Corticosteroid responses of snakes to toxins from toads (bufadienolides) and plants (cardenolides) reflect differences in dietary specializations

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ABSTRACT

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.

Keywords: Bufadienolide, Snake, Corticosteroids, Corticosterone, Aldosterone, Na^+/K^+ -ATPase

Abbreviations: Bufadienolide (BD), cardenolide (CD), bufophagous and resistant (BR), generalist and resistant (GR), non-bufophagous and non-resistant (NN)

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. 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 cardiotoxic steroid

100 ingestion. As such, enlargement of the adrenal glands would be beneficial by allowing
101 higher-level production of these hormones. It is not yet known which of the adrenal
102 tissues is responsible for the overall enlargement of the glands in bufophagous snakes,
103 although the effect of BDs on Na^+/K^+ -ATPase suggests that the interrenal tissue is a
104 likely candidate.

105 Corticosterone is the primary glucocorticoid metabolite in reptiles (Norris, 2006;
106 Moore & Jessop 2003), and it plays important roles in mediating trade-offs during the
107 stress response (Sapolsky et al., 2000). Accordingly, plasma corticosterone levels are
108 used as a standard hormonal measure of organismal stress in a wide range of experiments
109 (Hopkins et al., 2016; French, 2008, 2007; Moore et al., 2000). Aldosterone is the
110 primary mineralocorticoid hormone and is essential for maintaining sodium
111 concentration, water balance, and blood pressure (Weber, 2001). The role of aldosterone
112 as a signal for the expression of Na^+/K^+ -ATPase has been widely investigated (Summa et
113 al., 2001; Ahmad and Medford, 1995; Ewart and Klip, 1995; Oguchi et al., 1993; Ikeda et
114 al., 1991; Verrey et al., 1987). This role suggests that adrenal enlargement in
115 bufophagous snakes might be a result of increased aldosterone production, which could
116 play a compensatory role in counteracting BDs by upregulating resistant Na^+/K^+ -
117 ATPases. Indeed, significant upregulation of resistant Na^+/K^+ -ATPases has been
118 observed in the cardiac tissues of snakes that were exposed to BDs (Mohammadi et al., in
119 review).

120 Diverse bufophagous snakes have also been found to possess adrenals that are not
121 only enlarged but also sexually dimorphic in size, with males possessing significantly
122 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 cardiotoxic 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 S1-S2).

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 S1). 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, 1.49×10^{-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 S1; 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 1.49×10^{-2} M/g of a commercially available BD, gamabufotalin (PiChemicals®, Lot # PI201406082068 and # PI1201206031583); a 1.49×10^{-2} M/g dose of a commercially available CD, ouabain (Acrōs Organics, Lot # A0343825); or a 1.49×10^{-1} M/g dose of gamabufotalin. The 1.49×10^{-2} and 1.49×10^{-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 S1) from the corticosterone assay and 47 snakes from the aldosterone assay (see Table S1). 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 S1) 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 Holm-Bonferroni corrections (Holm, 1969) 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 1-2 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 2A-B and 3 for graphs of raw data represented by this analysis.

Table 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 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

344

345 3.3. Corticosterone response to HIGH gamabufotalin challenge (resistant species groups 346 only)

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

351

352 **Table 3. Summary of ANCOVA results for the analysis of HIGH doses of**
353 **gamabufotalin on corticosterone response.** Factors that had significant effects on
354 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

355

356 3.4. Aldosterone response to LOW gamabufotalin and ouabain challenges

There was a significant effect of species group on aldosterone response (Tables 4-5), 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 4). See Figures 4A-B and 5 for graphs of raw data represented by this analysis.

Table 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. 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

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 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 for raw data represented by this analysis.

Table 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

4. 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. 2; Table 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 and Zhou, 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. 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. 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. 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. 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, 2014). 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. 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 4 and 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. 3). Although we were not able to test these differences statistically for the aldosterone responses, Figure 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 S1). 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 cardiotoxic 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 cardiotoxic steroids.

6. Author contributions

SM, AS, SF, EB, AM, and LN designed and coordinated the study. SM, LN, YK, AS, and AM collected snakes and provided additional research support. SM, LN, and YK performed experiments and conducted assays. SM and SD analyzed the data. SM, AS, and SD wrote the manuscript. All authors reviewed and edited the manuscript and approved the final version.

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Figure 1. Molecular structures of a bufadienolide (gamabufotalin) and a cardenolide (ouabain), redrawn from PubChem (2016), 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 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 1-3 for results of statistical analyses of these data. Error bars represent standard error.

Figure 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 1-2 for results of statistical analyses of these data. Error bars represent standard error.

Figure 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 4-6 for results of statistical analyses of these data. Error bars represent standard error.

Figure 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)

938 snakes challenged with a LOW dose of ouabain. Control challenge was 5% DMSO. See
939 Tables 4-5 for results of statistical analyses of these data. Error bars represent standard
940 error.

molecular weight = 402.52

molecular weight = 402.52

molecular weight = 728.27

molecular weight = 728.27