawal et al., 2012). As with BDs, certain animals have evolved resistance to CDs, and a number of insect species can sequester the compounds for use in their own defense (reviewed in Opitz and Müller, 2009). Although the molecular structures of BDs and CDs are similar (Fig. 5-1; Córdova et al., 2016; Agrawal et al., 2012), there is evidence that the two classes of compounds have differing degrees of toxicity, at least in mammals (Barrueto et al., 2006). Both BDs and CDs exert their toxic effects by binding to an extracellular pocket of the membrane-bound enzyme Na^+/K^+-ATPase, rendering the molecule nonfunctional (Laurson et al., 2015; Schoner and Scheiner-Bobis, 2007; Krenn and Kopp, 1998). Furthermore, the discovery of endogenous CDs and BDs in mammals has shed new light on the significance of these

compounds, and the endogenous compounds are regarded as serving a hormonal function in the regulation of blood pressure (Dmitrieva et al. 2000; Dvela et al., 2007).

Comparisons of genetic sequences coding for the binding pocket have revealed that lowered affinity to binding of BDs in reptiles is conferred by at least two co-occurring amino acid substitutions on the first extracellular loop of the Na^+/K^+-ATPase (Ujvari et al., 2015). However, the presence of those amino acid substitutions is widespread among snakes (Mohammadi et al., 2016a), suggesting that target-site insensitivity may not be the only adaptation that confers resistance in snakes that prey heavily on toads. Insects that consume CDs exhibit several mechanisms of resistance, including detoxification by enzymes, excretion, and exclusion, in addition to target-site insensitivity conferred by mutations (Petschenka et al., 2013a; Petschenka et al., 2013b; Després et al., 2007; Scudder and Meredith, 1982). It is likely that a high level of bufophagy also requires more than one mechanism of resistance. Consumption of highly toxic prey is advantageous to a predator because it provides access to an underutilized dietary resource and in some cases permits sequestered chemical defense (Hutchinson et al., 2007; Darst et al., 2005; Nishida 2002). However, those advantages may be accompanied by the physiological stress of metabolizing the toxins, or they might require upregulation of the target proteins, perhaps mediated by hormones. To understand the origin and retention of an adaptation such as bufophagy, it is essential to determine whether there are physiological trade-offs associated with expression of the adapted phenotype. Therefore, it is necessary to combine genetic screens with physiological assays. This can be achieved by comparing physiological responses to toxin exposure in species that do and do not exhibit the dietary adaptation.

Vertebrates possess a suite of physiological responses to external challenges (Neuman-Lee et al., 2016a; Love and Williams, 2008; McEwen, 2007; Råberg et al., 1998), and the hypothalamic-pituitary-adrenal (HPA) axis constitutes one of the most conserved mechanisms underlying these responses (Wingfield, 2005; Wingfield and Romero, 2001). The HPA axis interacts with other physiological systems by activating and/or suppressing a physiological cascade that stimulates hormonal secretion from the adrenal glands (Dhabhar, 2009; Norris, 2006; Sapolsky et al., 2000). Because enlargement of the adrenals is associated with bufophagy in several independent lineages of snakes (Mohammadi et al., 2013), investigation of the adrenal response to BD consumption is warranted. Adrenal enlargement in some vertebrates has been linked to chronic stress, which results in hypertrophy and increased activity of the tissues that produce stress-related corticosteroids (Ulrich-Lai et al., 2006; Fickess, 1963). Adrenal tissues of amniotes consist of chromaffin cells, which produce catecholamines, and interrenal cells, which produce glucocorticoids and mineralocorticoids (Norris, 2006). These hormones may play a role in offsetting the toxic effects of cardiotonic steroid ingestion. As such, enlargement of the adrenal glands would be beneficial by allowing higher-level production of these hormones. It is not yet known which of the adrenal tissues is responsible for the overall enlargement of the glands in bufophagous snakes, although the effect of BDs on Na^+/K^+-ATPase suggests that the interrenal tissue is a likely candidate.

Corticosterone is the primary glucocorticoid metabolite in reptiles (Norris, 2006; Moore & Jessop 2003), and it plays important roles in mediating trade-offs during the stress response (Sapolsky et al., 2000). Accordingly, plasma corticosterone levels are

used as a standard hormonal measure of organismal stress in a wide range of experiments (Hopkins et al., 2016; French, 2008, 2007; Moore et al., 2000). Aldosterone is the primary mineralocorticoid hormone and is essential for maintaining sodium concentration, water balance, and blood pressure (Weber, 2001). The role of aldosterone as a signal for the expression of Na^+/K^+-ATPase has been widely investigated (Summa et al., 2001; Ahmad and Medford, 1995; Ewart and Klip, 1995; Oguchi et al., 1993; Ikeda et al., 1991; Verrey et al., 1987). This role suggests that adrenal enlargement in bufophagous snakes might be a result of increased aldosterone production, which could play a compensatory role in counteracting BDs by upregulating resistant Na^+/K^+-ATPases. Indeed, significant upregulation of resistant Na^+/K^+-ATPases has been observed in the cardiac tissues of snakes that were exposed to BDs (Mohammadi et al., 2017).

Diverse bufophagous snakes have also been found to possess adrenals that are not only enlarged but also sexually dimorphic in size, with males possessing significantly larger adrenal glands than females (Mohammadi et al., 2013). This pattern of sexual dimorphism has not been observed in snakes with non-toad-specialized diets (Mohammadi et al., 2013). To date, there is no explanation for this dimorphism, although Mohammadi et al. (2013) suggested that female bufophagous snakes might be provisioning BDs to their offspring, thereby reducing the degree to which the tissues of females are exposed to BDs. At least one species, *Rhabdophis tigrinus*, is known to provision its embryos with BDs (Hutchinson et al., 2008).

Here we compare acute hormonal responses to cardiotonic steroid exposure in select species of snakes representing three groups, classified by the amount of

physiological BD exposure the group typically encounters in the wild: highly bufophagous and BD-resistant (BR), generalist predator and BD-resistant (GR), and non-bufophagous and genetically non-resistant (NN). We measured circulating corticosterone and aldosterone levels following exposure to either a low dose of a BD (gamabufotalin), a low dose of a CD (ouabain), or a high dose of the BD. We designed this study to be both exploratory and, in part, hypothesis-driven. Our predictions are based on three main hypotheses: (1) corticosterone and aldosterone responses differ based on the degree of dietary specialization on toads; (2) responses differ between sex in highly bufophagous snakes; and (3) BDs and CDs elicit different responses in all three dietary groups. We predicted that corticosterone would be elevated in those snakes that are non-resistant relative to those resistant taxa, and that aldosterone would be elevated in the species that most frequently ingests toads. We also predicted that the aldosterone levels would be elevated in males compared to females in the toad-specialized species. Finally, we predicted that BDs and CDs would elicit different hormonal responses in all taxa.

2. Materials and Methods

2.1 Study species and functional groups

The six species used in this study were assigned to three groups according to their diets and their genetic tolerance of toad toxins. Logistic constraints on the availability of specimens precluded sufficient sample size for testing each species separately, so species were pooled into functional groups for analysis. Limits on the availability of species that could be collected from the wild also precluded replication of one of these functional

groups. Nonetheless, species was treated as a random-effects factor in the statistical analysis. *Rhabdophis tigrinus* was categorized as bufophagous and genetically resistant (BR). Although bufonids comprise a relatively small proportion of the species' diet (Kojima and Mori, 2015), *R. tigrinus* sequesters BDs from toads for use in its own defense, thereby exposing at least some of its tissues to BDs for an extended period (Hutchinson et al., 2007). Furthermore, females of this species are known to forage preferentially for toads during the season when vitellogenesis occurs (Kojima and Mori, 2015). The generalist and resistant group (GR) included the congeners *Thamnophis sirtalis*, *T. elegans*, and *T. radix*. *T. sirtalis* and *T. elegans* occasionally include toads in their varied diets, whereas *T. radix* primarily consumes earthworms (Tuttle and Gregory, 2009; Dalrymple and Reichenbach, 1981; Kephart and Arnold, 1982; Hart, 1979; Arnold, 1978; Gregory and Stewart, 1975). All species, however, possess mutations of the Na^+/K^+-ATPase gene that confers target-site resistance (Mohammadi et al., 2016a). The BD-susceptible species *Pituophis catenifer* and *Elaphe quadrivirgata*, which prey primarily on mammals and non-bufonid anurans, respectively (Mori and Moriguchi, 1988; Rodríguez- Robles, 2002; Hamanaka et al., 2014), lack the mutations that confer target-site insensitivity and thus were classified as non-bufophagous and non-resistant (NN). A total of 89 snakes were used in this study, although missing data reduced sample sizes for specific analyses (Tables 5-7 and 5-8).

2.2. Snake collection and animal care

Live snakes were collected from the field in Utah (Cache and Tooele Counties; permit no. 1COLL9134), Nebraska (Garden County; permit no. 477), and Japan (Kyoto, Okayama, Shizuoka, Shiga, Shimane, and Wakayama Prefectures; no permit required) (Table 5-7). Snakes were cared for and handled in accordance with Utah State University Institutional Animal Care and Use Committee (IACUC) regulations (protocol #2078), and with Kyoto University Graduate School of Science procedures (protocol #H2605). Snakes from Utah and Nebraska were brought to Utah State University (Logan, Utah, USA), housed individually in 37.8 L glass terraria with newspaper substrate, and provided with water *ad libitum* and a hide shelter. Snakes from Japan were brought to Kyoto University (Kyoto, Kyoto Prefecture, Japan) and housed separately in plastic terraria (18.9 L) with newspaper substrate, water *ad libitum*, and a hide shelter. *Rhabdophis tigrinus* and *Elaphe quadrivirgata* were fed frogs (*Pelophylax nigromaculatus*) collected from the field. *R. tigrinus* were fed twice per week due to this species' unusually rapid starvation rate (unpublished), whereas *E. quadrivirgata* were fed once per week. All other snakes were fed commercially purchased thawed mice (RodentPro.com, LLC) once per week.

2.3. Toxin challenges: hormonal responses to bufadienolide and cardenolide exposure

Body mass of each snake was measured one week prior to the start of the experiment and was used for calculations of mass-adjusted dosage. Individual BR and GR snakes were randomly assigned to one of three doses, regardless of sex: 1.49×10^{-2} M/g BD, 1.49×10^{-2} M/g CD, or 1.49×10^{-1} M/g BD, and NN snakes were randomly assigned to one of two doses: 1.49×10^{-2} M/g BD or 1.49×10^{-2} M/g CD. The 1.49×10^{-2} M/g

dose was selected because it represents the minimum lethal concentration for non-resistant snakes (Mohammadi et al., 2016b), and we were interested in comparing physiological effects between resistant and non-resistant species groups. A 10-fold higher dose, $1.49x10^{-1}$ M/g BD, was administered only to the two resistant species groups, to approximate more closely the amount of BD a snake might encounter from consumption of a whole toad (Mohammadi et al., 2016b; Córdova et al., 2016; Hutchinson et al., 2012; Zelnik, 1965).

A total of 89 adult snakes (Table 5-7; 34 females and 55 males) were tested two times in a repeated measures design, first for a control challenge and later for a treatment challenge (these constituted two levels of response measurements in the experimental design). Snakes were left in their respective terraria in a quiet room to acclimate undisturbed, except when fed, for one week. Following the acclimation week, control dose challenges were administered. Each snake was removed from its cage, and blood was collected from the caudal vein within three minutes (Romero and Butler, 2007) for measurement of baseline hormone levels. Immediately following the blood draw, the snake was administered a mass-adjusted intraperitoneal injection (delivered to the posterior region of the peritoneal cavity) of 5% dimethyl sulfoxide (DMSO; Fisher Scientific, Inc.) and then returned to its cage. DMSO served as the solvent for subsequent BD and CD doses and therefore represented the control challenge (Moon et al., 2004; Santos et al., 2003). Following the injection, the snakes were observed visually for 90 minutes, for signs of a behavioral response. At 30 minutes post-injection (Neuman-Lee et al., 2016a; Romero and Butler, 2007) the snake was removed from its cage a second time, and blood was collected from the caudal vein for a measurement of response levels of

hormones. We note that the temporal scale of the blood sampling limited our ability to identify differences in temporal responses to the stressors between different species. Due to the exploratory nature of this study, we opted to use a single standardized time for comparisons across species groups. All blood samples were stored on ice for less than two hours. Plasma was then separated from blood cells by centrifugation and stored at -20°C until assayed. Following the control dose challenge, snakes were left to re-acclimate in their terraria for an additional week. Following the re-acclimation week, the procedures were repeated with an injection of one of the three treatments, dissolved in 5% DMSO. Snakes were administered either $1.49x10^{-2}$ M/g of a commercially available BD, gamabufotalin (PiChemicals ®, Lot # PI201406082068 and # PI1201206031583); a $1.49x10^{-2}$ M/g dose of a commercially available CD, ouabain (Acrōs Organics, Lot # A0343825); or a $1.49x10^{-1}$ M/g dose of gamabufotalin. The $1.49x10^{-2}$ and $1.49x10^{-1}$ M/g doses will henceforth be referred to as the LOW and the HIGH doses, respectively.

2.4. Hormone assay

Circulating corticosterone concentrations were determined by radioimmunoassay (RIA), using a previously described and validated method (Neuman-Lee et al., 2015; French et al., 2010). Circulating aldosterone concentrations were determined using a commercially purchased ELISA kit (Enzo Life Science Aldosterone ELISA, Lot # 05151502B, 06231403, 06231403B, and 06231403C). Limited plasma volume resulted in the omission of three snakes (Table 5-7) from the corticosterone assay and 47 snakes from the aldosterone assay (see Table 5-7). Thus, of the 89 total snakes studied, 86

samples (33 females and 53 males) were assayed for corticosterone and 52 (24 females and 28 males) were assayed for aldosterone.

Steroid hormones were extracted from samples using 30% ethyl acetate:isooctane, dried, and re-suspended in a buffer solution. Samples were assayed in duplicate for both corticosterone (MP Biomedicals, Lot # 3R3PB-19E) and aldosterone. The averages of the duplicates were used as the final value for statistical analyses. For the corticosterone RIA, the antibody cross-reactivity for corticosterone was 100%, with the next highest cross-reactivity (deoxycorticosterone) occurring at 2.3% and all others <0.05%. The aldosterone ELISA cross-reactivity for aldosterone was 100%, with the next highest cross-reactivity (11-deoxycorticosterone) at 0.3% and all others <0.2%. We validated the ELISA with plasma samples and found a linearity of $R^2 = 0.966$, and we tested for interference caused by samples with known quantities of aldosterone (5 pg/ml, 50 pg/ml, 150 pg/ml). For each assay, we used aliquots of known hormone concentration to measure extraction recoveries. These recoveries were used to adjust final sample concentration values for any losses during the extraction procedures. Standards of known value and negative controls were included in every assay as references, to ensure accuracy. The mean intra-assay variation for corticosterone was 12.1% and accuracy was 108.9%. The mean intra-assay variation for aldosterone was 8.05%, and accuracy was 103.4%.

2.5. Statistical analyses

One outlier (SM134; Table 5-7) was removed from the analyses because its baseline corticosterone values were abnormally high, and it was likely experiencing

physiological distress from an unknown source. Hormone measurements of 85 snakes (33 females and 52 males) were thus used in the statistical analyses of corticosterone. Fifteen of these snakes (11 females and 4 males) were treated with HIGH BD and analyzed separately. Hormone measurements from 52 snakes (24 females and 28 males) were used in the statistical analyses of aldosterone. Sixteen of these snakes (10 females and 6 males) were treated with HIGH BD dose and analyzed separately. We calculated the Pearson's correlation between baseline corticosterone and aldosterone levels and between response corticosterone and aldosterone levels, from plasma samples that were assayed for both hormones, to determine whether changes in circulating levels of one hormone influenced the other. There was a positive relationship between baseline corticosterone and aldosterone levels ($r^2 = 0.45$, $P = 0.0009$, n = 49, CIs = 0.20-0.65). However, there was no relationship between response corticosterone and aldosterone levels ($r^2 = 0.23$, $P = 0.1012$, n = 49, CIs = -0.04-0.48). We therefore concluded that changes in circulating hormone levels in response to treatments were independent of one another.

Baseline (i.e., pre-toxin injection) and response (i.e., post-toxin injection) hormone concentrations were log-transformed prior to analysis, to better meet assumptions of normality, homogeneity of variance, and linearity. The effects on circulating hormone concentrations in three groups of snakes of two toxins relative to solvent (5% DMSO) controls were assessed using an analysis of covariance in a split-plot design. Species group (BR, GR, or NN), treatment (LOW ouabain or gamabufotalin), sex (male or female) and challenge level (control or treatment) were incorporated as categorical fixed-effects factors. Due to low sample size and unbalanced sex distribution, sex was not included in the model for the analysis of aldosterone response to

gamabufotalin and ouabain. Individual snakes, nested within species, comprised the random-effects factor (i.e., experimental unit) for species group, treatment, and sex. Repeated measures on each snake comprised the random-effects factor associated with challenge level. Baseline hormone concentration prior to injection in each level was included as a fixed effects covariate to adjust for variable baseline levels among snakes. Using the ordinary least square method, we regressed mass against snout-vent-length for each species group and used the output residuals as body condition indices (Schulte-Hostedde et al., 2005). Body condition indices were included as a fixed effects covariate to adjust for body condition variation among snakes. There was no evidence for interactions of mass with other fixed factors when included in the model, so we report here on a more parsimonious model that does not include those interactions. We used pre-planned contrasts with -Bonferroni corrections (Holm, 1979) when necessary to estimate the effects of treatment, relative to control, within each species group and between species groups.

The models used to analyze corticosterone and aldosterone responses to HIGH gamabufotalin included only species group and challenge level as categorical fixed effects factors. Low sample size and unbalanced sex distribution precluded the inclusion of sex in these models. Other model details were as described above.

Significance level was set at $\alpha = 0.05$. All statistical analyses were performed using JMP 12.2.0 (SAS Institute Inc., 2015).

3. Results

3.1. Behavioral responses to treatment challenges

All NN snakes challenged with LOW gamabufotalin displayed mouth-gaping and muscular tetany, followed by death within 40 minutes of dose administration. Similar responses were observed in NN snakes challenged with LOW ouabain, although the onset of these symptoms was delayed by approximately 30 minutes. Conversely, no BR snakes challenged with gamabufotalin or ouabain showed any behavioral signs of distress at any time following dose administration during the 90 minutes that they were monitored. Similarly, no GR snakes showed behavioral signs of distress in response to the gamabufotalin challenge. However, although all GR snakes survived the ouabain challenge, they reacted with occasional mouth gaping, body inversion, and cloacal eversion for the full 90 minutes of monitoring following exposure.

3.2 Corticosterone response to LOW gamabufotalin and ouabain challenges

There were significant effects of species group, treatment, challenge level, sex, and the interaction of species group and treatment on corticosterone responses, and contrasts of factors revealed significant differences between several comparisons (see Tables 51 and 52 for a summary of these results). GR snakes had a higher corticosterone response than the other two species groups, which were not statistically distinguishable. Gamabufotalin elicited a stronger corticosterone response overall than did ouabain. The treatment challenge level elicited a stronger corticosterone response than the control challenge level. BR males had a higher corticosterone response than did females. See Figures 5-2A and 5-2B and 3 for graphs of raw data represented by this analysis.

3.3. Corticosterone response to HIGH gamabufotalin challenge (resistant species groups only)

There was a significant effect of species group on corticosterone response to the higher gamabufotalin dose (Table 5-3), with GR snakes having a higher response than BR snakes. There was no significant effect of challenge level or the interaction of challenge level and species group. See Figure 5-2C for raw data represented by this analysis.

3.4. Aldosterone response to LOW gamabufotalin and ouabain challenges

There was a significant effect of species group on aldosterone response (Tables 5-4 and 5-5), with BR snakes having a higher response than GR or NN snakes. However, there were no significant effects of treatment, challenge level, sex, or the interactions of these factors on aldosterone response (Table 5-4). See Figures 5-4A and 5-4B and 5-5 for graphs of raw data represented by this analysis.

3.5. Aldosterone response to HIGH gamabufotalin challenge (resistant species groups only)

There was a significant effect of species group on aldosterone response to the higher dose of gamabufotalin (Table 5-6), with BR snakes having a higher response. There were no significant effects of challenge level or interactions of species group with challenge level. See Figure 5-5 for raw data represented by this analysis.

4. Tables and Figures

Table 5-1. Summary of ANCOVA results for the analysis of LOW doses of gamabufotalin and ouabain on corticosterone response. Factors and interactions of factors that had significant effects on corticosterone response are bolded.

Effect Tests	**df**	**F ratio**	**p-value**
baseline	**1, 112**	**15.5405**	**0.0001**
body condition	1, 60	0.0018	0.9662
species group	**2, 56**	**18.8195**	**<0.0001**
treatment	**1, 57**	**7.7644**	**0.0072**
challenge level	**1, 64**	**10.7958**	**0.0017**
sex	**1, 61**	**9.7322**	**0.0027**
species group*treatment	**2, 56**	**3.6123**	**0.0335**
species group*challenge level	2, 65	2.413	0.0975
species group*sex	2, 58	2.2569	0.1137
treatment*challenge level	1, 63	2.2937	0.1349
treatment*sex	1, 55	3.7673	0.0573
species group*treatment*challenge level	2, 65	2.2255	0.1162
species group*treatment*sex	2, 56	0.539	0.5863

Table 5-2. Summary of contrasts for the analysis of LOW doses of gamabufotalin and ouabain on corticosterone response. Patterns of interest observed in raw data prompted contrasts of species group* treatment*challenge level despite non-significant p-value for the interaction effect (see Table 1). P-values for contrast comparisons were adjusted using Holm-Bonferroni corrections. Contrast comparisons that were significantly different are bolded.

Contrasts	adjusted p-value
BR vs. GR	**<.0001**
BR vs. NN	0.1948
NN vs. GR	**<.0001**
BR – gamabufotalin vs. ouabain	0.2958
GR – gamabufotalin vs. ouabain	0.6336
NN – gamabufotalin vs. ouabain	**0.0003**
gamabufotalin – BR vs. NN	**0.0012**
gamabufotalin – BR vs. GR	**<0.0001**
gamabufotalin – GR vs. NN	**0.0484**
ouabain – BR vs. NN	0.5640
ouabain – BR vs. GR	**0.0120**
ouabain – GR vs. NN	**0.0002**
BR – males vs. females	**0.0132**
BR – gamabufotalin control vs. treatment	1.0000
BR – ouabain control vs. treatment	1.0000
GR – gamabufotalin control vs. treatment	1.0000
GR – ouabain control vs. treatment	**0.0001**
NN – gamabufotalin control vs. treatment	0.9256
NN – ouabain control vs. treatment	0.1940

Table 5-3. Summary of ANCOVA results for the analysis of HIGH doses of gamabufotalin on corticosterone response. Factors that had significant effects on corticosterone response are bolded.

Effect Tests	df	F ratio	p-value
baseline	**1, 16**	**59.6986**	**<.0001**
body condition	1, 11	2.225	0.1648
species group	**1, 11**	**11.276**	**0.0067**
challenge level	**1, 13**	3.5542	0.0819
species group*challenge level	1, 13	0.107	0.7489

Table 5-4. Summary of ANCOVA results for the analysis of LOW doses of gamabufotalin and ouabain on aldosterone response. Factors and interactions of factors that had significant effects on corticosterone response are bolded.

Effect Tests	df	F ratio	p-value
baseline	**1, 45**	**9.8024**	**0.0031**
body condition	**1, 26**	**8.0125**	**0.0088**
species group	**2, 26**	**8.0471**	**0.0019**
treatment	1, 25	0.8467	0.3662
challenge level	1, 35	0.3289	0.5700
sex	1, 25	0.3133	0.5806
species group*treatment	2, 26	0.9804	0.3888
species group*challenge level	2, 35	0.6090	0.5496
species group*sex	2, 26	0.3874	0.6826
treatment*challenge level	1, 35	0.0142	0.9058
species group*treatment*challenge level	2, 34	0.4159	0.6631

Table 5-5. Summary of contrasts for the analysis of LOW doses of gamabufotalin and ouabain on aldosterone response. P-values for contrast comparisons were adjusted using Holm-Bonferroni corrections. Contrast comparisons that were significantly different are also bolded.

Contrasts	adjusted p-value
BR vs. GR	0.2196
BR vs. NN	**0.0015**
NN vs. GR	0.3255

Table 5-6. Summary of ANCOVA results for the analysis of HIGH dose of gamabufotalin on aldosterone response. Factors and interactions of factors that had significant effects on corticosterone response are bolded.

Effect Tests	df	F ratio	p-value
baseline	**1, 22**	**6.5177**	**0.0181**
body condition	1, 12	1.6835	0.2183
species group	**1, 13**	**32.7168**	**<.0001**
challenge level	1, 14	0.6974	0.4173

species group*challenge level 1, 14 0.5822 0.4585

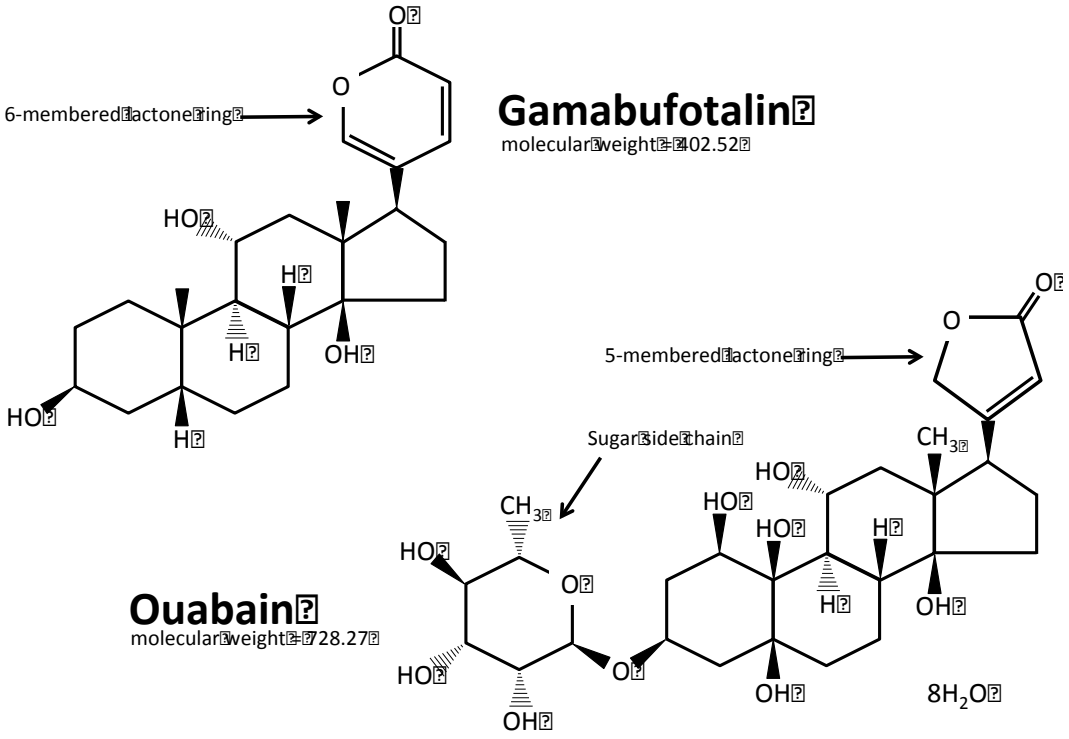

Figure 5-1. Molecular structures of a bufadienolide (gamabufotalin) and a cardenolide (ouabain), redrawn from PubChem (2016a, b), depicting similarities and differences between the two cardiotonic steroid classes. Both compounds consist of a steroidal skeleton with a lactone ring attached to C17. Bufadienolides of toads differ from cardenolides by the presence of an additional carbon on the lactone ring and absence of a sugar moiety commonly attached to C3 in cardenolides. However, some bufadienolides of toads, known as bufotoxins, do have a suberylarginine side chain attached to C3.

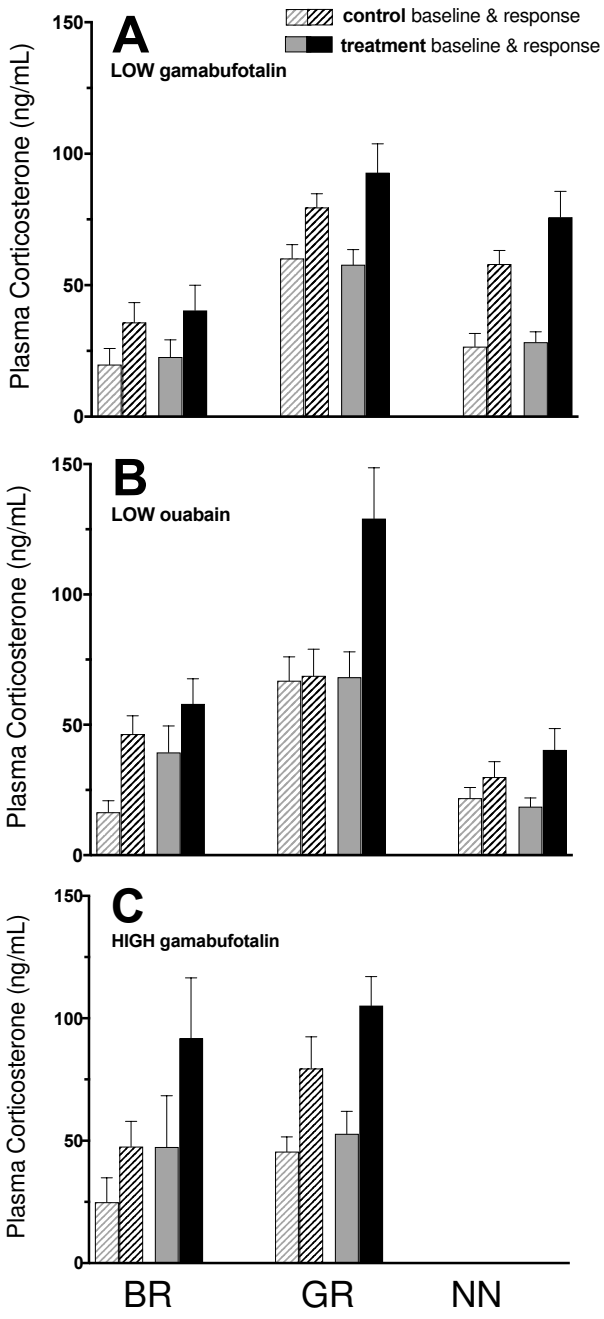

Figure 5-2. Mean baseline (grey-hatched and solid grey bars) and response (black-hatched and solid black bars) levels of corticosterone in (A) bufophagous and resistant (BR) (n = 8), generalist and resistant (GR) (n = 17), and non-resistant and non-bufophagous (NN) (n = 15) snakes challenged with LOW dose of gamabufotalin; (B) BR (n = 7), GR (n = 14), and NN (n = 9) snakes challenged with a LOW dose of ouabain; (C) BR (n = 7) and GR (n = 8) snakes challenged with a HIGH dose of gamabufotalin. Control challenge was 5% DMSO. See Tables 5-1 and 5-3 for results of statistical analyses of these data. Error bars represent standard error.

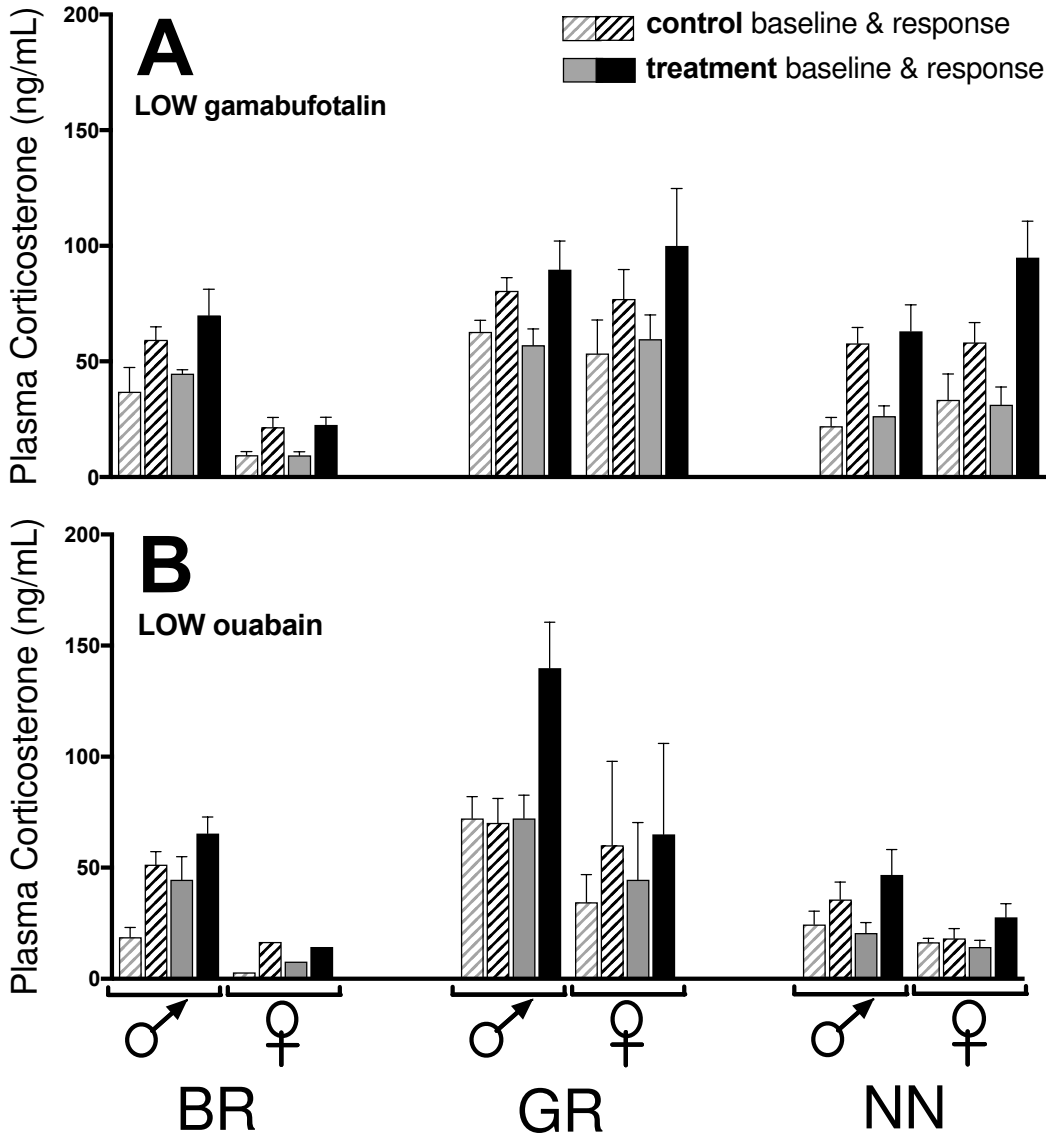

Figure 5-3. Mean baseline (grey-hatched and solid grey bars) and response (black-hatched and solid black bars) levels of corticosterone in (A) male and female bufophagous and resistant (BR) (male n = 3; female n = 5), generalist and resistant (GR) (male n = 12; female n = 5), and non-resistant and non-bufophagous (NN) (male n = 9; female n = 6) snakes challenged with a LOW dose of gamabufotalin and (B) male and female BR (male n = 6 female n = 1), GR (male n = 12; female n = 2), and NN (male n = 6; female n = 3) challenged with a LOW dose of ouabain. Control challenge was 5% DMSO. See Tables 5-1 and 5-2 for results of statistical analyses of these data. Error bars represent standard error.

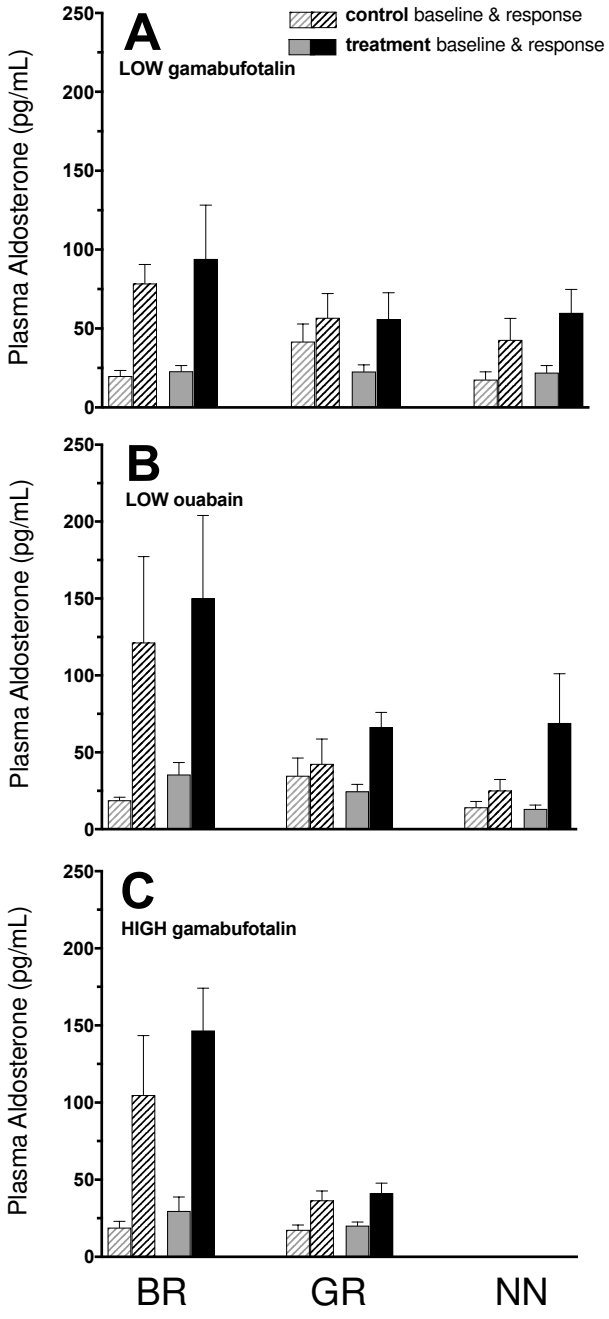

Figure 5-4. Mean baseline (grey-hatched and solid grey bars) and response (black-hatched and solid black bars) levels of aldosterone in (A) bufophagous and resistant (BR) (n = 6), generalist and resistant (GR) (n = 9), and non-resistant and non-bufophagous (NN) (n = 10) snakes challenged with a LOW dose of gamabufotalin; (B) BR (n = 5), GR (n = 2), and NN (n = 4) snakes challenged with a LOW dose of ouabain; (C) BR (n = 6) and GR (n = 10) snakes challenged with a HIGH dose of gamabufotalin. Control challenge was 5% DMSO. See Tables 5-4 to 5-6 for results of statistical analyses of these data. Error bars represent standard error.

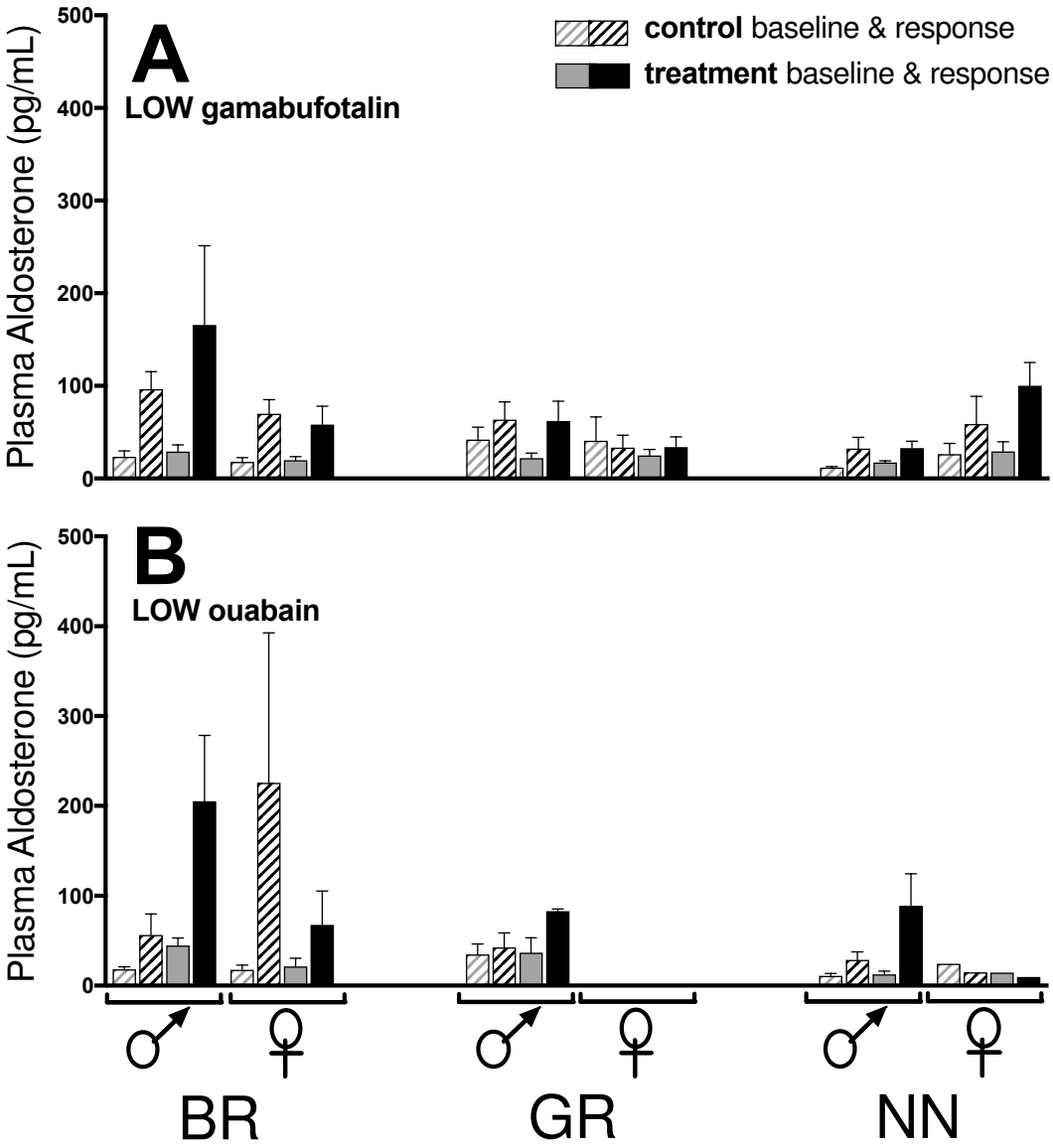

Figure 5-5. Mean baseline (grey-hatched and solid grey bars) and response (black-hatched and solid black bars) levels of aldosterone in (A) male and female bufophagous and resistant (BR) (male n = 2; female n = 4), generalist and resistant (GR) (male n = 7; female n = 2), and non-resistant and non-bufophagous (NN) (male n = 4; female n = 6) snakes challenged with a LOW dose of gamabufotalin and (B) male and female BR (male n = 3; female n = 2), GR (male n = 2; female n = 0), and NN (male n = 3; female n = 1) snakes challenged with a LOW dose of ouabain. Control challenge was 5% DMSO. See Tables 5-4 and 5-5 for results of statistical analyses of these data. Error bars represent standard error.

Table 5-7. List of individuals with associated corticosterone and aldosterone measurements used in this study. Hormones that were not assayed for a given individual are indicated by a dot.

	ID	Species	Locality	Species Group	Sex	Treatment	Treatment Level	Baseline Cort (ng/mL)	Response Cort (ng/mL)	Baseline Ald (pg/mL)	Response Ald (pg/mL)
1	2015-020	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g GB	control	27.976	53.196	29.91	77.38
	2015-020	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g GB	toxin	43.132	86.045	36.40	251.32
2	2015-046	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	control	5.097	18.932	•	•
	2015-046	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	toxin	13.463	33.742	•	•
3	2015-047	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	control	12.883	30.278	31.62	100.24
	2015-047	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	toxin	5.177	20.968	17.89	85.38
4	2015-048	*Rhabdophis tigrinus*	Wakayama, JP	BR	M	1.49x10-2 M/g GB	control	57.904	70.668	•	•
	2015-048	*Rhabdophis tigrinus*	Wakayama, JP	BR	M	1.49x10-2 M/g GB	toxin	48.128	75.727	•	•
5	2015-051	*Rhabdophis tigrinus*	Wakayama, JP	BR	M	1.49x10-2 M/g GB	control	24.764	54.384	17.15	114.93
	2015-051	*Rhabdophis tigrinus*	Wakayama, JP	BR	M	1.49x10-2 M/g GB	toxin	43.109	47.846	21.64	80.30
6	2015-052	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	control	10.908	32.733	12.73	90.26
	2015-052	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	toxin	11.033	21.84	12.35	24.60
7	2015-053	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	control	11.884	12.631	12.10	39.92
	2015-053	*Rhabdophis tigrinus*	Wakayama, JP	BR	F	1.49x10-2 M/g GB	toxin	6.173	13.598	19.19	23.55
8	2016-068	*Rhabdophis tigrinus*	Shimane, JP	BR	F	1.49x10-2 M/g GB	control	6.957	13.631	15.54	48.82
	2016-068	*Rhabdophis tigrinus*	Shimane, JP	BR	F	1.49x10-2 M/g GB	toxin	11.252	23.214	30.12	99.30
9	2015-065	*Rhabdophis tigrinus*	Sizuoka, JP	BR	F	1.49x10-2 M/g OB	control	2.899	16.605	12.83	392.63
	2015-065	*Rhabdophis tigrinus*	Sizuoka, JP	BR	F	1.49x10-2 M/g OB	toxin	7.855	14.254	12.56	30.29
10	2015-028	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g OB	control	22.689	60.047	17.05	102.45
	2015-028	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g OB	toxin	70.197	75.739	42.44	105.44
11	2015-036	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-2 M/g OB	control	25.302	52.538	•	•
	2015-036	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-2 M/g OB	toxin	23.214	46.354	•	•
12	2015-008	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-2 M/g OB	control	7.029	38.452	•	•

(Table 5-7 continued.)

	2015-008	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-2 M/g OB	toxin	21.477	56.926	•	•
13	2015-009	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-2 M/g OB	control	8.743	29.942	14.50	39.96
	2015-009	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-2 M/g OB	toxin	66.043	74.704	60.17	348.28
14	2015-013	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g OB	control	13.391	67.096	•	•
	2015-013	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g OB	toxin	19.797	46.401	•	•
15	2015-016	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g OB	control	35.148	60.333	23.30	27.24
	2015-016	*Rhabdophis tigrinus*	Okayama, JP	BR	M	1.49x10-2 M/g OB	toxin	67.096	91.584	32.07	161.92
16	2015-015	*Rhabdophis tigrinus*	Okayama, JP	BR	F	1.49x10-1 M/g GB	control	10.853	40.041	13.35	31.79
	2015-015	*Rhabdophis tigrinus*	Okayama, JP	BR	F	1.49x10-1 M/g GB	toxin	13.754	32.098	29.81	134.14
17	2015-084	*Rhabdophis tigrinus*	Kyoto, JP	BR	F	1.49x10-1 M/g GB	control	8.964	24.548	23.01	106.87
	2015-084	*Rhabdophis tigrinus*	Kyoto, JP	BR	F	1.49x10-1 M/g GB	toxin	16.524	68.45	8.25	175.25
18	2015-085	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-1 M/g GB	control	5.473	19.69	15.89	105.20
	2015-085	*Rhabdophis tigrinus*	Kyoto, JP	BR	M	1.49x10-1 M/g GB	toxin	24.36	103.961	19.12	145.46
19	2015-086	*Rhabdophis tigrinus*	Kyoto, JP	BR	F	1.49x10-1 M/g GB	control	11.104	25.743	22.92	58.96
	2015-086	*Rhabdophis tigrinus*	Kyoto, JP	BR	F	1.49x10-1 M/g GB	toxin	21.932	61.92	30.65	105.17
20	2015-039	*Rhabdophis tigrinus*	Kyoto, JP	BR	F	1.49x10-1 M/g GB	control	78.681	83.18	21.63	62.59
	2015-039	*Rhabdophis tigrinus*	Kyoto, JP	BR	F	1.49x10-1 M/g GB	toxin	158.363	202.928	65.54	119.58
21	2015-029	*Rhabdophis tigrinus*	Okayama, JP	BR	F	1.49x10-1 M/g GB	control	41.726	83.722	33.61	251.77
	2015-029	*Rhabdophis tigrinus*	Okayama, JP	BR	F	1.49x10-1 M/g GB	toxin	86.31	150.087	45.23	255.04
22	2015-011	*Rhabdophis tigrinus*	Okayama, JP	BR	F	1.49x10-1 M/g GB	control	16.982	56.269	9.89	72.98
	2015-011	*Rhabdophis tigrinus*	Okayama, JP	BR	F	1.49x10-1 M/g GB	toxin	10.908	23.333	10.15	50.56
23	989	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g GB	control	22.595	60.314	14.23	51.30
	989	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g GB	toxin	38.653	64.63	11.64	28.46
24	990	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	control	41.94	63.189	21.56	35.18
	990	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	toxin	25.043	37.593	9.43	21.95
25	995	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g GB	control	41.18	103.275	•	•

(Table 5-7 continued.)

	995	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g GB	toxin	36.949	50.629	•	•
26	997	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g GB	control	33.893	35.69	•	•
	997	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g GB	toxin	62.85	80.428	•	•
27	1003	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	control	66.422	94.444	100.21	162.11
	1003	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	toxin	46.376	64.096	26.24	34.69
28	1006	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	control	56.952	74.894	•	•
	1006	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	toxin	60.367	74.673	•	•
29	1007	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	control	93.958	94.444	•	•
	1007	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	toxin	92.215	198.553	•	•
30	SM83	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	control	56.362	67.079	86.23	57.83
	SM83	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	toxin	37.177	96.305	13.43	31.99
31	SM86	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	F	1.49x10-2 M/g GB	control	103.907	93.958	66.52	46.58
	SM86	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	F	1.49x10-2 M/g GB	toxin	65.885	113.912	31.41	44.88
32	SM91	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	control	74.673	98.351	•	•
	SM91	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	toxin	99.774	92.603	•	•
33	SM93	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	control	51.096	124.492	•	•
	SM93	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	toxin	56.655	114.485	•	•
34	SM97	*Thamnophis radix*	Garden Co., NE, USA	FR	F	1.49x10-2 M/g GB	control	65.885	92.215	15.23	19.85
	SM97	*Thamnophis radix*	Garden Co., NE, USA	FR	F	1.49x10-2 M/g GB	toxin	93.958	190.042	18.60	23.14
35	SM99	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	control	58.458	58.378	30.59	30.35
	SM99	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	toxin	27.677	45.399	47.93	176.78
36	SM100	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	control	77.307	82.239	•	•
	SM100	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g GB	toxin	87.737	115.125	•	•
37	SM130	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	control	43.711	58.449	13.64	13.91
	SM130	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	toxin	45.637	100.336	12.30	47.80
38	SM133	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	control	85.78	85.235	27.23	93.58
	SM133	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g GB	toxin	58.949	84.526	33.00	94.00

(Table 5-7 continued.)

39	SM101	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g OB	control	117.847	113.912	•	•
	SM101	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g OB	toxin	160.789	246.459	•	•
40	SM94	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g OB	control	51.618	48.202	•	•
	SM94	*Thamnophis radix*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g OB	toxin	51.884	61.315	•	•
41	SM92	*Thamnophis radix*	Garden Co., NE, USA	FR	F	1.49x10-2 M/g OB	control	22.046	22.482	•	•
	SM92	*Thamnophis radix*	Garden Co., NE, USA	FR	F	1.49x10-2 M/g OB	toxin	18.918	24.091	•	•
42	SM84	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g OB	control	76.971	62.794	46.35	58.72
	SM84	*Thamnophis sirtalis*	Garden Co., NE, USA	FR	M	1.49x10-2 M/g OB	toxin	51.369	69.772	53.26	85.46
43	976	*Thamnophis sirtalis*	Cache Co UT, USA	FR	M	1.49x10-2 M/g OB	control	61.691	78.837	•	•
	976	*Thamnophis sirtalis*	Cache Co UT, USA	FR	M	1.49x10-2 M/g OB	toxin	84.808	167.232	•	•
44	SM131	*Thamnophis sirtalis*	Garden Co, NE, USA	FR	M	1.49x10-2 M/g OB	control	122.126	81.984	•	•
	SM131	*Thamnophis sirtalis*	Garden Co, NE, USA	FR	M	1.49x10-2 M/g OB	toxin	67.749	169.53	•	•
45	1004	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	52.062	59.852	•	•
	1004	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	51.884	97.823	•	•
46	1005	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	84.808	99.774	•	•
	1005	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	90.535	291.681	•	•
47	SM132	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	121.005	153.516	•	•
	SM132	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	101.09	164.171	•	•
48	SM134	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	87.667	315.133	•	•
	SM134	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	266.318	469.046	•	•
49	SM135	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	48.393	64.977	•	•
	SM135	*Thamnophis sirtalis*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	46.53	53.439	•	•
50	SM105	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	14.587	15.273	•	•
	SM105	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	17.729	73.918	•	•
51	991	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	51.618	35.991	•	•
	991	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	81.491	111.371	•	•
52	992	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	68.662	38.055	23.30	26.35

(Table 5-7 continued.)

	992	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	64.939	115.879	20.52	80.83
53	993	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	control	44.322	53.947	•	•
	993	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-2 M/g OB	toxin	42.159	107.978	•	•
54	998	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g OB	control	46.995	97.823	•	•
	998	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-2 M/g OB	toxin	70.322	105.815	•	•
55	KM31	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	control	41.851	36.229	18.88	17.05
	KM31	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	toxin	41.851	90.677	18.49	35.04
56	KM32	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	control	26.419	46.686	17.83	21.44
	KM32	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	toxin	52.991	106.914	18.13	40.49
57	KM33	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	control	54.399	116.47	15.36	73.03
	KM33	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	toxin	16.892	114.448	19.55	42.62
58	KM34	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	control	•	•	8.13	20.16
	KM34	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	toxin	•	•	12.68	17.71
59	KM35	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	control	49.498	122.494	15.36	61.04
	KM35	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	toxin	68.276	99.902	16.42	14.68
60	KM36	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	control	43.597	75.708	13.97	41.45
	KM36	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	toxin	81.759	118.901	31.92	48.77
61	KM37	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	control	73.517	106.914	43.83	43.80
	KM37	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	toxin	73.896	141.966	17.68	47.56
62	KM38	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	control	20.028	34.244	17.31	40.44
	KM38	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	toxin	71.575	134.643	34.60	79.15
63	KM39	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	control	55.046	97.363	14.17	17.19
	KM39	*Thamnophis elegans*	Cache Co., UT, USA	FR	F	1.49x10-1 M/g GB	toxin	15.2	33.915	20.98	65.79
64	KM40	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	control	•	•	10.30	31.18
	KM40	*Thamnophis elegans*	Cache Co., UT, USA	FR	M	1.49x10-1 M/g GB	toxin	•	•	10.88	20.42
65	SM110	*Pituophis catenifer*	Tooele Co., UT, USA	NR	F	1.49x10-2 M/g GB	control	62.183	68.317	•	•
	SM110	*Pituophis catenifer*	Tooele Co., UT, USA	NR	F	1.49x10-2 M/g GB	toxin	38.729	50.416	•	•

(Table 5-7 continued.)

66	SM140	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	control	12.94	67.332	15.46	12.59
	SM140	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	toxin	25.116	48.061	9.60	12.27
67	SM141	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	control	12.446	35.338	8.53	17.86
	SM141	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	toxin	38.668	60.681	17.17	19.39
68	SM143	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	control	16.667	43.849	12.86	20.96
	SM143	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	toxin	38.403	22.105	16.01	52.40
69	SM144	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g GB	control	9.475	22.703	•	•
	SM144	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g GB	toxin	15.387	68.283	•	•
70	SM145	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	control	17.628	59.267	13.22	19.18
	SM145	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	toxin	41.784	62.744	18.10	21.72
71	SM67	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	control	37.261	51.356	•	•
	SM67	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g GB	toxin	16.261	20.168	•	•
72	2015-055	*Elaphe quadrivirgata*	Kyoto, JP	NR	F	1.49x10-2 M/g GB	control	8.921	45.181	10.42	29.74
	2015-055	*Elaphe quadrivirgata*	Kyoto, JP	NR	F	1.49x10-2 M/g GB	toxin	40.945	133.79	22.07	35.34
73	2015-058	*Elaphe quadrivirgata*	Wakayama, JP	NR	M	1.49x10-2 M/g GB	control	10.498	57.894	•	•
	2015-058	*Elaphe quadrivirgata*	Wakayama, JP	NR	M	1.49x10-2 M/g GB	toxin	5.549	123.798	•	•
74	2015-062	*Elaphe quadrivirgata*	Wakayama, JP	NR	F	1.49x10-2 M/g GB	control	65.974	78.155	57.23	147.36
	2015-062	*Elaphe quadrivirgata*	Wakayama, JP	NR	F	1.49x10-2 M/g GB	toxin	57.653	62.331	60.44	88.80
75	2015-063	*Elaphe quadrivirgata*	Wakayama, JP	NR	M	1.49x10-2 M/g GB	control	42.496	66.742	•	•
	2015-063	*Elaphe quadrivirgata*	Wakayama, JP	NR	M	1.49x10-2 M/g GB	toxin	17.407	99.945	•	•
76	2015-071	*Elaphe quadrivirgata*	Shimane, JP	NR	M	1.49x10-2 M/g GB	control	25.993	102.706	12.31	91.58
	2015-071	*Elaphe quadrivirgata*	Shimane, JP	NR	M	1.49x10-2 M/g GB	toxin	37.781	81.365	24.65	39.39
77	2015-081	*Elaphe quadrivirgata*	Kyoto, JP	NR	M	1.49x10-2 M/g GB	control	22.548	36.375	7.97	30.49
	2015-081	*Elaphe quadrivirgata*	Kyoto, JP	NR	M	1.49x10-2 M/g GB	toxin	16.791	49.175	18.00	53.07
78	2015-082	*Elaphe quadrivirgata*	Kyoto, JP	NR	F	1.49x10-2 M/g GB	control	10.366	62.999	7.40	23.07
	2015-082	*Elaphe quadrivirgata*	Kyoto, JP	NR	F	1.49x10-2 M/g GB	toxin	5.433	125.432	13.99	145.41
79	2015-083	*Elaphe quadrivirgata*	Kyoto, JP	NR	F	1.49x10-2 M/g GB	control	44	72.585	30.73	34.95

(Table 5-7 continued.)

	2015-083	*Elaphe quadrivirgata*	Kyoto, JP	NR	F	1.49x10-2 M/g GB	toxin	0.531	129.484	20.48	131.69
80	SM142	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g OB	control	•	•	24.37	14.92
	SM142	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g OB	toxin	•	•	14.60	9.79
81	SM108	*Pituophis catenifer*	Tooele Co., UT, USA	NR	F	1.49x10-2 M/g OB	control	14.74	9.585	•	•
	SM108	*Pituophis catenifer*	Tooele Co., UT, USA	NR	F	1.49x10-2 M/g OB	toxin	9.73	15.425	•	•
82	SM146	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	control	21.547	34.298	15.67	18.70
	SM146	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	toxin	34.879	19.201	11.44	17.70
83	SM147	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	control	36.522	29.777	•	•
	SM147	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	toxin	21.429	56.867	•	•
84	SM148	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g OB	control	19.951	20.745	•	•
	SM148	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g OB	toxin	19.918	31.222	•	•
85	SM62	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	control	8.877	26.364	•	•
	SM62	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	toxin	15.976	21.552	•	•
86	SM65	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	control	36.949	35.69	•	•
	SM65	*Pituophis catenifer*	Garden Co., NE, USA	NR	M	1.49x10-2 M/g OB	toxin	22.482	31.893	•	•
87	SM66	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g OB	control	14.9775	24.219	•	•
	SM66	*Pituophis catenifer*	Garden Co., NE, USA	NR	F	1.49x10-2 M/g OB	toxin	13.621	35.997	•	•
88	2015-087	*Elaphe quadrivirgata*	Shiga, JP	NR	M	1.49x10-2 M/g OB	control	37.361	71.876	10.09	46.53
	2015-087	*Elaphe quadrivirgata*	Shiga, JP	NR	M	1.49x10-2 M/g OB	toxin	27.998	91.129	19.40	123.38
89	2015-088	*Elaphe quadrivirgata*	Shiga, JP	NR	M	1.49x10-2 M/g OB	control	5.701	16.708	7.27	20.63
	2015-088	*Elaphe quadrivirgata*	Shiga, JP	NR	M	1.49x10-2 M/g OB	toxin	1.147	59.877	7.63	125.48

Table 5-8. Number of individuals used in experiment and statistical analyses. Individuals are divided by species group (BR, GR, NN), then species (*Rhabdophis tigrinus*, *Thamnophis sirtalis*, *T. elegans*, *T. radix*, *Elaphe quadrivirgata*, *Pituophis catenifer*), and finally treatment (LOW BD, LOW CD, HIGH BD) for (A) corticosterone measurements and (B) aldosterone measurements.

A	**BR (n = 22)**		**GR (n = 39)**									**NN (n = 24)**			
Rhabdophis tigrinus			*Thamnophis sirtalis*			*Thamnophis elegans*			*Thamnophis radix*			*Elaphe quadrivirgata*		*Pituophis catenifer*	
LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	LOW BD	LOW CD
8	7	7	7	5	0	6	6	8	4	3	0	8	2	7	7
B	**BR (n = 17)**		**GR (n = 21)**									**NN (n = 14)**			
Rhabdophis tigrinus			*Thamnophis sirtalis*			*Thamnophis elegans*			*Thamnophis radix*			*Elaphe quadrivirgata*		*Pituophis catenifer*	
LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	HIGH BD	LOW BD	LOW CD	LOW BD	LOW CD
6	5	6	4	1	0	3	1	10	2	0	0	6	2	4	2

5. Discussion

Previous studies of the evolution of cardiotonic steroid resistance in vertebrates have focused largely on the comparative target-site sensitivity of Na^+/K^+-ATPase (Mohammadi et al., 2016a; Ujvari et al., 2015; Ujvari et al., 2013; Moore et al., 2009), and there have been few investigations of potential differences at the organismal scale. The results of this study demonstrate for the first time the effects of exposure to cardiotonic steroids on select endocrine responses of snakes. Our results also provide evidence for the presence of physiological adaptations beyond target-site insensitivity in *Rhabdophis tigrinus*, which has evolved dietary specialization involving consumption of toxic prey. Although the availability of experimental subjects limited us to a single toad-eating specialist and required us to pool species in the GR and NN groups, we nonetheless observed variations in corticosteroid responses among the functionally different species groups. Future studies would benefit from testing these responses in additional toad-specialized species to determine whether our observations on *R. tigrinus* are associated with a dietary specialization on toads or other interspecific variation.

An increase in corticosterone in response to a chemical stressor is not unusual and has been documented in a variety of species in response to various chemicals (Neuman-Lee et al., 2016b; Adams et al., 2009; Franceschini et al., 2008; Hopkins et al., 1997). Differences in these responses may reflect species-specific physiological adaptations (Hopkins et al., 2016; Neuman-Lee et al., 2016a). We have shown that exposure to low concentrations of gamabufotalin elicits an increase in corticosterone in all three species groups. Although the treatment responses did not increase significantly compared to the

control responses (i.e., to 5% DMSO alone) within each group, the responses differed significantly between species groups (Fig. 5-2; Table 5-1).

Contrary to our predictions, the stress responses to gamabufotalin and ouabain were not highest in NN snakes, which did not survive either treatment, but rather in GR snakes. Given the ubiquity of Na^+/K^+-ATPase among tissues, the effect on NN snakes may have been so rapid and profound that slower corticosterone responses were pre-empted. The HPA axis is regulated by a potent negative feedback loop, and in some cases of disruption of physiological homeostasis the HPA axis can be suppressed (Dhabhar, 2009; Miller et al., 2007; Rich and Romero, 2005). Such suppression of the HPA axis may have resulted from these acute stressors (Narayan and Hero, 2014). The ouabain response in the NN snakes was much lower than the gamabufotalin response (Fig. 5-2). This discrepancy might be explained by the fact that ouabain exposure took twice as long as gamabufotalin to elicit fatality in these snakes. It is thus possible that the low ouabain response of NN snakes reflected an inappropriately short post-injection effect window (i.e., 30 minutes post-injection) for this treatment in this species group. It is also possible that the stress response reached its maximum attainable level at these treatment concentrations (Hews and Baniki, 2013; Robert et al., 2009), masking any differences between the effects of BDs and CDs. Increasing the gamabufotalin dose 10-fold did not elicit a statistically significant response in GR and BR snakes. However, this treatment did cause an increased corticosterone response in the BR snakes that was not discernable at the lower dose (Fig. 5-2).

Perhaps the most unexpected finding of this study was the strong behavioral response and elevated corticosterone level of GR snakes exposed to ouabain. Despite

high baseline corticosterone levels, which are normally associated with lowered corticosterone responses (Rich and Romero, 2005; Moore and Jessop, 2003), GR snakes exhibited a very high stress response to ouabain (Fig. 5-2). CDs differ from BDs by the possession of five, rather than six, carbons on the C17 lactone ring and often by the presence of a sugar moiety at C3 (Fig. 5-1). The sugar moiety significantly increases the polarity of the molecule, which can affect uptake and transport of the compound through the body (King and Mainwaring, 1974). CDs with different polarities have been shown to elicit significantly different cardiovascular effects in mammals (reviewed in Erdmann et al., 2013). Generally, non-polar CDs are absorbed more quickly by cells (Rasmann and Agrawal, 2011; Cavet et al., 1996), which suggests that BDs should be taken up more readily than CDs. However, the polarity of the steroids also affects their interactions with circulating binding globulins (Antolovic et al., 1998). It is conceivable that the composition and concentration of binding globulins in snakes that are specialized to prey on toads differ from those in snakes that simply exhibit target-site insensitivity but do not feed frequently on toads. Although there has been significant progress in our understanding of the physiological consequences of mammalian endogenous cardiotonic steroids, no comparable work has been conducted in reptiles. Future studies of that kind would greatly advance our understanding of the evolution of BD resistance in snakes.

The results of the aldosterone assays revealed consistently higher responses in BR snakes than in either of the other species groups, regardless of treatment and challenge level (Fig. 5-4). BR snakes also had a higher aldosterone response compared to their corticosterone response, whereas the other two species groups had higher corticosterone responses than aldosterone. Furthermore, the 5% DMSO control dose seems to have had

a large effect on the aldosterone response of BR snakes (Figures 5-4 and 5-5), but that response was not observed in the other species groups. DMSO can cause vasodilation and decreased blood pressure (Jacob and Herschler, 1986) and can also affect renal function in mammals (Santos et al., 2003). The association between DMSO and osmoregulation suggests it may affect aldosterone, which plays a significant role in maintaining ion balance and blood pressure (Norris, 2006; Weber, 2001; Tuck et al., 1981; Laragh, 1973). However, it remains puzzling that these effects were absent in GR and NN snakes. The high aldosterone response of BR snakes could reflect the presence of more numerous aldosterone-producing interrenal cells in the adrenal glands. Importantly, aldosterone is strongly associated with regulation of Na^+/K^+-ATPase (Salyer et al., 2013; Ikeda et al., 1991; Verrey et al., 1987). Ikeda et al. (1991) conducted a series of *in vitro* experiments in which they added aldosterone to cardiac cells. Those cells experienced a three-fold increase in Na^+/K^+-ATPase mRNA within six hours and reached a plateau in 12 hours, suggesting that aldosterone causes an increase in Na^+/K^+-ATPase content in cell membranes. It is conceivable that aldosterone plays a role in suppressing the toxic effects of dietary BDs in BR snakes by signaling the expression of the mutated Na^+/K^+-ATPase isoform (*ATP1a3*) in certain tissues, notably the heart and kidneys. The role of the latter in osmoregulation makes kidneys a likely target of increased aldosterone production. To confirm this relationship, the effects of aldosterone injections on expression levels of *ATP1a3* should be tested in various tissues.

Another interesting result of this study was the difference in corticosterone responses of male versus female BR snakes (Figs. 5-3). Although we were not able to test these differences statistically for the aldosterone responses, Figure 5-5 suggests that the

same pattern might hold true for this hormone. Sex differences in corticosterone levels in response to stress are known for other species of snakes (Neuman-Lee et al., 2016b; Sykes and Klukowski, 2009; Moore and Jessop, 2003). Comparisons of adrenal mass in bufophagous and non-bufophagous snakes not only revealed significant enlargement in most bufophagous species, including *R. tigrinus*, but also demonstrated sexual dimorphism in size, with males of those species having significantly larger adrenal glands than females (Mohammadi et al., 2013). The observed responses of both corticosterone and aldosterone to gamabufotalin in BR snakes are consistent with such sexual dimorphism, with hormonal responses of males being higher than those of females. Male responses to ouabain were also high, but female responses to both ouabain and to the DMSO control were equally high (whereas males did not exhibit high levels in response to DMSO). However, we note that the sample size for that group was small (n = 2), and the high response was driven by a single individual (2015-065; Table 5-7). Thus, the observed response may not represent an accurate measurement of that group's response to the DMSO control. Although we do not yet know which adrenal tissues are enlarged in bufophagous snakes, the results of this study suggest that corticosteroid-producing interrenal tissues may be hypertrophied in both sexes and further enlarged in males.

To date, there is no explanation for the sexual dimorphism in adrenal gland mass in BR snakes, nor for the sexual differences in hormonal responses that we observed. It is possible that, in addition to sequestering BDs from toads in their nuchal glands, males may be storing exogenous BDs in their adrenal glands, whereas females may transfer much of those BDs to their ova. In *R. tigrinus*, the BR species of this study, females can provision their offspring with exogenous BDs by transfer to the yolk and through direct

transfer to developing embryos *in utero* (Hutchinson et al., 2008). The adrenal glands are known to secrete and to store endogenous cardiotonic steroids in mammals (Manunta et al., 2009; Schoner, 2002; Kitano et al., 1998; Laredo et al., 1995; Laredo et al., 1994; Boulanger et al., 1993), so the tissue may have a particular affinity for these compounds. Indeed, Kitano et al. (1998) showed that exogenous ouabain, obtained from diet, accumulates in the adrenal glands of rats, and a similar mechanism might occur in BR snakes. Further work on the physiology of adrenal glands in snakes is needed to clarify these issues. Furthermore, given the ability of exogenous BDs from the female's diet to accumulate in the nuchal glands of embryonic *R. tigrinus*, the effect of such compounds on the developing adrenals should also be investigated.

In summary, this study reveals strong differences in corticosteroid hormone responses to cardiotonic steroids between a snake that exhibits dietary specialization on toads, snakes that possess genetic resistance to toad toxins but do not specialize on toads, and snakes that are not resistant to toad toxins. Whether these differences reflect dietary specialization alone or are also influenced by other interspecific differences remains to be determined. Furthermore, high aldosterone responses in the toad-specialized species suggest the occurrence of complex physiological adaptations beyond Na^+/K^+-ATPase target-site insensitivity, which has been the primary focus of previous studies on bufadienolide resistance in snakes. Although more comprehensive sampling of species is necessary to determine the extent of these patterns, this study provides an important basis for more expansive work. Furthermore, we have demonstrated increased adrenal hormone activity in response to acute cardiotonic steroid exposure in snakes. Chronic stress can have depressive effects on the HPA axis (Dhabhar, 2009; Rich and Romero, 2005), and

expanding this study to examine the effects of chronic BD exposure in snakes may provide results that are more ecologically relevant to the responses of toad-eating species to cardiotonic steroids.

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CHAPTER 6

THE EFFECTS OF CARDIOTONIC STEROID EXPOSURE ON CARDIAC CONTRACTILITY OF SNAKES THAT POSSESS RESISTANCE-CONFERRING MUTATIONS TO NA^+/K^+-ATPASE

Resistance to toad toxins (bufadienolides) in snakes is conferred by mutations of Na^+/K^+-ATPase, which lower the target enzyme's affinity to the toxins. Previous studies have shown that, despite such genetic resistance, ingestion or exposure to bufadienolides can have negative physiological consequences in certain snakes. Bufadienolides are one of two classes of cardiotonic steroids, which have been used extensively throughout history, including in modern medicine, for their cardioactive properties. A recent upsurge of studies investigating the evolutionary origins of cardiotonic steroid resistance in a wide range of organisms has focused on the molecular mechanisms of this adaptation. Few studies have investigated the physiological responses to consumption of dietary cardiotonic steroids in vertebrates. A recent study revealed that the cardiac tissue of a genetically resistant species of snake expresses significantly higher levels of resistant Na^+/K^+-ATPase mRNA than other tissues, indicating the importance of the heart in resistance physiology. Here we tested the cardiac response of two species of genetically resistant natricine snakes (*Thamnophis sirtalis* and *Rhabdophis tigrinus*) to two classes of cardiotonic steroids (gamabufotalin, a bufadienolide, and ouabain, a cardenolide). We found that the two species responded differently to the two compounds. The bufadienolide had an overall depressive effect on the heart rate of *T. sirtalis*, whereas the cardenolide caused irregular heart rates. Neither compound had a strong effect on the

heart rate of *R. tigrinus*. This result is not surprising, inasmuch as *R. tigrinus* sequesters bufadienolide toxins in high concentrations in its defensive nuchal glands.

INTRODUCTION

Cardiotonic steroids include a wide variety of animal- and plant-derived toxins that affect cardiac contractility by binding to the α-subunit of the Na^+/K^+-ATPase of cell membranes and disabling the enzyme (Krenn and Kopp, 1998; Barrueto et al., 2006; Dvela et al., 2007). The deactivation of membrane Na^+/K^+-ATPases causes a cascade of intracellular ion imbalances, which result in strengthened and more frequent cardiac contractions, often with lethal consequences (Barry and Bridge, 1993; Rose and Valdes, Jr., 1994). Humans have exploited this mechanism for over 3,000 years, using these compounds to treat ailments of the heart (Krenn and Kopp, 1998; Schoner, 2002; Mijatovic et al., 2007). All cardiotonic steroids share the common structure of a steroidal skeleton with a lactone ring on C17 (Dvela et al., 2007). Primarily plant-derived cardiotonic steroids such as digitalis and ouabain, which contain a five-carbon lactone ring, are known as cardenolides (CDs) and have been widely used in contemporary Western pharmaceuticals (Schoner and Scheiner-Bobis, 2007; Prassas and Diamandis, 2008). Primarily animal-derived cardiotonic steroids, which contain a six-carbon lactone ring, are known as bufadienolides (BDs; Krenn and Kopp, 1998). BDs are best known from toads (Bufonidae), but they also occur in fireflies (Lampyridae; Eisner, et al. 1978; Krenn and Kopp, 1998; Dvela et al., 2007), and a few plants (Krenn and Kopp, 1998). BDs are used in traditional Chinese medicine for purposes similar to the Western use of CDs (Gowda et al., 2003; Ma et al., 2012). Furthermore, both BDs and CDs are produced

by the adrenal cortex in mammals (Shaikh et al., 1991; Dmitrieva et al., 2000). Although previously regarded solely as defensive compounds, the ubiquity of cardiotonic steroids in organisms has shifted attention to their role as cardiovascular hormones (Dvela et al., 2007).

Cardiotonic steroids affect Na^+/K^+-ATPases of all cells, but the effects on the heart have been traditionally deemed important because that is where the main therapeutic and lethal effects are known to occur (Barry and Bridges, 1993; Rose and Valdes 1994). In the heart, Na^+/K^+-ATPases act as indirect regulators of contractions by maintaining intracellular (Na^+) levels. In a properly functioning cell, intracellular Ca^{2+} ions are taken up by the sarcoplasmic reticulum, then released in controlled levels to sarcoplasm, where they bind to the protein complex troponin, causing a conformational change in actin and myosin filaments, which produces a contraction (Barry and Bridge, 1993). When Na^+/K^+-ATPases are disabled, the membrane stops transporting Na^+ out of the cell. The resulting elevated intracellular Na^+ level deactivates the Ca^{2+} /Na^+ exchanger, which compensates for high Na^+ levels by terminating its import into the cell. However, by doing so, the Ca^{2+} /Na^+ exchanger also terminates the export of Ca^{2+} out of the cell, causing an increase in intracellular Ca^{2+} . To rectify this further shift away from homeostasis, the cell moves the excess Ca^{2+} to the sarcoplasmic reticulum where, to regain equilibrium, it is then released to sarcoplasm in higher concentrations and at faster rates (Barry and Bridge, 1993). In myocardial cells, this shift can enhance contractile strength and heart rate. Thus, in low doses cardiotonic steroids can have beneficial effects on the heart. However, when Na^+/K^+-ATPases are disabled for a prolonged period, these symptoms can lead to arrhythmia and eventual cardiac failure

(Rose and Valdes, 1994). Many plants and animals produce or sequester lethal concentrations of these compounds as a means of chemical defense (Krenn and Kopp, 1998; Hutchinson et al., 2007).

Although the quantities of BDs produced for chemical defense by toads are lethal to most organisms, certain species of snakes have evolved target-site insensitivity through mutations of Na^+/K^+-ATPase (Ujvari et al., 2015, Mohammadi et al., 2016a) and can tolerate the normally lethal effects of BDs (Mohammadi et al., 2016b). Target-site insensitivity has been identified in one of three isoforms of the Na^+/K^+-ATPase in snakes (Ujvari et al., 2015; Mohammadi et al., 2016a), and in at least one resistant species of snake the gene that encodes that isoform (*ATP1a3*) is expressed at significantly higher levels in the heart than in any other tissue (Mohammadi et al., 2017a). Physiological investigations of the trade-offs of exposure to cardiotonic steroids revealed significantly higher endocrine hormonal responses to cardiotonic steroids in resistant species of snakes compared to nonresistant species (Mohammadi et al., 2017b). These hormonal responses differed according to the snake's potential level of toxin exposure in the wild and whether the snake was exposed to a BD or a CD. Snakes of the genus *Thamnophis* had a strong stress response to the CD, ouabain, whereas the BD-sequestering species *Rhabdophis tigrinus* did not, and neither had a strong stress response to the BD, gamabufotalin (Mohammadi et al., 2017b). On the other hand, *R. tigrinus* had a very strong aldosterone response to both the CD and BD, whereas the *Thamnophis* spp. did not (Mohammadi et al., 2017b). Furthermore, enlargement of the adrenal glands has been associated with snakes whose diets consist heavily (>80%) of toads (Mohammadi et al., 2013). Although these studies have indicated the presence of physiological consequences of cardiotonic

steroid exposure in resistant species, the exact physiological pathways involved in dealing with the toxins, beyond target-site insensitivity, remain to be elucidated.

To understand the physiology and trade-offs associated with evolved resistance to cardiotonic steroids in snakes, it is important to test the cardiac effects of the toxins in resistant species. One previous study investigated this question by measuring the effects of BDs on cardiac contractility of a resistant snake, *Thamnophis sirtalis* (Licht and Low, 1968). The study revealed that at very high BD concentrations, *T. sirtalis* responded with cardiac arrhythmia, ventricular fibrillation, and tachycardia, which resulted in cardiac arrest 16 hours after exposure (Licht and Low, 1968). Although *T. sirtalis* is resistant and will feed on toads occasionally or heavily depending on the population, location, and season, they have a highly variable diet and do not specialize on toads (Gregory and Stewart, 1975; Gregory, 1978; Kephart and Arnold, 1982). Furthermore, Licht and Low (1968) tested exposure to crude toad BD extract from the cane toad, *Rhinella marina*, a genus of toad that the *T. sirtalis* does not normally encounter, and the highest concentration administered to the snakes exceeded the amount that could be collected from a single toad. We expanded upon that study by testing the cardiac response to both a BD and a CD in two species of resistant snakes. *T. sirtalis* is considered resistant but not chronically exposed to BDs under natural conditions, due to its highly variable diet. In contrast, the Japanese tiger keelback, *R. tigrinus*, is considered both resistant and chronically exposed to BDs. Although both species are usually occasional predators of toads (Gregory and Stewart, 1975; Arnold, 1978; Tanaka and Ota, 2002; Hirai, 2004), *R. tigrinus* stores dietary bufadienolides acquired from toads in its tissues (Hutchinson et al., 2007), and we thus considered that species to be chronically exposed to the toxins under

natural conditions. We expected that *T. sirtalis* would respond to the toxins with a significant increase in heart rate, whereas *R. tigrinus* would not respond as strongly.

MATERIALS AND METHODS

Experimental animals

Snakes were collected from the field in Utah (Cache County; permit no. 1COLL9134), Nebraska (Garden County; permit no. 477), and Japan (Kyoto, Okayama, Shimane, and Wakayama Prefectures; no permit required). A total of 27 snakes from two species (*Thamnophis sirtalis* and *Rhabdophis tigrinus*) were used in this experiment (Table 1).

All snakes were housed and cared for and handled in accordance with Utah State University Institutional Animal Care and Use Committee (IACUC) regulations (protocol #2078) and with Kyoto University Graduate School of Science (protocol #H2605). Snakes from Utah and Nebraska were brought to Utah State University (Logan, Utah, USA), housed individually in 37.8 L glass terraria with newspaper substrate, and provided with a hide shelter and water *ad libitum*. Snakes from Japan were brought to Kyoto University (Kyoto, Kyoto Prefecture, Japan) and housed separately in plastic terraria (18.9 L) with newspaper substrate, water *ad libitum*, and a hide shelter. *R. tigrinus* were fed frogs (*Pelophylax nigromaculatus*) collected from the field, twice per week, due to their unusually rapid rate of starvation (unpublished). *T. sirtalis* were fed commercially purchased thawed mice (RodentPro.com, LLC) once per week.

Heart rate monitoring

Snakes were removed from their terraria and palpated to determine the location of their heart. For each snake, small incisions of the skin were made on either side of the heart and either side of the trunk near the cloaca. Flat-tipped copper alligator clips were used as electrodes and were applied subcutaneously at each incision site, and the snake was then lightly taped to the bottom of a plastic container (54.5 cm l x 36.5 cm w x 14.5 cm h) to restrain movement, which can cause fluctuations in heart rate and dislodge the electrodes. When the snake was secured, the leads from a heart monitor (CardiMax FX-2111) were attached to the copper alligator clips, and the monitor was turned on to measure heart rate. The containers were covered with a thin cloth to provide shelter, and the snakes were left undisturbed to acclimate for one hour. Following acclimation, heart rate was recorded manually from the digital readout every minute for 20 minutes to collect an average baseline beats per minute (bpm) for each individual. At 21 minutes, each snake was given a randomly assigned, mass adjusted dose of either a control fluid (5% dimethyl sulfoxide (DMSO; Fisher Scientific, Inc.), 0.149 M/g of the BD gamabufotalin (GB; PiChemicals ®, Lot # PI201406082068 and # PI1201206031583), or 0.149 M/g of the CD ouabain (OB; Acrōs Organics, Lot # A0343825) by intraperitoneal injection in the posterior trunk region (Mohammadi et al., 2016b). DMSO served as the solvent for the BD and CD solutions. The BD dose was selected based on previously published experiments and represents 100 times the intravenous LD_{50} of mice and approximately 10 times the intraperitoneal lethal dose for a non-resistant snake (Yoshida et al., 1976; Mohammadi et al., 2016b). Following injection, each snake was left to recover from the handling/injection stress for five minutes, and then the post-injection

heart rate was recorded every minute for 80 minutes. All experiments were performed at 24-25°C.

Statistical analysis

Data were analyzed for the effects of fixed and random factors on heart rate using a mixed-model repeated-measures ANOVA (treatment: control versus BD versus OB; species: *R. tigrinus* versus *T. sirtalis*; injection time: pre-injection baseline versus post-injection response) x 101 (minutes: 1-101). Heart rate data were log-transformed to better meet the assumptions of homogeneity of variance and linearity. Individuals were considered a random factor nested within species. Pre-planned contrasts with Holm-Bonferroni corrections were used to compare differences between factor levels. The statistical analysis was performed using JMP 12.2.0 (SAS Institute Inc., 2015). To account for missing data in some time series we also used a random coefficient model (Bliese and Ployhart, 2002) in R (R Core Team 2015) using the mixed model package nlme (Pinheiro and Bates, 2000). Our models analyzed the change in heart rate over time, with fixed effects for treatment and body mass and random effects for each individual snake nested within species. The significance level was set at $\alpha < 0.05$ for all statistical comparisons.

RESULTS

The mean baseline heart rates of *T. sirtalis* were 41 bpm prior to the control injection (5% DMSO), 46 (± 3.24 S.E.) bpm prior to OB, and 46 (± 2.09 S.E.) bpm prior to GB. Mean baseline heart rates of *R. tigrinus* were 55 (± 1.73 S.E.) bpm prior to the control injection (5% DMSO), 47 (± 1.83 S.E.) bpm prior to OB, and 44 (± 2.83) bpm

prior to GB. Mean post-injection heart rates of *T. sirtalis* were 37 (± 1.44 S.E.) bpm for control (5% DMSO), 47 (± 3.26 S.E.) bpm for OB, and 40 (± 1.04 S.E.) bpm for GB. Mean post-injection heart rates of *R. tigrinus* were 57 (± 2.32 S.E.) bpm for control (5% DMSO), 49 (± 1.77 S.E.) bpm for OB, and 47 (± 2.55 S.E.) bpm for GB. There was no indication of prolonged post-injection changes in heart rate in response to the control (5% DMSO) in either species (figs. 6-1A and 6-2A). OB exposure caused strong fluctuations in heart rate in *T. sirtalis* but not in *R. tigrinus* (fig. 6-1B and 6-2B). Furthermore, OB caused strong body movement responses in *T. sirtalis* that interfered with heart rate measurements, thereby significantly reducing the amount of data we were able to collect at each time point for that treatment. There was an overall depression of heart rate in GB-treated *T. sirtalis* (fig. 6-1C). *R. tigrinus* exhibited prolonged variations in heart rate following injection of GB and decreased heart rate following injection of OB (fig. 6-2B and 6-2C).

The mixed-model repeated-measures ANOVA revealed significant effects of species, injection time, and time on heart rate. Significant two-way interactions of species x treatment, species x injection time, and treatment x injection time, and significant three-way interactions of treatment x injection time x time on heart rate were also detected. See Table 6-2 for a summary of test results. Contrast analyses revealed significant differences between control and post-injection OB responses of *T. sirtalis*, and between control–OB and control–GB responses of *R. tigrinus.* Contrast analyses also revealed significant differences between pre-injection and post-injection heart rates of *T. sirtalis* exposed to OB and *R. tigrinus* exposed to OB. See Table 6-2 for a summary of contrast results.

Results from random coefficient models were in agreement with the above results. Our model showed that heart rate decreased over time in *T. sirtalis* given GB, with a significant three-way interaction among treatment, species, and time (Table 6-3; $t = 3.59_{(2440)}$, $p = 0.0003$)

TABLES AND FIGURES

Table 6-1. List of snakes used for this experiment, including locality, sex, morphometrics, treatment, mass of toxin administered, and volume of final solution.

ID	Species	Locality	Sex	Mass (g)	SVL (cm)	TL (cm)	Toxin administered	Mass toxin administered (mg)	Volume injected (mL)
2015-019	*Rhabdophis tigrinus*	Tsuyama, Okayama	F	70	652	829	0.149 M/g GB	7	1.167
2015-045	*Rhabdophis tigrinus*	Shizuhala, Kyoto	F	118	771	951	0.149 M/g GB	11.8	1.967
2015-005	*Rhabdophis tigrinus*	Oheyama, Kyoto	F	132	805	1017	0.149 M/g GB	13.2	2.2
2015-047	*Rhabdophis tigrinus*	Nachikatsuura, Wakayama	F	48	566	701	0.149 M/g OB	4.8	0.8
2015-068	*Rhabdophis tigrinus*	Hikawa, Shimane	F	100	774	974	0.149 M/g OB	10	1.667
2015-046	*Rhabdophis tigrinus*	Nachikatsuura, Wakayama	F	122	794	1031	0.149 M/g OB	12.2	2.033
2015-052	*Rhabdophis tigrinus*	Nachikatsuura, Wakayama	F	140	798	1038	0.149 M/g OB	14	2.333
2015-053	*Rhabdophis tigrinus*	Nachikatsuura, Wakayama	M	118	655	902	0.149 M/g OB	11.8	1.967
2015-051	*Rhabdophis tigrinus*	Nachikatsuura, Wakayama	M	45	528	732	0.149 M/g OB	4.5	0.75
2015-084	*Rhabdophis tigrinus*	Tauta, Kyoto	F	130	72.3	90.2	5% DMSO Control	-	0.433
2015-011	*Rhabdophis tigrinus*	Tsuyama, Okayama	F	120	55.7	67.7	5% DMSO Control	-	0.4
2015-029	*Rhabdophis tigrinus*	Tsuyama, Okayama	F	75	71.1	89.9	5% DMSO Control	-	0.25
2015-015	*Rhabdophis tigrinus*	Tsuyama, Okayama	F	41	22.2	41	5% DMSO Control	-	0.137
2015-003	*Rhabdophis tigrinus*	Sakyo Kyoto	F	180	89.4	110.1	5% DMSO Control	-	0.6
2015-085	*Rhabdophis tigrinus*	Tauta, Kyoto	M	90	77.8	92.8	5% DMSO Control	-	0.3
BR6	*Thamnophis sirtalis*	Cache Co., Utah	M	32	47	64	0.149 M/g GB	3.195	1.065
BR12	*Thamnophis sirtalis*	Cache Co., Utah	M	18	40	56	0.149 M/g GB	1.79	0.298
BR16	*Thamnophis sirtalis*	Cache Co., Utah	M	19	42	58	0.149 M/g GB	1.85	0.308
1192	*Thamnophis sirtalis*	Cache Co., Utah	M	52	520	750	0.149 M/g GB	5.237	0.873
1188	*Thamnophis sirtalis*	Cache Co., Utah	M	39	470	610	0.149 M/g GB	3.85	0.642
1181	*Thamnophis sirtalis*	Cache Co., Utah	F	146	680	810	0.149 M/g OB	14.58	2.43
BR3	*Thamnophis sirtalis*	Cache Co., Utah	M	48	51	69	0.149 M/g OB	4.82	0.803

(Table 6-1 continued.)

1189	*Thamnophis sirtalis*	Cache Co., Utah	M	35	440	600	0.149 M/g OB	3.47	0.578
BR1	*Thamnophis sirtalis*	Cache Co., Utah	M	30	47	63	5% DMSO Control	-	0.1
BR9	*Thamnophis sirtalis*	Cache Co., Utah	M	23	43	60	5% DMSO Control	-	0.077
BR14	*Thamnophis sirtalis*	Cache Co., Utah	M	22	41	55	5% DMSO Control	-	0.075
BR20	*Thamnophis sirtalis*	Cache Co., Utah	M	20	41	55	5% DMSO Control	-	0.068

Table 6-2. Summary of repeated measures ANOVA results and contrast comparisons for the analysis of OB and GB effects on heart rate. Factors and interactions of factors that had significant effects on heart rate are bolded. Contrast comparisons that were significantly different are also bolded. P-values for contrast comparisons were adjusted using Holm-Bonferroni corrections.

Effect Tests	**df**	**F ratio**	**p-value**
mass	1, 20	2.4403	0.1341
species	**1, 26**	**20.9044**	**0.0001**
treatment	2, 30	0.3765	0.6894
injection time	**2, 2508**	**4.6285**	**0.0315**
time	**1, 2507**	**37.1572**	**<0.0001**
species*treatment	**2, 30**	**8.1965**	**0.0015**
species*injection time	**1, 2508**	**7.5822**	**0.0059**
treatment*injection time	**2, 2508**	**4.5922**	**0.0102**
species*time	1, 2507	0.0057	0.9400
treatment*time	2, 2507	2.3707	0.0936
injection time*time	1, 2507	0.5514	0.4578
species*treatment*injection time	2, 2508	2.8205	0.0598
species*treatment*time	2, 2507	0.6430	0.5258
species*injection time*time	1, 2507	3.4647	0.0628
treatment*injection time*time	**2, 2507**	**7.3820**	**0.0006**
species*treatment*injection time*time	2, 2507	2.4033	0.0906

Contrasts	**adjusted p-value**
T. sirtalis – control vs. OB	0.1181
T. sirtalis – control vs. GB	0.1002
***R. tigrinus* – control vs. OB**	**0.0056**
***R. tigrinus* – control vs. GB**	**0.0186**
T. sirtalis control – pre vs. post-injection	1.0000
T. sirtalis OB – pre vs. post-injection	0.1980
***T. sirtalis* GB – pre vs. post-injection**	**0.0160**
R. tigrinus control – pre vs. post-injection	1.0000
***R. tigrinus* OB – pre vs. post-injection**	**0.0012**
R. tigrinus GB – pre vs. post-injection	0.1688

Table 6-3. Summary of best-fitting (ΔAIC = 1902) random coefficient model. Regression coefficient for intercept represents the average heart rate (bpm) of a *Rhabdophis tigrinus* of average mass in the control treatment at time 0. The model includes random effects for time (SD = 2.52), nested within snake ID (SD = 4.39), nested within species (SD = 4.39; residual variance = 1.68). Factors and interactions of factors that had significant effects on heart rate are bolded.

Term	Regression coefficient	S.E.	df	t-value	p-value
intercept	**64.89**	**0.84**	**2440**	**77.17**	**<0.0001**
time	**-0.06**	**0.01**	**99**	**-4.80**	**<0.0001**
GB	**-9.23**	**0.91**	**2440**	**-10.13**	**<0.0001**
OB	**-7.58**	**0.75**	**2440**	**-10.17**	**<0.0001**
T. sirtalis	**-23.01**	**0.89**	**2440**	**-25.77**	**<0.0001**
mass (g)	**-0.05**	**0.00**	**2440**	**-12.40**	**<0.0001**
time*GB	-0.02	0.02	2440	-1.14	0.25
time*OB	-0.02	0.01	2440	-1.78	0.08
time**T. sirtalis*	0.01	0.01	2440	0.39	0.69
GB**T. sirtalis***	**18.38**	**1.26**	**2440**	**14.59**	**<0.0001**
OB**T. sirtalis***	**15.52**	**1.37**	**2440**	**11.34**	**<0.0001**
time*GB**T. sirtalis***	**-0.10**	**0.02**	**2440**	**-4.53**	**<0.0001**
time*OB**T. sirtalis***	**0.10**	**0.03**	**2440**	**3.59**	**0.0003**

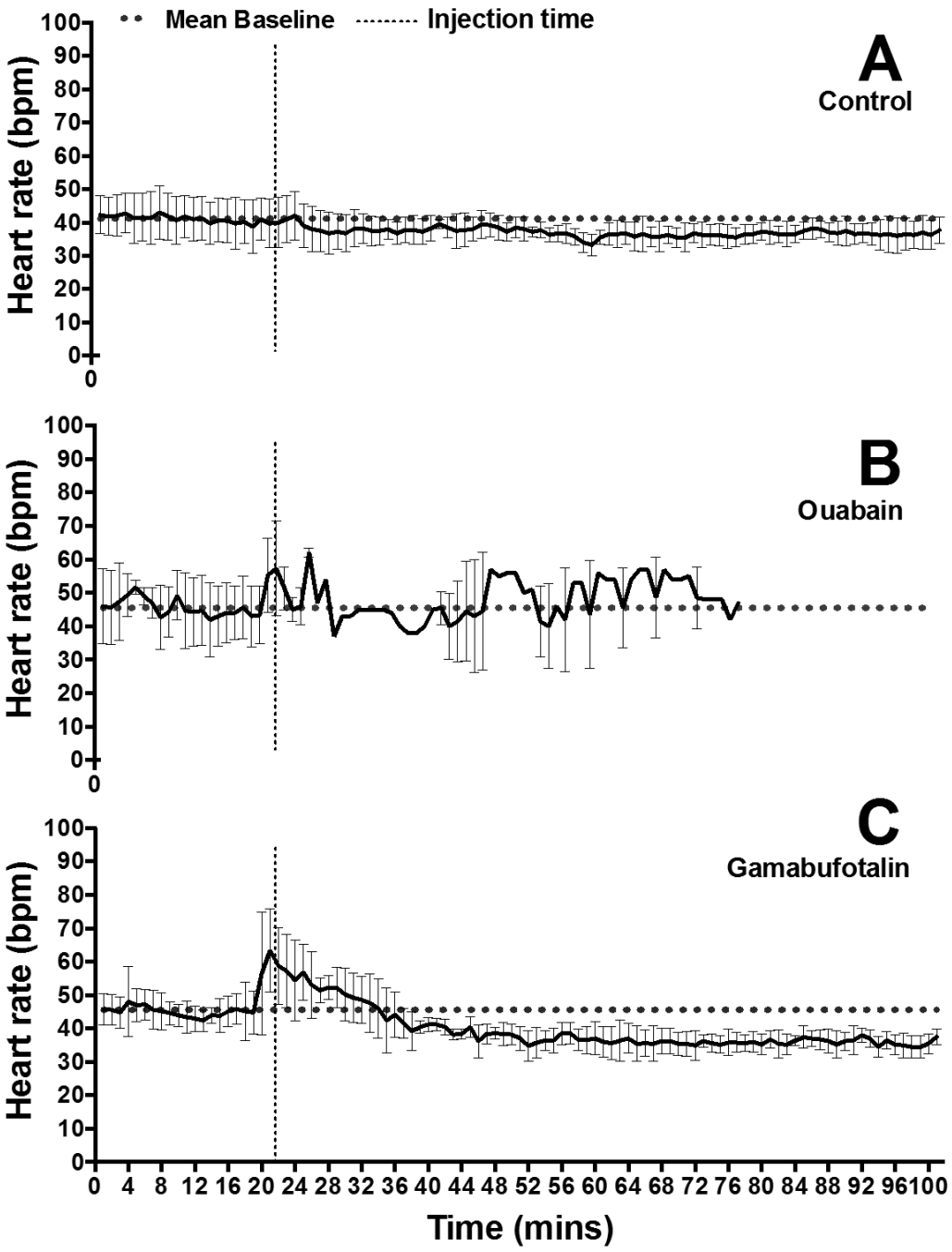

Figure 6-1. Mean post-injection heart rates over time of *Thamnophis sirtalis* in response to (A) control (5% DSMO; n = 4), (B) ouabain (n = 3), and (C) gamabufotalin (n = 6) injections. Mean baseline heart rate for each treatment (A-C) is represented by a red, dotted line. Minutes 21-28 represent the time immediately after snakes received treatment injections and are typically followed by an increase in heart rate as a response to the physical handling involved with the injection. Error bars represent standard error (± 1).

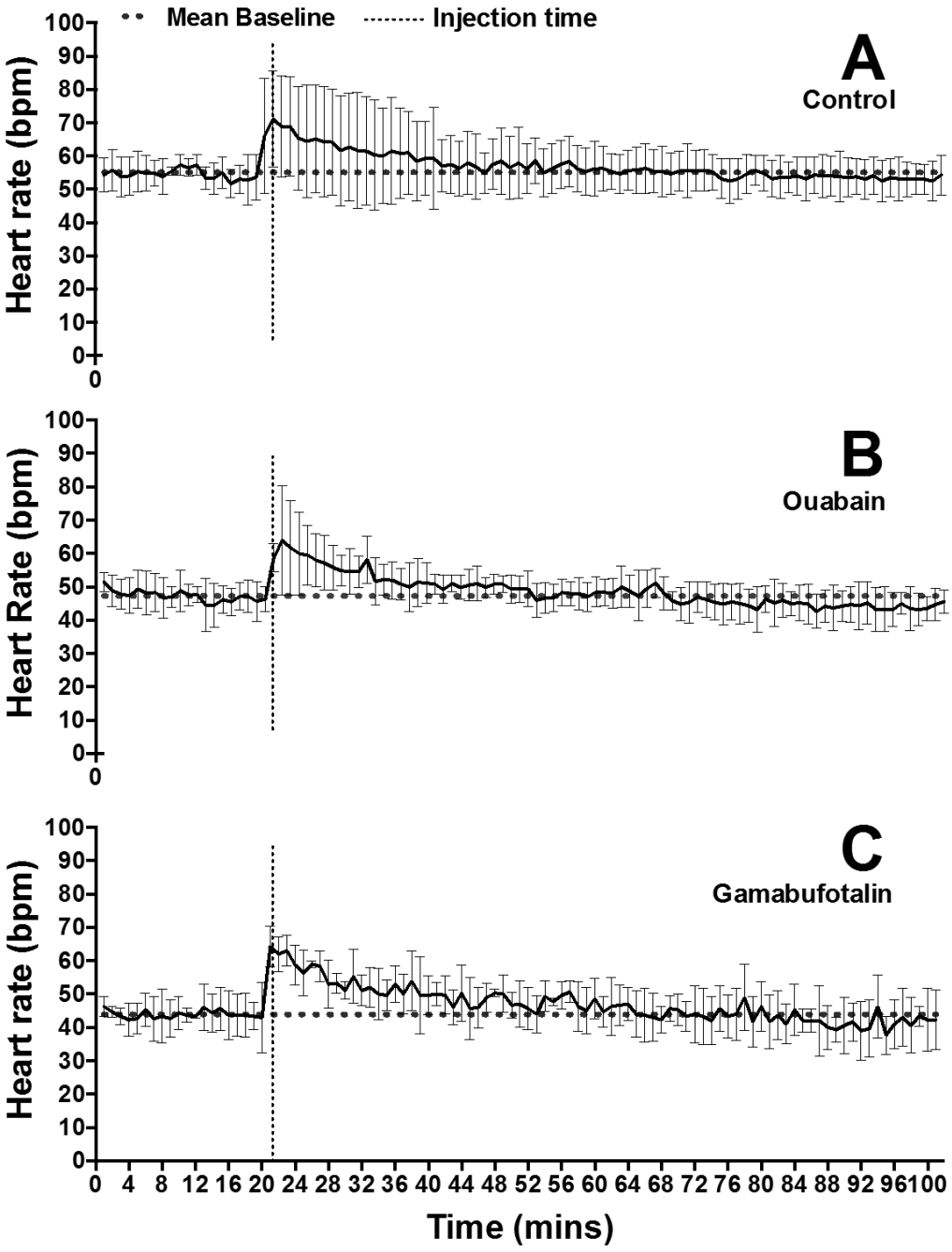

Figure 6-2. Mean post-injection heart rates over time of *Rhabdophis tigrinus* in response to (A) control (5% DSMO; n = 6), (B) ouabain (n = 6), and (C) gamabufotalin (n = 3) injections. Mean baseline heart rate for each treatment (A-C) is represented by a red, dotted line. Minutes 21-28 represent the time immediately after snakes received treatment injection and are typically followed by an increase in heart rate as a response to the physical handling involved with the injection. Error bars represent standard error (± 1).

DISCUSSION

The molecular mechanisms and evolutionary origins of cardiotonic steroid resistance among organisms have been, and continue to be, widely investigated (Dobler et al., 2012; Zhen et al., 2012; Ujvari et al., 2013; Dalla et al., 2013; Ujvari et al., 2015; Aardema and Andolfatto, 2016; Pierce et al., 2016; Mohammadi et al., 2016). Although physiological investigations of insects have revealed resistance-complementing functions, such as detoxification and compartmentalization of ingested toxins (Scudder and Meredith, 1982; Despres et al., 2007; Petschenka et al., 2013a; Petschenka et al., 2013b; Bramer et al., 2016), such studies are lacking in vertebrate systems. This study provides important insight into the physiology of cardiotonic steroid-resistant vertebrates. We have found that exposure to GB does not significantly affect the heart rate of *R. tigrinus* but causes a significant depression in heart rate of *T. sirtalis.* Exposure to OB caused significant changes in heart rate in both species (Figs. 6-1B and 6-2B). However, behavioral responses, including frequent body movements, prevented us from obtaining consistent post-injection heart rate measurements in OB-exposed *T. sirtalis*. We therefore acknowledge that our conclusions regarding OB-exposed *T. sirtalis* are based upon limited data. Additional study using wireless ECG system would be informative. Although the experimental constraints precluded a robust statistical comparison, we nevertheless observed a pattern of strong variation in heart rate and behavioral distress. This finding aligns with those of Mohammadi et al. (2017b), who showed that snakes of the genus *Thamnophis* had a significantly higher stress response (as measured by corticosterone) to OB exposure than to GB exposure. Mohammadi et al. (2017b) further showed that high stress response did not occur in *R. tigrinus* exposed to either of the two

toxins. Although we observed a statistically significant overall decrease in heart rate of *R. tigrinus* exposed to OB, there was no significant change in heart rate when exposed to GB (Fig. 6-2B and 6-2C). Conversely, *T. sirtalis* experienced a significant decrease in heart rate when exposed to GB (Fig. 6-1C).

Licht and Low (1968) conducted a similar experiment with *T. sirtalis*, using orally administered crude parotoid gland secretion from cane toads (*Rhinella marina*). Their results showed that at low doses (3 mg/g body mass and 10 mg/g body mass), the heart rate of *T. sirtalis* generally increased. At the highest dose (20 mg/g body mass) the heart rate increased, and then the snake died. Key differences in experimental procedures make comparison of our results with those of Licht and Low (1968) difficult. First, the exact composition of BDs and other compounds in the crude parotoid secretions was unknown. Furthermore, the highest dose administered far exceeded the amount that could be collected from a single toad and therefore represented a much higher dose than any snake would normally ingest at one time. Finally, we do not yet know how uptake of cardiotonic steroids differs between oral and intraperitoneal administration. The toxicity of a compound can vary between these two delivery routes, depending on the compound. Some are more toxic when ingested, whereas others are more toxic when administered by intraperitoneal injection (Stump et al., 1999; Abdel-Barry and Hakiem, 2000; Aune et al., 2002).

R. tigrinus sequesters dietary BDs in specialized defensive glands (Hutchinson et al., 2007; Mori et al., 2012). These snakes possess enlarged adrenal glands and produce high levels of aldosterone, a hormone secreted by the adrenal glands that signals the expression of Na^+/K^+-ATPases (Ikeda et al., 1991; Mohammadi et al., 2013; Mohammadi

et al., 2017a). It is therefore conceivable that *R. tigrinus* possesses physiological mechanisms that help it better deal with chronic exposure to the toxins. Although adrenal enlargement and increased adrenal hormone production suggest the possible existence of such mechanisms, further research is needed to elucidate what those mechanisms may be.

Both CDs and BDs have similar modes of action, although BDs are often considered more toxic. In a review of human case reports, Barrueto et al. (2006) found that out of 924 individuals reported to have ingested cardiotonic steroids, the mortality rate for CD ingestion was 6%, whereas that for BD ingestion was 29.6%. However, these data were compiled from individual case reports that did not address such variables as dose or body absorbance rates. Furthermore, BDs are most commonly ingested by humans in the form of *Chan Su*, a traditional Chinese medicinal tea stone whose BD concentration is not readily controlled (Chan et al., 1995; Ma et al., 2012; Barrueto et al., 2006). Differences in the form in which BDs and CDs are administered in traditional medicine may therefore influence their perceived toxicities.

Studies on the physiological effects of specific cardiotonic steroids in mammals have revealed that CDs generally cause depression of heart rate, whereas BDs cause an increase (reviewed in Dvela et al., 2007). However, these effects are more compound-specific than they are class-specific. For example, the CD ouabain and the BD bufalin both shorten the duration of the action potential of a cardiac contraction, whereas the CD digoxin does not have this effect (Kieval et al., 1988; Ruch et al., 2003; Dvela et al., 2007). The only established cellular receptor for cardiotonic steroids is the α-subunit of the Na^+/K^+-ATPase (Krenn and Kopp, 1998; Barrueto et al., 2006; Dvela et al., 2007). Differences in the physiological effects of various cardiotonic steroids are therefore most

likely explained by the way differing molecular structures affect the toxins' binding characteristics. Furthermore, the structure and distribution of various α-isoforms will also influence the physiological response. Therefore, a multitude of pathways can influence the biological responses of various cardiotonic steroids. We do not yet know the relative distribution of α-isoforms in snakes, with the exception of α-3, which contains resistance-conferring mutations in some species and is expressed at significantly higher levels in the heart than any other tissue (Mohammadi et al., 2017b). However, α-1 and α-2 remain to be investigated in snakes.

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CHAPTER 7

CONCLUSION

The preceding five chapters provide evidence that target-site insensitivity of the Na+/K+-ATPase (NKA) is necessary to survive acute toxicity of bufadienolides but it is not sufficient to explain all physiological mechanisms involved in tolerance of the toxins on a chronic basis. A broad survey of NKA genes in diverse lineages of snakes revealed that genetic resistance to bufadienolides is widespread among snakes, especially in the natricine and dipsadine lineages, which are almost entirely composed of resistant taxa. However, many species that exhibit molecular resistance are not, in fact, predators of toads, revealing that bufophagy and resistance to bufadienolides are not tightly coupled, contrary to the conclusions of previous studies.

A closer look at the tissue-specific distribution of sensitivity to the toxins in resistant species revealed that there is variation in different organs. Whereas the heart and kidneys are highly resistant, the brain, liver, and gut are sensitive. Organ-specific variation in bufadienolide sensitivity of resistant snakes suggests that there may be physiological mechanisms that offset the need for resistance in the sensitive organs. Indeed, the blood-brain barrier of snakes prevents ingested toxins from reaching the brain, which may explains the lack of resistance in that tissue. However, the mechanisms responsible for reduced resistance in the gut and liver remain to be elucidated.

Measurements of gene expression levels of *ATP1a3* mRNA, the NKA α-subunit paralog with resistance-conferring mutations, have revealed that the heart expresses significantly higher levels than any other tissue. Conversely, gut and kidney expressed

relatively low levels of *ATP1a3* mRNA, and the liver expressed almost undetectable levels. Furthermore, heart was the only tissue that significantly upregulated expression of *ATP1a3* in response to a snake's exposure to a bufadienolide. These results mirror those of the tissue-sensitivity assay, except in the case of kidney, which showed high resistance in the sensitivity assay, but low *ATP1a3* mRNA expression. It is possible that the kidneys express an alternate paralog of the NKA, one that might also carry resistance-conferring mutations. Although snakes possess three paralogs of the NKA α-subunit, including *ATP1a1*, *ATP1a2*, and *ATP1a3*, only one (*ATP1a3*) has been investigated. Future research should investigate the sequences of *ATP1a1* and *ATP1a2* and determine their tissue-specific expression levels.

Investigations of select physiological responses to cardiotonic steroid exposure have revealed evidence that bufophagy is accompanied with negative physiological consequences and that toad-specialized resistant species respond differently from nontoad-specialized but resistant species. The presence of physiological consequences of bufophagy could explain why it was secondarily lost in many lineages of snakes that retained resistance-conferring mutations. Testing the effects of cardiotonic steroids on heart rates in *Rhabdophis tigrinus* and *Thamnophis sirtalis* revealed strong fluctuations of heart rate in the former and significant depression of heart rate in the latter. These species differ in the amount of bufadienolides they encounter in their natural diets. Whereas *R. tigrinus* feeds regularly on toads and sequesters bufadienolides in its specialized nuchal glands, *T. sirtalis* does not often feed on toads. It is possible that a high degree of dietary specialization on toads is associated with differing physiological mechanisms for dealing with chronic exposure to the toxins, which might explain the difference in heart rate

responses observed between the two species. However, in order to confirm these associations, further replication through testing of additional, phylogenetically independent species is required.

Testing the effects of cardiotonic steroid exposure on the adrenal hormone responses of select snakes revealed that *R. tigrinus* possesses a strong aldosterone response. Because aldosterone acts in part as a signal for the expression of NKA, it is possible that these snakes produce high levels of the hormone to counteract the negative effects of chronic bufadienolide exposure. By increasing the number of resistant NKAs in certain tissues that receive high exposure to ingested bufadienolides, a snake may increase protection of that tissue. Future research should test the effects of aldosterone exposure on snakes' NKA (*ATP1a3*) gene expression to confirm this hypothesis. Testing the effects of cardiotonic steroids on the hormonal response of snakes also revealed that snakes of the genus *Thamnophis* have a significantly elevated stress response to a cardenolide, whereas they did not have that response when exposed to a bufadienolide. This suggests that the two classes of cardiotonic steroids have differing effects on the physiologies of these snakes, and it is interesting that we do not see the same pattern of responses in *R. tigrinus,* which did not exhibit a significant stress response to either toxin. Overall, the hormonal responses further indicate that the physiological mechanisms involved in dealing with the toxins differs between snakes that are adapted to feed regularly on toads and those that are resistant to, but do not feed regularly on, such toxic prey.

The studies comprising this dissertation provide an initial view of a complex adaptation. Although they advance our understanding of the mechanisms underlying

some of snakes' physiological capacity to consume toads, expansion of these studies, compare a greater diversity of species, will be necessary to explain many of the newly discovered patterns.

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Utah State University
Department of Biology

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Education

Ph.D. Utah State University – Biology **2011-2017**
Dissertation: Molecular and physiological mechanisms of toxin resistance in toad-eating snakes.
Supervisor: Alan H. Savitzky

M.S. Old Dominion University – Biological Sciences **2008-2011**
Thesis: A comparison of adrenal glands in toad-eating and nontoad-eating snakes.
Supervisor: Alan H. Savitzky

B.S. George Mason University – Biology **2002-2007**
Honors: Dean's List – Spring 2006

Research Statement

I use a highly integrative, diverse toolbox of skills to answer questions pertaining to molecular biology, physiology, and biochemistry in the context of evolutionary biology. My work is currently focused on understanding the molecular and physiological mechanisms of toxin resistance and toxin susceptibility in snakes and tracing the evolutionary origins of these adaptations.

Professional Appointments

Utah State University, Graduate Instructor Jan 2017-May 2017
Department of Biology, Logan UT
Supervisor: Alan H. Savitzky; savitzky@usu.edu; 435-797-1909

Utah State University, Graduate Teaching Assistant Aug 2011-May 2017
Department of Biology, Logan UT
Supervisor: Alan H. Savitzky; savitzky@usu.edu; 435-797-1909

Universität Hamburg, Visiting Research Fellow Apr-Jun 2016
Department of Zoology, Hamburg, Hamburg, Germany
Supervisor: Susanne Dobler; susanne.dobler@uni-hamburg.de

NSF East Asia and Pacific Summer Institute, Fellow Jun-Aug 2015
Kyoto University, Sakyo, Kyoto, Japan
Supervisor: Akira Mori; gappa@ethol.zool.kyoto-u.ac.jp

Smithsonian Encyclopedia of Life Rubenstein Program, Fellow Jan-Dec 2011
Smithsonian NMNH, Washington DC
Supervisor: Roy W. McDiarmid; mcdiarmidr@si.edu; 202-357-1932

Old Dominion University, Graduate Research Assistant Jan 2008- May 2011
Department of Biological Sciences, Norfolk VA
Supervisor: Alan H. Savitzky; savitzky@usu.edu; 435-797-1909

Field Museum of Natural History, Field Assistant (Thailand) Jun 2007-Aug 2007
Sakaerat Biosphere Reserve, Thailand
Supervisor: Jacques G. Hill; ngookhiew@yahoo.com

Smithsonian National Museum of Natural History, Research Student May 2005-2010
Smithsonian NMNH, Washington DC
Supervisor: Roy W. McDiarmid; mcdiarmidr@si.edu; 202-357-1932

Integrated Taxonomic Information System, Contractor Jun 2004- Jun 2006
Smithsonian NMNH, Washington DC
Supervisors: Thomas M. Orrell; orrellt@si.edu; 202-294-0514

U.S. Geologic Survey/Smithsonian, Intern Jul 2004-Jun 2005
Smithsonian NMNH, Washington DC
Supervisor: Robert P. Reynolds; reynolds@si.edu; 202-357-1932

Peer-Reviewed Publications **undergraduate coauthor*

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12. **Mohammadi, S.**, S.S. French, L.A. Neuman-Lee, Y. Kojima, A. Mori, E.D. Brodie, Jr., Y. Kojima, A. Mori, and A.H. Savitzky. *In press*. Corticosteroid responses of snakes to toxins from toads (bufadienolides) and plants (cardenolides) reflect differences in dietary specializations. ***General and Comparative Endocrinology***.

11. **Mohammadi, S.**, A.H. Savitzky, J. Lohr, and S. Dobler. 2017. Toad toxin-resistant snake (*Thamnophis elegans*) expresses high levels of mutant Na+/K+-ATPase mRNA in cardiac muscle. ***GENE.*** 614:21-25.

10. **Mohammadi, S.**, Z. Gompert , J. Gonzalez*, H. Takeuchi, A. Mori, and A.H. Savitzy. 2016. Mutant isoforms of the Na+/K+-ATPase are necessary but not sufficient to explain dietary specialization of snakes on toxic toads. ***Proceedings of the Royal Society B.*** 283:20162111 **[Featured cover article]**

9. Hayes, M.M., **S. Mohammadi**, A.E. Nicholson. 2016. Students of the American Society of Ichthyologists and Herpetologists. ***Copeia.*** 104:778-781. **[Invited contribution]**

8. Hopkins, G.R., E.D. Brodie, Jr., L.A. Neuman-Lee, **S. Mohammadi**, G.A. Brusch IV, Z.M. Hopkins, and S.S. French. 2016. Physiological responses to salinity vary with proximity to the ocean in a coastal amphibian. ***Physiological and Biochemical Zoology***. 89:322-330.

7. **Mohammadi, S.**, L.A. Neuman-Lee, E.D., Brodie, Jr., and A.H. Savitzky. 2016. Mutations to the cardiotonic steroid binding site of Na+/K+-ATPase are associated with high level of resistance to gamabufotalin in a natricine snake. ***Toxicon***. 114:13-15.

6. Smith, G.D., G.R. Hopkins, **S. Mohammadi**, H. Skinner, T. Hansen*, E.D. Brodie, Jr., and S.S. French. 2015. Effects of temperature on embryonic and early larval growth and development of the rough-skinned newt (*Taricha granulosa*). ***Journal of Thermal Biology***. 51:89-95.

5. **Mohammadi, S.**, B.M. Kluever, T. Tamashiro*, Y. Amano*, and J.G. Hill. 2014. Spatial and thermal observations of a Malayan Krait (*Bungarus candidus*) from Thailand. ***Tropical***

Natural History. 14:21-26.

4. **Mohammadi, S**., K.A. McCoy, D.A. Hutchinson, D.T. Gauthier, and A.H. Savitzky. 2013. Independently evolved toad-eating snakes exhibit sexually dimorphic enlargement of adrenal glands. ***Journal of Zoology***. 290:237-245. **[Featured cover article]**

3. E.E. Ferry*, G.R. Hopkins, A.N. Stokes, **S. Mohammadi**, E.D. Brodie, Jr., and B.G. Gall. 2013. Do all portable cases constructed by caddisfly larvae function in defense? ***Journal of Insect Science***. 13:1-9.

2. **Mohammadi, S.** and J.G. Hill. 2012. Dietary and behavioral notes on the red-necked keelback (*Rhabdophis subminiatus)* from northeast Thailand. ***Tropical Natural History***. 12:123-125.

1. Streicher, J.W. and **S. Mohammadi.** 2012. *Leptodactylus poecilochilus*. Geographic Distribution. ***Herpetological Review***. 43:97.

Other publications

1. **Mohammadi, S.** and C.D. McMahan. 2013. A review of : Venomous Reptiles of the United States, Canada, and Northern Mexico Volume 2: *Crotalus.* ***Copeia***. 2013:177-182.

Manuscripts in preparation

Mohammadi, S., G. Petschenka, A. Mori, Susannah S. French, and A.H. Savitzky. *In prep*. Tissue-specific variation in cardiotonic steroid sensitivity of Na+/K+-ATPase. Target journal: ***Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology***.

Mohammadi, S., T. Eggertsen*, N. Aardema*, Y. Kojima, A. Mori, D.A. Hutchinson, and A.H. Savitzky. *In prep.* Exposure to cardiac glycosides decreases cardiac contractility in snakes that possess resistance-conferring mutations of Na+/K+-ATPase. Target journal: ***Journal of Experimental Zoology.***

Hill, J.G, **S. Mohammadi**, B.M. Kluever, T. Tamashiro*, D. Bagsby*, Y. Amano*, D.R. Karns, K.Thirakupt. and H.K. Voris. *In prep*. Natural history of the big eyed pit viper (*Cryptelytrops macrops*): a tropical, arboreal pitviper, in northeast Thailand. Target journal: ***Copeia.***

Fellowships, Grants, and Awards *Total = $66,538*

2016 Utah State University, Department of Biology Doctoral Researcher of the Year
2016 ASIH Clark Hubbs Travel Award **($600**)
2016 USU RGS Dissertation Enhancement Grant (**$9,448**)
2015 USU College of Science ZoBell Scholarship for academic achievement **($1,000**)
2015 Joseph E. Greaves Scholarship in Biology (**$15,000**)
2015 USU Ecology Center Graduate Research Award **($3,800**)
2015 NSF East Asia and Pacific Summer Institute Fellowship **($10,000**)
2014 USU Ecology Center Travel Award (**$500**)
2014 USU Center for Women and Gender Travel Award **($500**)
2014 ASIH Clark Hubbs Travel Award (**$300**)
2014 International Society of Chemical Ecology Student Presentation Award (**$300**)

2014 USU Ecology Center Graduate Research Award (**$2,000**)
2013 Hansen Life Science Retreat Outstanding Poster Presentation Award (**$50**)
2013 USU Graduate Enhancement Award (**$4,000**)
2012 USU Research and Projects Grant (**$1,000**)
2012 USU Center for Women and Gender Research Grant (**$500**)
2012 SICB Grants-in-aid of Research (**$1,000**)
2011 Smithsonian Encyclopedia of Life Rubenstein Fellowship (**$8,000**)
2011 Virginia S. Bagley Scholarship (**$8,500**)

Teaching Experience

Courses taught as Instructor

Non-majors General Biology Lecture – Utah State University — Spring 2017
Herpetology Lab – Utah State University — 2014-2015
Human Physiology Lab – Utah State University — 2015

Guest lectures

Human Physiology (cardiovascular physiology) – Utah State University — 2014-2016
Comparative Animal Physiology Lab (snake physiology) – Utah State University — 2015
Herpetology (snake evolution and radiation) – Utah State University — 2015
Biology (evolution of dinosaurs) – Venture Academy (high school) — 2014
Evolution (snake evolution and phylogenetics) – Old Dominion University — 2010
Ecology (frog diversity in Costa Rica) – Northern VA Community College — 2006

Courses taught as Graduate Teaching Assistant

Human Physiology Lab – Utah State University — 2013-2016
Evolutionary Biology (science writing) – Utah State University — 2012-2016
Human Anatomy Lab – Utah State University — 2012-2013
General Biology Lab - Utah State University — 2011

Mentoring Experience

Mentored 11 undergraduates and 1 high school student
Includes 7 women and 4 under-represented minority students

Old Dominion University: Sarah Barns♀, Lucille Brunner, Donna Bucci, Amanda Bieber, Mandy Pearson, Marjorie Tabango, Michael Graham

Utah State University: Jonathan Gonzalez*✦♀, Dylan Bills*, Taylor Eggerston*, Niklas Ardema*, Savannah Knight (high school student)

**student presented or published their work ✦student acquired funding of their own ♀student went on to (or plans to go on to) graduate school*

Professional Societies, Workshops, and Training

Societies (within last 5 years): International Society of Chemical Ecology, American Society of Ichthyologists and Herpetologists, Society of Integrative and Comparative Biology, Society for the Study of Evolution.
Workshops: "How Can I Improve My Teaching Technique" (2016), USU Radiation Safety Training (2013-2015), Agilent Technologies HPLC Master Class (2014), Getting Started as a Successful Proposal Writer and Academician (2014), Quartzy Lab Management System (2014), Social Media and Academia Applications (2014), College Teaching Seminar (2011).

Notable Field Work

Kyoto Prefecture, Japan June-Aug 2015
Project: Physiological mechanisms of toad toxin resistance in *Rhabdophis tigrinus.*
Principal Investigators: Alan H. Savitkzy, Akira Mori

Sakaerat Biosphere Reserve, Nakon Ratchasima, Thailand May-Aug 2007
Project: Ecology and thermal biology of the big-eyed pit viper (*Trimeresurus macrops*)
Principal Investigators: Harold K. Voris, Jacques G. Hill

Braulio Carrillo National Park, Costa Rica Dec-Jan 2005
Project: Survey frog populations and collect specimens for the US National Museum amphibian and reptile collection.
Principal Investigator: myself

Braulio Carrillo National Park, Costa Rica Dec-Jan 2003
Project: Insect diversity study for the Smithsonian Arthropods of La Selva (ALAS) project.
Principal Investigators: David G. Furth

Professional Presentations and Seminars

** poster ** invited talk*

2016** **Mohammadi, S.** Molecular and physiological mechanisms of toxin resistance in snakes. Department of Biology, Whitman College, Walla Walla, WA. *[Departmental seminar]*

2016** **Mohammadi, S.** Molecular mechanisms and physiological consequences of toxin resistance in snakes. Department of Zoology, Universitàt Hamburg. Hamburg, Germany. *[Departmental seminar]*

2016** **Mohammadi, S.** Molecular mechanisms and physiological consequences of toxin resistance in snakes. Department of Biology, Georgia Southwestern State University, Americus, GA. *[Departmental seminar]*

2015* Gonzalez, J., **S. Mohammadi**, and A.H. Savitzky. The molecular basis for bufadienolide resistance in toad-eating snakes. Society for Advancing Chicanos/Hispanics and Native Americans in Science National Conference. Washington, DC. *[National STEM promotion-focused scientific meeting]*

2015* **Mohammadi, S.**, A.H. Savitzky ,G. Petschenka, and J. Gonzalez. Tissue-specific sensitivity to cardiotonic steroids in resistant and non-resistant snakes. Hansen Life Science Retreat. Logan, UT. *[Local biochemical scientific meeting]*

2015** **Mohammadi, S.** The evolution of bufadienolide resistance in snakes. Department of Zoology, Kyoto University. Sakyo, Kyoto, Japan. *[Departmental seminar]*

2014* **Mohammadi, S.**, A.H. Savitzky ,G. Petschenka, and J. Gonzalez. Tissue-specific sensitivity to cardiotonic steroids in resistant and non-resistant snakes. Hansen Life Science Retreat. Logan, UT. *[Local biochemical scientific meeting]*

2014 **Mohammadi, S.,** A.H. Savitzky, L. Neuman-Lee, and G. Petschenka. Molecular and physiological mechanisms of bufadienolide-resistance in Toad-Eating Snakes. JMIH. Chattanooga, TN. *[National scientific meeting]*

2014** **Mohammadi, S.,** A.H. Savitzky, and G. Petschenka. Molecular and Physiological Mechanisms of Bufadienolide-resistance in toad-eating snakes. International Society of Chemical Ecology Annual Meeting. Champaign-Urbana, IL. *[International scientific meeting]*

2013* **Mohammadi, S.**, D. Springthorpe, D. Bills, and A.H. Savitzky. A miniature transmitter for recording cardiac function in reptiles. Hansen Life Science Retreat. Logan, UT. *[Local biochemical scientific meeting]*

2012* **Mohammadi, S**., A. Mori, D.A. Hutchinson, and A.H. Savitzky. The evolution of toxin resistance in toad-eating snakes. World Congress of Herpetology. Vancouver, Canada. *[International scientific meeting]*

2010 **Mohammadi, S.,** A.H. Savitzky, and K.A. McCoy. A comparison of adrenal glands in toad eating and non-toad eating snakes. JMIH. Providence, RI. *[National scientific meeting]*

2010 Hill, J.G., D.S. McLeod, K.M. Hesed, **S. Mohammadi,** T. Artchawakom. Herpetofaunal diversity at Sakaerat Environmental Research Station, northeast Thailand: revisiting a historically important site. JMIH. Providence, RI. *[National scientific meeting]*

2009* **Mohammadi, S.,** K.A. McCoy, A.H. Savitzky. A comparison of adrenal glands in toad eating and non-toad eating snakes. JMIH. Portland, OR. *[National scientific meeting]*

Professional Services

2015-present	Science Advisory Board, Bioinformatics LLC member
2014-present	ASIH Web Content Committee member
2014-present	ASIH Long Range Planning Committee member
2016	USU Department of Biology and Ecology Center Candidate Search Committee
2014-2015	USU Biology Graduate Student Silent Auction organizer
2014-2015	USU Biology Graduate Student Plant Sale organizer
2014-2016	ASIH Centennial Planning Committee member
2014-2015	USU Graduate Student Fundraising Committee chair
2014	ASIH Graduate Student Committee chair
2013	ASIH Graduate Student Committee chair-elect
2012	USU Biology Graduate Student Association president
2011	USU Biology Graduate Student Association vice president
2011	ASIH Graduate Student Workshop Committee co-organizer
2010	Encyclopedia of Life online snakes and lizards curator

2009 ASIH Graduate Student Workshop organizer
Reviewer for: *Asian Herpetological Research*

Press

2016 NSF - https://nsf.gov/news/news_summ.jsp?cntn_id=190535&org=NSF&from=news
2016 AAAS Eureka Alert! - https://www.eurekalert.org/pub_releases/2016-11/usu-fwf112216.php
2016 ScienceDaily.com - https://www.sciencedaily.com/releases/2016/11/161122193506.htm
2016 Phys.org - http://phys.org/news/2016-11-feast-scientist-snake-species-resist.html
2016 Utah State Today - http://www.usu.edu/today/index.cfm?id=56330&nl=500
2016 Utah Public Radio - http://upr.org/post/snakes-resist-toad-toxin

Outreach and Volunteer Work

My public outreach efforts involve exhibiting live animals and demonstrating research materials to the general public with the aim of increasing science de-mystification and bridging the gap between scientists and the public.

2016 USU "Science Unwrapped" public outreach, Logan UT (**400+ visitors**)
2015 USU "Science Unwrapped" public outreach, Logan UT **(300+ visitors)**
2014 USU "Science Unwrapped" public outreach, Logan UT **(350+ visitors)**
2014 Venture Academy student outreach presentation, Ogden UT **(20+ students)**
2014 Thomas Edison Charter School science fair judge, Logan UT **(50+ entries)**
2013 Bear River M. Bird Refuge "Sense of Wonder Day", Brigham City, UT **(300+ visitors)**
2013 Edith Bowen School Science Night public outreach, Logan UT **(200+ visitors)**
2011 USU "Science Unwrapped" public outreach, Logan UT **(400+ visitors)**
2011 Smithsonian NMNH "The Scientist Is In" public outreach, Washington DC **(350 visitors)**
2007 Public school outreach tour about local herpetofauna, Thailand **(500+ students)**
2006 Clearing and staining specimen demonstration, Smithsonian NMNH (**20 summer interns**)

Languages

English (fluent)
Farsi (fluent)
French (fluent)