To stress or not to stress: Physiological responses to tetrodotoxin in resistant gartersnakes vary by sex

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Abstract

The activation of the hypothalamic pituitary adrenal (HPA) axis is one of the most important physiological processes in coping with any deviation in an organism's homeostasis. This activation and the secretion of glucocorticoids, such as corticosterone, allow organisms to cope with perturbations and return to optimal physiological functioning as quickly as possible. In this study, we examined the HPA axis activation in Common gartersnakes (*Thamnophis sirtalis*) as a response to a natural toxin, tetrodotoxin (TTX). This neurotoxin is found in high levels in the Rough-skinned Newt (*Taricha granulosa*), which is a prey item for these snakes. To consume this toxic prey, these snakes have evolved variable resistance. We hypothesized that the more resistant individuals would show a lower HPA axis response than less resistant individuals, as measured by corticosterone (CORT) and bactericidal ability, which is a functional downstream measurement of CORT's activity. We determined "resistance level" for tetrodotoxin from each individual snake by determining the dose which reduced race speed by 50%. Individuals were injected them with an increasing amount of tetrodotoxin (10, 25, and 50 MAMUs) to determine this value. Thirty minutes after every injection, we gathered blood samples from each snake. Our results show that, while there were no significant differences among individual CORT levels in a dose-dependent manner, female snakes did have a larger stress response when compared to both males and juveniles. Different life-histories could explain why females were able to mount a higher HPA axis response. However, TTX had no downstream effects on bactericidal ability, although juveniles had consistently lower values than adults. Our research shows a possible dichotomy between how each sex manages tetrodotoxin and gives way for a more comprehensive analysis of tetrodotoxin in an ecological context.

Introduction

Organisms have a suite of physiological responses to various external challenges, most of which are considered adaptive (Love and Williams, 2008; McEwen, 2007; Råberg et al., 1998). One of the most important and conserved of these responses is the initiation of the activation of hypothalamic-pituitary-adrenal axis (HPA; Wingfield and Romero, 2001; Wingfield, 2005). This system is responsible for interacting with other physiological systems by activating and/or suppressing functions, dependent upon context (Dhabhar, 2009; Sapolsky et al., 2000). While this activation has been examined in organisms for both anthropogenic and natural stressors, many questions remain as to the adaptive value of the HPA axis response and resulting physiological cascades (Dickens and Romero, 2013). There is a high amount of variation in both behavioral and neuroendocrine responses which are likely based on both genetic and environmental factors (Øverli et al., 2007; reviewed in Koolhaas et al., 1999; Korte et al., 2005).

Within this context, is the question of how organisms are adapted to respond to deviations from homeostasis that are regularly encountered within their specific environment. Theoretically, there should be an "optimal response," by which an organism does not over- or under-respond to these deviations (McEwen, 2007; Korte et al., 2005; Charmandari et al., 2004), especially if the challenge is frequently faced.

It would likely be beneficial for organisms to modulate their response based on evolutionary history with factors that might otherwise cause dramatic alterations in the homeostatic balance (Romero et al., 2009; Wingfield and Romero, 2001). Theoretical examinations of the HPA axis provide evidence that its responses are likely influenced by evolutionary pressures (Ellis et al., 2006). Further, these examinations are supported by an ever-growing body of work that provides tangible examples. Variation exists between individuals, but

also occurs between sexes and across life history stages (Lattin et al., 2012; Wada et al., 2008; Crespi et al., 2013; Moore and Jessop, 2003). These variances between sexes within species is especially important as the sexes often have disparate life history strategies due to the inherent cost of reproduction for females (Stearns, 1989; Madsen, 1987) and sex hormones which greatly influence the action of the HPA axis (von der Ohe and Servheen, 2002; Kajantie and Phillips, 2006; Wingfield and Sapolsky, 2003).

However, measuring HPA activation requires an examination of multiple factors (Breuner et al., 2013; Sapolsky et al., 2000). While glucocorticoids, such as corticosterone (CORT) in reptiles (Moore and Jessop, 2003), are most frequently used, these values have proven to be extremely complex (Dickens and Romero, 2013). Therefore, examining multiple measurements of HPA activation, such as downstream functional effects of CORT and the health of the animal, is necessary (Breuner et al., 2008). One increasingly common method is to analyze an organism's immune function (French et al., 2008). Acute increases in CORT typically correspond to increases in immune function, especially innate immunity (Dhabhar, 2002; Dhabhar and McEwen, 1997). However, studies in reptilian ecoimmunology have not shown consistent patterns as to the relationship between bactericidal ability and acute increases in CORT (Refsnider et al., 2015; Neuman-Lee and French, 2014; French et al., 2010), possibly because much is unknown about the reptilian immune system (Zimmerman et al., 2010). Finally, examining the physical condition of animals is important when assessing HPA responses because this process is engaged to mobilize energy (Sapolsky et al., 2000). Energetic stores, as measured by body condition, can have a large impact on the resulting HPA activation (Jaatinen et al., 2013; Moore et al., 2005; Smith et al., 1994; Moore et al., 2000).

To examine the potential variation of the HPA response between individuals, sexes, and ontogenetic stages, a system with measured variation that could correspond to HPA activation and downstream effects should be tested. Gartersnakes (*Thamnophis sirtalis*) have adapted the ability to consume the highly toxic Rough-skinned Newt (*Taricha granulosa*), however, this trait is highly variable across populations and even within populations (Brodie and Brodie, 1990). For example, across populations, resistance varies from very high (>90 MAMU; San Mateo, California) to very low (<4 MAMU; Bear Lake, UT) (Brodie and Brodie, 1990; Brodie et al., 2002). This is due to the fact that sympatric newt populations also have a high amount of variance in toxicity, which matches the snake resistance to toxicity (Hanifin et al., 1999). This pattern is evidence of an evolutionary arms race which results in increasing toxicity of the newts by the concurrent increase in toxicity resistance by the snakes (Brodie and Brodie, 1990; Brodie et al., 2002). However, there is variation in both the population of newts (Hanifin et al., 1999) and snakes (Brodie and Brodie, 1990) in toxicity and resistance, respectively. This system makes testing whether there is variation in the HPA response among individuals and between sexes and ontogenetic stages in relation to exposure to the toxin.

Rough-skinned Newts secrete the potent neurotoxin tetrodotoxin (TTX) when threatened (Brodie, 1968). Tetrodotoxin binds competitively to sodium ion channels in muscle tissue and causes paralysis, which leads to suffocation and death (reviewed in Narahashi, 2001). Many populations of gartersnakes have evolved changes in the proteins of the sodium channel, which blocks the binding of TTX and therefore resists the effects (Geffeney et al., 2005). Because the muscle tissue is affected, resistance to TTX can be measured using the behavioral metric of sprint speed (Brodie and Brodie, 1990). Thus a snake's resistance can be measured by determining how fast it moves with varying concentrations of TTX relative to no TTX.

In this study, we wanted to determine the individual snake's resistance to TTX and relate this resistance to its stress and immune response. We tested reproductive-age male and females and juveniles to examine potential sex and ontogenetic differences in the response in a dose-response manner. We hypothesized that individuals with higher resistance would have a lower CORT response to TTX exposure, but that the response would be dose-dependent. We further hypothesized that innate immunity would be positively correlated with increasing levels of CORT. Finally, we predicted that these relationships would be consistent across both sex and ontogenetic stage.

Methods

Collection

Common gartersnakes (*Thamnophis sirtalis*) were hand collected in April 2014 in Benton County, OR (n = 41; Females = 21, Males = 12, Juveniles = 8), and transported in individual opaque bags to Utah State University within 10 days of capture. Each individual was housed separately in a glass aquaria (37.8L) with newspaper substrate, water dish, and plastic hide box with moist sphagnum moss. Air temperatures were maintained at 27°C, snakes were allowed a thermal gradient using heat tape, and light was on a 12:12 on:off cycle. Snakes were all offered one meal of thawed mice (approximately 10% of their body mass) six days after arriving in the laboratory. All uneaten food was removed and recorded. Fifteen days after arrival at the laboratory, each snake was weighed to the nearest tenth of a gram, the snout-vent length was determined (SVL, distance from snout to cloaca), and the number of follicles in females was counted using an ultrasound (Sonosite, Micromaxx). All procedures were approved by the Utah State University IACUC (Protocol #2299).

Racing and determining resistance level

This bioassay was conducted as described by Brodie and Brodie (1990). Two days after measurements, we began the testing. All animals were tested on the same day in the same manner so that animals had been housed in captivity for the same amount of time. Briefly, twenty-four hours prior to racing, all snakes were removed from heat tape to ensure that each individual was the same temperature, as temperature affects racing speed (Brodie and Russell, 1999). To test maximal racing speed, each individual was removed from the aquaria and placed on a three-meter racetrack lined with astroturf. The investigator then lightly taps the tail of the snake to simulate a predator to ensure that the snake moves as quickly as possible. Four half-meter sections are measured using a digital timer and the fastest time is used. For the baseline measurement, this process was repeated twice, four hours apart, and the speeds averaged. All future racing speeds were calculated as a percentage of this maximal speed. Previous work has shown that snake speed is slowed linearly with TTX injections (Brodie and Brodie, 1990; Brodie et al., 2002). Therefore, by determining the dose at which the speed of the snake is reduced by 50% of the control run, individual resistance level can be calculated. Hereafter this is referred to as "resistance level."

Dosing and collecting blood samples

The day after the baseline race, each snake was given an intra-coelomic injection with a mass-adjusted dose of Ringer's solution. The snake was promptly put back into its aquarium for exactly 30 min. At 30 min, the snake was removed and raced down the track as described above. A blood sample was then collected via the caudal vein within one minute. All samples were stored on ice for less than one hr and centrifuged at 2400 rpms. The plasma was separated from the red blood cells and stored at -80°C until further processing.

Two days later, the snakes were injected with 10 mass-adjusted mouse units (MAMUs, i.e. the amount of TTX required to kill one gram of mouse; (Brodie and Brodie, 1990). This inital dose was selected due known resistance from previous investigations of this population. After 30 minutes, snakes were raced and bled as described before. The same process was repeated two days later using 25 MAMUs and then two more days later using 50 MAMUs. The maximum time that this took an individual was eight days. Because of the variation within the population, if an individual snake proved to be less resistant than 50% at any of the doses (its speed was decreased more than 50% at a given dose), it was not given the next highest dose to prevent killing it. In these cases, the snake's blood was not used in the statistical means given that it did not receive the same dose. The following are number of animals tested at Baseline: Females (F) = 21, Males (M) = 12, Juveniles (J) = 8); 10 MAMUs: F = 21, M = 12, J = 8; 25 MAMUs: F = 17, M = 11, J = 4; 50 MAMUs: F = 11, M = 8, J = 4.

Radioimmunoassay

Circulating corticosterone levels were determined using a previously described protocol (French et al., 2010; French et al., 2006; Moore, 1986). Samples were extracted using isooctane: ethyl acetate, dried, and resuspended in PBS buffer. Samples were assayed in duplicate for CORT (MP Biomedicals, Lot #3R3PB-19E) and the mean of the two were used in analysis. For each sample, we used an aliquot of the resuspended fractions to measure individual recoveries following extraction and chromatography. These recoveries were used to adjust final sample concentration values to account for any losses during these procedures. Standards of known value and negative controls were included in every assay as a reference to ensure accuracy. All samples were run in a single assay. Intra-assay variation was 12.1% and accuracy was 103.6%.

Bactericidal Ability

We performed the bactericidal assay (BKA) to measure innate immune function, following the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1:4 dilution of plasma with CO_2-Independent media (Gibco, Grand Island, NY) plus 4nM L-glutamine (Sigma-Aldrich), and 10^5 CPU (colony producing unit) *Escherichia coli* (EPowerTM Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN), and agar broth on a 96-well microplate. We calculated the background absorbance using BioRad xMark microplate reader. After a 12 hour incubation, we again read the absorbance and calculated the bactericidal ability by dividing the mean absorbance for each sample (run in duplicate) by mean absorbance for the positive controls (containing only media and bacterial solution), and multiplying by 100. This provides the percent bacteria killed relative the positive controls. Negative controls (containing media only) were also run to ensure contamination was absent. Inter-assay variation between plates was 1.1%.

Statistical Analyses

Body condition was calculated by taking the residual of mass and SVL (Neuman-Lee et al., 2015b) for each individual. Linear regressions of the residual against physiological measures were calculated for all snakes and within each sex class. Further, the number of follicles was regressed against the residuals for females.

Corticosterone was not normally distributed, so we $\log_{10}$-transformed the data for all analyses. We first measured possible differences between the sexes and juveniles (Male, female, juvenile) at each time-point (increasing values of TTX) using an analysis of variance (ANOVA). When appropriate, we compared the groups using a Tukey's pairwise comparison. To measure the effect of time on the sexes, we conducted a repeated measures ANOVA (rmANOVA). We repeated this procedure for the bactericidal ability, although the data were not normal and no

transformations were successful. Therefore, we conducted a Wilcoxon/Kruskal-Wallis to determine sex effects and a Wilcoxon each pair test for pair-wise comparisons.

We measured the possible differences in resistance level between the sexes and juveniles by $\log_{10}$-transforming the resistance values and conducting an ANOVA. To determine the possible effect of individual resistance level on corticosterone and bactericidal ability, we conducted linear regressions of the individual resistance level against the $\log_{10}$ CORT. We could not meet the assumption of normality for the bactericidal ability data, thus we conducted a Spearman's test. Bonferroni corrections were made to all linear regressions to reduce a Type I error and for these tests, the $\alpha = 0.003$.

Females were separated by those with verifiable follicles against those that were non-reproductive. A t-test was conducted then conducted for CORT and resistance level, a Wilcoxon test for bactericidal ability. All analyses were conducted in JMP 11.0 (SAS Institute, Inc, 2013).

Results

Body Condition

Mass was significantly different between sex classes ($F_{(2,38)} = 80.97$, $p < 0.0001$), with females being the largest and juveniles being the smallest (g ± 1 standard error; Females 51.79 ± 5.38, n = 21; Males 31.32 ± 2.33, n = 12; Juveniles 6.95 ± 0.49, n = 8).

Body condition was significantly different among the three groups (Male, female, juvenile; $F_{(2,38)} = 18.84$, $p < 0.0001$), with no significant difference between the female and juveniles, but a difference between both of them and males. However, this was likely due to females having a higher condition due to developing follicles and thus made the females have larger mass. The number of follicles each female had was strongly positively related with female body condition ($R^2 = 0.72$, $p < 0.0001$).

Body condition had varying relationships with corticosterone. Overall, body condition did not affect corticosterone concentrations at any time point ($p > 0.09$). When each sex was analyzed, body condition was negatively correlated with CORT only for males at 25 MAMU ($R^2 = 0.68$, $F_{(1,10)} = 21.32$, $p = 0.001$). Body condition was not related to bactericidal ability at any time point ($p > 0.1$).

Reproduction

At the onset of the study, 14 of the 21 females had verifiable follicles. No factors (CORT, BKA, resistance level) were influenced by follicle presence ($p > 0.15$).

Corticosterone

At baseline (after saline injection), there was no difference in CORT concentration between the sexes (Male, Female, Juvenile; Figure 1, $F_{(2,38)} = 0.921$, $p = 0.41$). The rmANOVA revealed a significant effect of sex and sex*time (Time: $F_{(3,95.8)} = 1.01$, $p = 0.39$; Sex: $F_{(2, 38.3)} = 12.62$, $p < 0.0001$; Sex*Time: $F_{(6, 95.7)} = 4.86$, $p = 0.0002$). After the first toxin injection (10 MAMU), female levels were significantly higher than either juveniles or males ($F_{(2,38)} = 11.84$, $p < 0.0001$). Males and juveniles did not significantly differ from each other. Female levels of CORT remained significantly higher for both the second ($F_{(2,37)} = 14.76$, $p < 0.0001$) and third ($F_{(2,20)} = 4.80$, $p = 0.02$) toxin injections.

Bactericidal ability

At baseline, there was a significant difference between the juveniles and either males or females, but not between males and females (Figure 2, $X^2 = 8.55$, $p = 0.014$, d.f. 2). Juvenile bactericidal ability continued to be significantly lower than either males or females for the remaining injections (1^{st}: $X^2 = 9.89$, $p = 0.007$; 2^{nd}: $X^2 = 12.96$, $p = 0.002$; 3^{rd}: $X^2 = 8.55$, $p = 0.014$). The rmANOVA reflected these analyses showing no significant effect of time, but only

of sex (Time: $F_{(3,85.4)} = 0.87$, $p = 0.46$; Sex: $F_{(2, 38.7)} = 11.39$, $p < 0.0001$; Sex*Time: $F_{(6, 85.1)} = 0.44$, $p = 0.85$).

Resistance

There was no difference among the sexes (Male, Female, Juvenile) in terms of resistance level ($F_{(2,38)} = 0.832$, $p = 0.44$; Resistance in MAMUs ± 1 standard error: Females 42.67 ± 10.80, Males 48.76 ± 8.82, Juveniles 45.86 ± 17.66). There was a high amount of variation between individuals (Figure 3). There was no relationship between individual resistance level and CORT or bactericidal ability, regardless of time-point (p values between 0.17 and 0.98). Further, none of the parameters were related to individual resistance level (p values between 0.13 and 0.91).

Discussion

The current study provides evidence that female gartersnakes may respond differently to TTX than either males or juveniles. Females had an elevated CORT response after the first TTX injection that remained throughout the next three injections, while the males and juveniles did not elevate their CORT levels above baseline at any dose. Individual TTX resistance was not related to CORT levels. This CORT response was not related to body condition in females, yet was negatively correlated to body condition for males at the second (25 MAMU) injection. Finally, TTX and CORT had no apparent effect on bactericidal ability, although overall juveniles had significantly lower innate immune function than adults.

Contrary to our predictions, our study showed no evidence that individual snakes modulated their CORT response to TTX based on their individual resistance, as a measure of homeostatic deviation. However, evidence that fitness and health of an organism is directly related to CORT has proven elusive (Bonier et al., 2009a; Bonier et al., 2009b; Dickens and Romero, 2013) and additional measurements, such as other immune metrics and corticosteroid

binding globulins, may be necessary to provide a more clear picture of the mechanisms behind the HPA axis functioning (Breuner et al., 2013).

When examining the additional measure of immune function, we found no effect of TTX in either sex. Juveniles had a consistently lower level of bactericidal ability, as is seen in many species where juveniles do not have a fully developed immune system. Bactericidal ability can be seen as a functional measurement of health given that organisms in the wild must frequently cope with pathogens (French and Neuman-Lee, 2012). In reptiles, bactericidal ability is especially useful given the high reliance on non-specific innate immune responses (Zimmerman et al., 2010).

The increase in CORT associated with TTX exposure for females, but not males or juveniles, may occur for many reasons, none of which are mutually exclusive: 1) It may be adaptive for females to have a stronger stress response, 2) Males may be energetically less able to mount a stress response after emergence and mating, 3) Females may be able to redirect energy from developing follicles or existing fat stores toward the stress response, 4) TTX may serve as an anticipatory signal for larger snakes (females) that a meal is about to be ingested, and/or 5) Other physiological mechanisms, such as sex steroids, play a role in enhancing the stress response.

A key component of most life-history trade-off theories is that a high amount of energetic investment is required to reproduce (Stearns, 1989; Wade and Schneider, 1992). However, in the vast majority of organisms, the female expends the bulk of this energy by producing eggs, carrying eggs/embryos, supplying nutrients to developing embryos, and/or providing nutrients after parturition (lactation (Wade and Schneider, 1992; Blackburn and Flemming, 2009)). Therefore, it is likely that there are major differences by which males and females have adapted

due to this inherent dissimilarity in energetic expenditure for reproduction. It may be beneficial for sexes to have a different reaction norm to a similar stressor. Females also may have a higher stress reactivity because they must have a more proactive coping style to ensure not only her survival, but that of her young (Kajantie and Phillips, 2006). However, in some species, females show a reduced stress response relative to males to prevent abandoning already vested eggs or offspring (Bokony et al., 2009). Females in this study, however, were still early in reproduction and therefore able to resorb follicles if necessary.

The second possibility for the lack of response in the males is that males had a negative energy balance and were therefore unable to mount a stress-response (Wingfield, 2005). Moore et al. (2000) provided evidence that males from this population have low body condition after emerging from overwintering, likely due to the lack of prey. Interestingly, this study found that males have the some of the lowest concentrations of CORT during this time frame as well. This study did not examine females, but another study with gartersnakes showed that females have a significantly higher fat body index than males throughout and after hibernation (Costanzo, 1985).

In conjunction with emerging from hibernation with a lower body condition, males also typically emerge earlier than females (Gregory, 1974; Shine et al., 2001), forcing them to use more energetic stores while waiting for females to emerge. Males may expend this valuable energy in courtship and vying for a female and will often remain at the hibernaculum site for a longer period of time, while females will disperse rapidly and likely begin foraging (Gregory, 1974; Shine et al., 2001). While gartersnakes do not display energetically costly combat as in other species (e.g. rattlesnakes), they still must compete to mate with the female (Shine and Mason, 2005). Throughout the entire mating period, male gartersnakes undergo a period of anorexia, further compounding their lower body condition (O'Donnell et al., 2004).

Thirdly, in capital breeders, such as snakes, the majority of the energetic requirements for reproduction is obtained prior to or early in reproduction (Doughty and Shine, 1997), allowing females the possibility to divert this energy away from developing follicles.

Our fourth explanation of the enhanced female reactivity comes from the nature of the source of TTX. Rough-skinned newts average 5.7-8.9 cm (Stebbins, 2003), and therefore only larger snakes may be regularly ingesting adult newts. There is a considerable amount of work which shows that CORT increases in individuals as an anticipatory hormone prior to eating (Stephan, 2002; Krieger, 1974; Honma et al., 1984). While this model has mostly been applied to lab rodents that are on a regular feeding schedule, the mechanism behind this repeated observation has not been fully determined (Stephan, 2002). When a snake first bites a newt, the newt begins secreting TTX, thus exposing the snake orally to the toxin. This may serve as a signal that food will be ingested shortly. However, this seems an unlikely scenario given that there is a high latency to eating in snakes (Rossman et al., 1996) and an inherent unpredictability for predators to find prey in the wild.

The fifth possibility states that other physiological mechanisms inherent in females may interact with and enhance CORT concentrations. A large body of research has revealed that females will often have increased levels of glucocorticoids after experiencing a stressor, likely due to elevated estradiol levels (reviewed in Kudielka and Kirschbaum, 2005). Estradiol levels are known to enhance corticotropin-releasing factor gene transcription in humans (Vamvakopoulos and Chrousos, 1993), increase both adrenocorticotropic hormone and glucocorticoids (Kitay, 1963), decrease receptor-mediated negative feedback (thereby increasing CORT responsiveness (Burgess and Handa, 1992)), and increases the production of

corticosteroid-binding globulins (Moore et al., 1978). Taken together, it is possible that the stress-response to TTX was, in part, enhanced by estradiol.

Given the design of the study, whereby we controlled for the effects of injection and antipredator behavior in order to isolate the effects of TTX on CORT, we were unable to take true baseline CORT measurements. However, males and juveniles did not mount an additional response to TTX when compared to vehicle control, whereas females did, providing strong evidence for sex differences in reactivity. Interestingly, we found a different result when we subjected a subset of adult individuals in this study to an acute handling stressor (baseline sample then 30 minutes after initial capture) in the field. From these data, we know that the sexes responded similarly to this acute handling stress (Males (n = 12) baseline CORT = 21.8 ± 6.8 and stress-induced CORT = 88.6 ± 18.9 and Females (n = 13) baseline CORT = 19.4 ± 6.8 and stress-induced CORT = 93.9 ± 18.9 Neuman-Lee, unpublished data). Moreover, in a different study testing the effects of an anthropogenic contaminant (polybrominated diphenyl ether), female snakes did not have elevated CORT throughout the study nor a significant change in magnitude compared to the control (Neuman-Lee et al., 2015a). An additional complication in our interpretation was the increasing dose of TTX with each snake. We may have seen different results had we tested snakes with only a higher dose instead of starting at a lower dose and then increasing the dosage.

We used behavior as a metric of resistance (standardized protocol in TTX research) and thus our study also tested possible relationships between antipredator response and CORT. While some studies have shown certain antipredator behaviors are associated with elevated levels of CORT (Thaker et al., 2009a, b), CORT may not always be correlated with behavior (Neuman-Lee et al., 2015). In this case, it is likely that the physiological effects of TTX (i.e. paralysis of

muscular tissue (Narahashi, 2001)) are the primary control mechanisms of racing antipredator behavior.

Our current study demonstrates a clear difference between sexes in responses to an ecologically and evolutionarily relevant toxin. This is surprising given that both sexes are known to ingest toxic newts in the wild, and that the sexes respond similarly to other controlled stressors (capture and handling stress). The difference between the sexes in the hormonal response to tetrodotoxin illustrates a potential divergence in strategies for coping with a toxic byproduct of a valuable food source. This divergence may be driven by classic evolutionary reproductive conflicts in energy use and requirements or physiological differences between the sexes. However, we did not see divergence in bactericidal ability between the sexes, indicating that TTX exposure may not be related to innate immune function. More work is needed testing responses to TTX across varying reproductive and energy states to disentangle the sex related differences that we observed.

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References

Blackburn D, Flemming A (2009) Morphology, development, and evolution of fetal membranes and placentation in squamate reptiles. J Exp Zool 312B (6):579-589

Bokony V, Lendvai AZ, Liker A, Angelier F, Wingfield JC, Chastel O (2009) Stress response and the value of reproduction: are birds prudent parents? The Amer Nat 173 (5):589-598

Bonier F, Martin PR, Moore IT, Wingfield JC (2009a) Do baseline glucocorticoids predict fitness? Trends Ecol Evol 24 (11):634-642

Bonier F, Moore IT, Martin PR, Robertson RJ (2009b) The relationship between fitness and baseline glucocorticoids in a passerine bird. Gen Comp Endocrinol 163 (1–2):208-213

Breuner CW, Delehanty B, Boonstra R (2013) Evaluating stress in natural populations of vertebrates: total CORT is not good enough. Funct Ecol 27 (1):24-36
Breuner CW, Patterson SH, Hahn TP (2008) In search of relationships between the acute adrenocortical response and fitness. Gen Comp Endocrinol 157 (3):288-295
Brodie E, Brodie E, Jr. (1990) Tetrodotoxin resistance in Garter Snakes: An evolutionary response of predators to dangerous prey. Evolution 44 (3):651-659
Brodie ED, III, Russell NH (1999) The consistency of individual differences in behaviour: Temperature effects on antipredator behaviour in garter snakes. Anim Behav 57 (2):445-451
Brodie ED, Jr. (1968) Investigations on the skin toxin of the adult rough-skinned newt, *Taricha granulosa*. Copeia (2):307-313
Brodie ED, Jr., Ridenhour BJ, Brodie ED, III (2002) The evolutionary response of predators to dangerous prey: Hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution 56 (10):2067-2082
Burgess LH, Handa RJ (1992) Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. Endocrin 131 (3):1261-1269
Charmandari E, Tsigos C, Chrousos G (2004) Endocrinology of the stress response. Annu Rev Physiol 67 (1):259-284
Costanzo JP (1985) The Bioenergetics of Hibernation in the Eastern Garter Snake *Thamnophis sirtalis sirtalis*. Physiol Zool 58 (6):682-692
Crespi EJ, Williams TD, Jessop TS, Delehanty B (2013) Life history and the ecology of stress: how do glucocorticoid hormones influence life-history variation in animals? Funct Ecol 27 (1):93-106
Dhabhar FS (2002) A hassle a day may keep the doctor away: Stress and the augmentation of immune function. Integr Comp Biol 42 (3):556-564
Dhabhar FS (2009) Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation 16 (5):300-317
Dhabhar FS, McEwen BS (1997) Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. Brain, Behav, and Imm 11 (4):286-306
Dickens MJ, Romero LM (2013) A consensus endocrine profile for chronically stressed wild animals does not exist. Gen Comp Endocrinol 191 (0):177-189
Doughty P, Shine R (1997) Detecting life history trade-offs: measuring energy stores in "capital" breeders reveals costs of reproduction. Oecologia 110 (4):508-513
Ellis BJ, Jackson JJ, Boyce WT (2006) The stress response systems: Universality and adaptive individual differences. Dev Rev 26 (2):175-212
French S, Fokidis H, Moore M (2008) Variation in stress and innate immunity in the tree lizard (*Urosaurus ornatus*) across an urban-rural gradient. J Comp Physiol B: Biochem, Systemic, Environ Physiol 178 (8):997-1005
French SS, DeNardo DF, Greives TJ, Strand CR, Demas GE (2010) Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (*Amblyrhynchus cristatus*). Horm Behav 58 (5):792-799
French SS, Neuman-Lee LA (2012) Improved ex vivo method for microbiocidal activity across vertebrate species. Biology Open 1 (5):482-487

Geffeney SL, Fujimoto E, Brodie ED, Jr, Ruben PC (2005) Evolutionary diversification of TTX-resistant sodium channels in a predator–prey interaction. Nature 434 (7034):759-763
Gregory PT (1974) Patterns of spring emergence of the red-sided garter snake (*Thamnophis sirtalis parietalis*) in the Interlake region of Manitoba. Can J Zool 52 (8):1063-1069
Hanifin C, Yotsu-Yamashita M, Yasumoto T, Brodie E, III, Brodie E, Jr. (1999) Toxicity of Dangerous Prey: Variation of Tetrodotoxin Levels Within and Among Populations of the Newt *Taricha granulosa*. J Chem Ecol 25 (9):2161-2175. doi:10.1023/a:1021049125805
Honma KI, Honma S, Hiroshige T (1984) Feeding-associated corticosterone peak in rats under various feeding cycles. Amer J Physiol - Regulatory, Integ Comp Physiol 246 (5):R721-R726
Jaatinen K, Seltmann MW, Hollmén T, Atkinson S, Mashburn K, Öst M (2013) Context dependency of baseline glucocorticoids as indicators of individual quality in a capital breeder. Gen Comp Endocrinol 191:231-238
Kajantie E, Phillips DIW (2006) The effects of sex and hormonal status on the physiological response to acute psychosocial stress. Psychoneuroendocrinology 31 (2):151-178
Kitay JI (1963) Effects of estradiol on pituitary-adrenal function in male and female rats 1. Endocrinol 72 (6):947-954
Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MAW, Blokhuis HJ (1999) Coping styles in animals: current status in behavior and stress-physiology. Neurosci Biobehav Rev 23 (7):925-935
Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005) The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci Biobehav Rev 29 (1):3-38
Krieger DT (1974) Food and water restriction shifts corticosterone, temperature, activity and brain amine periodicity. Endocrinol 95 (5):1195-1201
Kudielka BM, Kirschbaum C (2005) Sex differences in HPA axis responses to stress: a review. Biol Psychol 69 (1):113-132
Lattin CR, Bauer CM, de Bruijn R, Michael Romero L (2012) Hypothalamus-pituitary-adrenal axis activity and the subsequent response to chronic stress differ depending upon life history stage. Gen Comp Endocrinol 178 (3):494-501
Love OP, Williams TD (2008) The adaptive value of stress-induced phenotypes: effects of maternally derived corticosterone on sex-biased investment, cost of reproduction, and maternal fitness. The Amer Nat 172 (4)
Madsen T (1987) Cost of reproduction and female life-history tactics in a population of Grass Snakes, *Natrix natrix*, in southern Sweden. Oikos 49 (2):129-132
McEwen BS (2007) Physiology and Neurobiology of Stress and Adaptation: Central Role of the Brain. Physiol Rev 87 (3):873-904
Moore DE, Kawagoe S, Davajan V, Nakamura RM, Mishell DR (1978) An in vivo system in man for quantitation of estrogenicity. Am J Obstet Gynecol 130 (4):475-481
Moore I, Greene M, Lerner D, Asher C, Krohmer R, Hess D, Whittier J, Mason R (2005) Physiological evidence for reproductive suppression in the introduced population of brown tree snakes (*Boiga irregularis*) on Guam. Biol Conserv 121 (1):91-98
Moore IT, Jessop TS (2003) Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Horm Behav 43 (1):39-47

Moore IT, Lerner JP, Lerner DT, Mason RT (2000) Relationships between annual cycles of testosterone, corticosterone, and body condition in male red-spotted garter snakes, *Thamnophis sirtalis concinnus*. Physiol Biochem Zool 73 (3):307-312
Narahashi T (2001) Pharmacology of tetrodotoxin. Toxin Reviews 20 (1):67-84
Neuman-Lee LA, Carr J, Vaughn K, French SS (2015a) Physiological effects of polybrominated diphenyl ether (PBDE-47) on pregnant gartersnakes and resulting offspring. Gen Comp Endocrinol 219:143-151
Neuman-Lee LA, Fokidis HB, Spence A, Van der Walt M, Smith GD, Durham S, French SS (2015b) Food restriction and chronic stress alter energy use and affect immunity in an infrequent feeder. Funct Ecol 29 (11):1453-1462
Neuman-Lee LA, French S (2014) Wound healing reduces stress-induced immune changes: evidence for immune prioritization in the side-blotched lizard. J Comp Physiol B 184 (5):623-629
Neuman-Lee LA, Stokes AN, Greenfield S, Hopkins GR, Brodie ED, Jr., French SS (2015) The role of corticosterone and toxicity in the antipredator behavior of the Rough-skinned Newt (*Taricha granulosa*). Gen Comp Endocrinol 213:59-64
O'Donnell RP, Shine R, Mason RT (2004) Seasonal anorexia in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. Behav Ecol Sociobiol 56 (5):413-419
Øverli Ø, Sørensen C, Pulman KGT, Pottinger TG, Korzan W, Summers CH, Nilsson GE (2007) Evolutionary background for stress-coping styles: Relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. Neurosci Biobehav Rev 31 (3):396-412
Råberg L, Grahn M, Hasselquist D, Svensson E (1998) On the adaptive significance of stress-induced immunosuppression. Proc Roy Soc Lond B: Biol Sci 265 (1406):1637-1641
Refsnider JM, Palacios MG, Reding DM, Bronikowski AM (2015) Effects of a novel climate on stress response and immune function in painted turtles (*Chrysemys picta*). Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 323 (3):160-168
Romero LM, Dickens MJ, Cyr NE (2009) The reactive scope model — A new model integrating homeostasis, allostasis, and stress. Horm Behav 55 (3):375-389
Rossman DA, Ford NB, Seigel RA (1996) The garter snakes: Evolution and ecology. University of Oklahoma Press, Norman
Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21 (1):55-89
Shine R, Elphick MJ, Harlow PS, Moore IT, LeMaster MP, Mason RT, Price AH (2001) Movements, mating, and dispersal of Red-Sided Gartersnakes (*Thamnophis sirtalis parietalis*) from a communal den in Manitoba. Copeia 2001 (1):82-91
Shine R, Mason RT (2005) Does large body size in males evolve to facilitate forcible insemination? A study on garter snakes. Evolution 59 (11):2426-2432
Smith GT, Wingfield JC, Veit RR (1994) Adrenocortical Response to Stress in the Common Diving Petrel, *Pelecanoides urinatrix*. Physiol Zool 67 (2):526-537
Stearns SC (1989) Trade-offs in life-history evolution. Funct Ecol 3 (3):259-268
Stebbins RC (2003) Western Reptiles and Amphibians. Peterson Field Guides, 3rd edn. Houghton Mifflin Company, New York, New York
Stephan FK (2002) The "other" circadian system: Food as a zeitgeber. J Biol Rhythms 17 (4):284-292

Thaker M, Lima SL, Hews DK (2009a) Acute corticosterone elevation enhances antipredator behaviors in male tree lizard morphs. Horm Behav 56 (1):51-57
Thaker M, Lima SL, Hews DK (2009b) Alternative antipredator tactics in tree lizard morphs: hormonal and behavioural responses to a predator encounter. Anim Behav 77 (2):395-401
Vamvakopoulos NC, Chrousos GP (1993) Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. Potential implications for the sexual dimophism of the stress response and immune/inflammatory reaction. J Clin Investig 92 (4):1896
von der Ohe CG, Servheen C (2002) Measuring stress in mammals using fecal glucocorticoids: opportunities and challenges. Wildl Soc Bull 3 (4):1215-1225
Wada H, Salvante KG, Stables C, Wagner E, Williams TD, Breuner CW (2008) Adrenocortical responses in zebra finches (*Taeniopygia guttata*): Individual variation, repeatability, and relationship to phenotypic quality. Horm Behav 53 (3):472-480
Wade GN, Schneider JE (1992) Metabolic fuels and reproduction in female mammals. Neurosci Biobehav Rev 16 (2):235-272
Wingfield JC (2005) The concept of allostasis: Coping with a capricious environment J Mammal 86 (2):248-254
Wingfield JC, Romero LM (2001) Adrenocortical responses to stress and their modulation in free-living vertebrates. Compreh Physiol
Wingfield JC, Sapolsky RM (2003) Reproduction and resistance to stress: When and how. J Neuroendocrinol 15 (8):711-724
Zimmerman LM, Vogel LA, Bowden RM (2010) Understanding the vertebrate immune system: insights from the reptilian perspective. J Exper Biol 213 (5):661-671

Table 1. Body condition (residual of snout-vent length and mass) vs CORT concentrations (log-transformed) in gartersnakes (*Thamnophis sirtalis*) after increasing doses of TTX. Asterisk indicates significance after Bonferroni correction ($\alpha = 0.003$).

	Baseline	**10 MAMU TTX**	**25 MAMU TTX**	**50 MAMU TTX**
All snakes	$R^2 = 0.002$ $F_{(1,39)} = 0.08$ $p = 0.78$	$R^2 = 0.05$ $F_{(1,39)} = 2.15$ $p = 0.15$	$R^2 = 0.07$ $F_{(1,38)} = 2.98$ $p = 0.09$	$R^2 = 0.13$ $F_{(1,20)} = 2.96$ $p = 0.10$
Male snakes	$R^2 = 0.22$ $F_{(1,10)} = 2.85$ $p = 0.12$	$R^2 = 0.35$ $F_{(1,10)} = 5.42$ $p = 0.042$	$R^2 = 0.68$ $F_{(1,10)} = 21.32$ $p = 0.001$*	$R^2 = 0.51$ $F_{(1,5)} = 5.17$ $p = 0.07$
Female snakes	$R^2 = 0.04$ $F_{(1,19)} = 0.84$ $p = 0.37$	$R^2 = 0.002$ $F_{(1,19)} = 0.05$ $p = 0.83$	$R^2 = 0.10$ $F_{(1,18)} = 2.07$ $p = 0.17$	$R^2 = 0.37$ $F_{(1,9)} = 5.38$ $p = 0.046$
Juvenile snakes	$R^2 = 0.50$ $F_{(1,6)} = 5.98$ $p = 0.05$	$R^2 = 0.01$ $F_{(1,6)} = 0.08$ $p = 0.79$	$R^2 = 0.15$ $F_{(1,2)} = 1.09$ $p = 0.34$	$R^2 = 0.57$ $F_{(1,2)} = 2.66$ $p = 0.244$

Figures

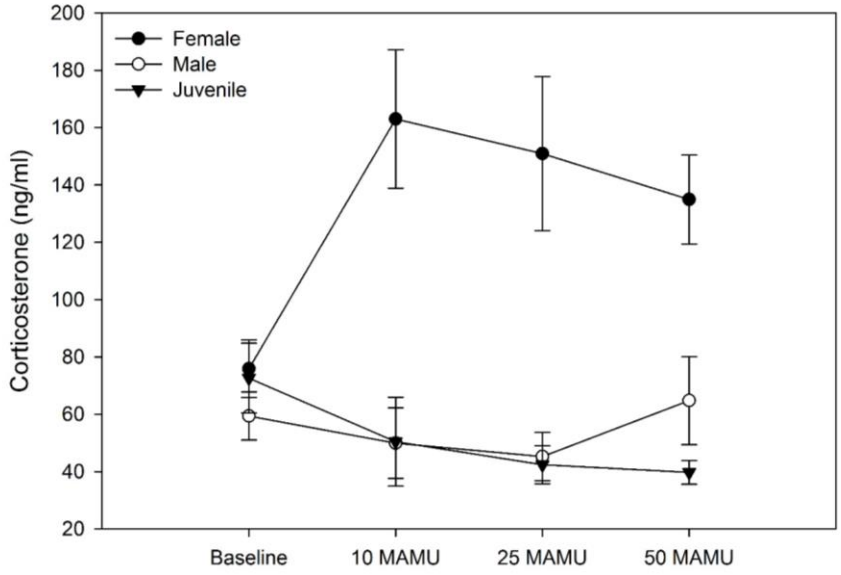

Figure 1. Corticosterone levels (ng/ml) in female, male, and juvenile gartersnakes (*Thamnophis sirtalis*) 30 min after exposure to increasing levels of TTX and immediately after racing. Error bars indicate ± 1 standard error. Sample sizes, in the following order: BL, 10 MAMU, 25 MAMU, and 50 MAMU, for females (21, 21, 17, 11), males (12, 12, 11, 7), and juveniles (8, 8, 4, 4).

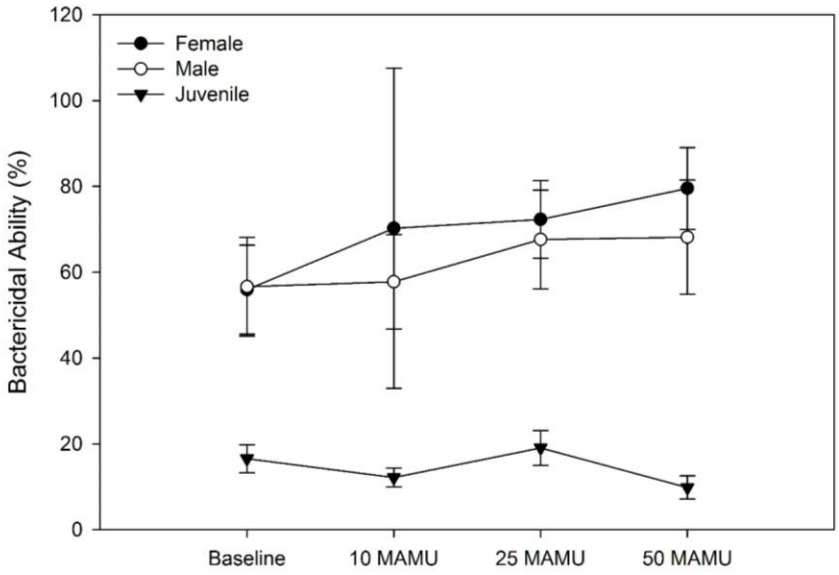

Figure 2. Bactericidal ability in female, male, and juvenile gartersnakes (*Thamnophis sirtalis*) 30 min after exposure to increasing levels of TTX and immediately after racing. Error bars indicate ± 1 standard error. Sample sizes, in the following order: BL, 10 MAMU, 25 MAMU, and 50 MAMU, for females (16, 16, 14, 10), males (12, 12, 11, 8), and juveniles (8, 5, 4, 4).

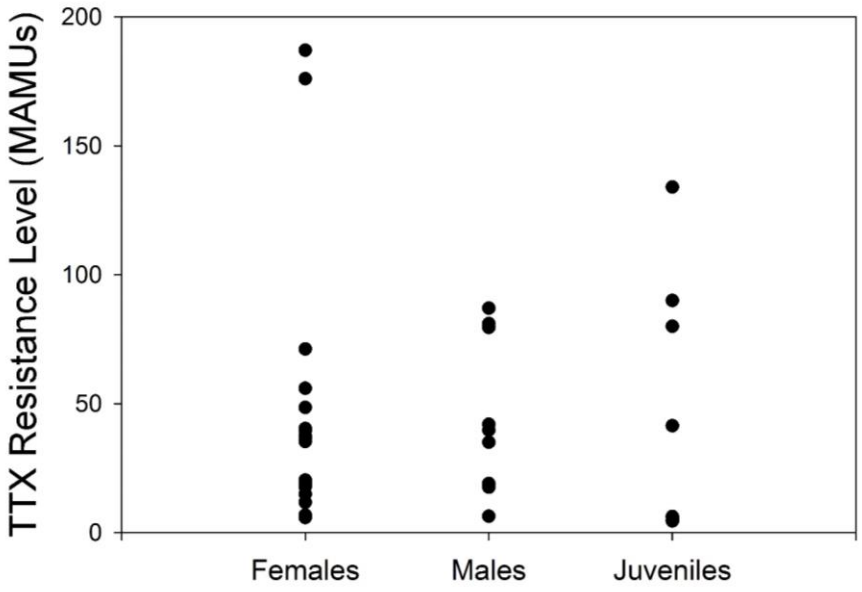

Figure 3. Resistance levels in individual gartersnakes (*Thamnophis sirtalis*) to tetrodotoxin (TTX) as calculated by the concentration of TTX which decreased their race speed by 50%. Units are given as mass-adjusted mouse units (MAMU), which is a standardized amount of TTX used in previous studies (see Brodie and Brodie, 1990).