RESEARCH ARTICLE

Comparing the Natural and Anthropogenic Sodium Channel Blockers Tetrodotoxin and Indoxacarb in Garter Snakes

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ABSTRACT

Synthetic chemicals, such as pesticides, are used in a variety of ways in the agricultural industry. Anthropogenic chemicals pose a unique challenge to organisms because of the lack of evolutionary history between the chemical and the organism. However, research has shown that some organisms have a resistance to these synthetic chemicals due to their evolved resistance to a natural compound with a similar structure or mode of action. Indoxacarb (INDOX) is a relatively new pesticide with a similar mode of action to that of tetrodotoxin (TTX). Tetrodotoxin is a naturally occurring toxin that is used as an antipredator defense in the rough-skinned newt (*Taricha granulosa).* Some populations of the common garter snake (*Thamnophis sirtalis)* have developed a resistance to tetrodotoxin. Here, we investigated the correlation between TTX and INDOX resistance in snakes. We hypothesized that INDOX would induce a much higher stress response than the naturally occurring TTX. We injected each snake with tetrodotoxin (1 mass-adjusted mouse unit). We did the same with mass-adjusted units of INDOX. We measured corticosterone, testosterone, and bactericidal ability. Our results show an acute stress response to INDOX, but not TTX through an elevate corticosterone and innate immune response, although there was no difference in testosterone concentration. These results suggest that, although INDOX may have a similar mechanism of action, garter snakes do not react in a similar manner as to TTX. This research gives a physiological perspective on the differences between naturally occurring compounds and synthetic compounds. *J. Exp. Zool. 325A:255–264, 2016.* © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

A wide variety of chemicals are permeating the environment at an unprecedented rate, which poses unique challenges to organisms. Synthetic chemicals are particularly problematic because organisms lack an evolutionary history with them. In numerous cases, organisms have evolved resistance to naturally occurring chemicals, even when these same chemicals are highly toxic to other organisms (Brodie and Brodie, '90; Mebs, '98; Hutchinson et al., 2007). If an organism has developed an evolutionary resistance to a natural toxin, it may provide a small reprieve whereby the organism has inadvertently evolved resistance to a synergistic chemical with a similar structure and/or a similar mode of action. This has been found in many species of insects that have proven to be resistant to both natural plant chemical defenses as well as pesticides with a similar mechanism of action (reviewed in Després et al., 2007).

One natural highly toxic chemical is tetrodotoxin (TTX). This defensive mechanism is found across the phylogenetic tree, from marine animals such as the puffer fish, to the western newts *Taricha granulosa*, and even some terrestrial invertebrates (Stokes et al., 2014). Structurally, TTX is a guanidine compound attached to an oxygenated carbon skeleton (Chau et al., 2011). TTX's mechanism of action is attaching itself to TTX-sensitive voltage-gated sodium channels, inhibiting them from carrying out action potentials in excitable nerve and muscle tissue. This results in paralysis of any tissue controlled by TTX-sensitive sodium channels. In mammals, it paralyzes the diaphragm (Brodie, '68).

This natural toxin is also unique because of the well-documented role it plays in a coevolutionary arms race between two North American vertebrates, the rough-skinned newt (*T. granulosa*), and the common garter snake (*Thamnophis sirtalis*). Newts are able to secrete TTX from granular glands on their dorsal surface as a defense mechanism (Brodie, '68). Through genetic mutation of the sodium ion channel, sympatric garter snakes have evolved resistance to TTX and are effective predators of newts (Brodie and Brodie, '90; Brodie et al., 2002; Feldman et al., 2009). While the level of toxin in newts and resistance in snakes varies across the species' ranges, in general populations of highly toxic newts are sympatric with populations of highly resistant snakes (Hanifin et al., '99; Brodie et al., 2002).

With a similar mode of action to TTX, the relatively new pesticide indoxacarb (INDOX) is used to control lepidopteran pests on fruits and vegetables (McCann et al., 2001). While little is known about the effects of this oxadiazine pesticide on vertebrates, research indicates that the mode of action is similar. In a study performed on rats, the effects indoxacarb on TTX-sensitive and TTX-resistant sodium channels were examined. This study found an irreversible inhibition in the action potential in both channels, but the magnitude was approximately twice as great in the TTX-sensitive channels (Zhao et al., 2003). Hematological, immunological, and behavioral effects of indoxacarb were also tested using mice and rats (Shit et al., 2008).

Given that virtually all organisms have a finite amount of energy that must be distributed to various functions, exposure to any form a stress can cause an imbalance in energy allocation (Wingfield and Romero, 2001; Wingfield, 2005). Organisms that are adapted to their environment should have an optimal reaction to normal perturbations within that environment, even chemical exposures (Romero et al., 2009). Therefore, testing the perceived magnitude of stress on an organism and the downstream effects can elucidate how well an individual reacts to changes in its environment (Walker et al., 2005; Angelier and Wingfield, 2013;). One of the potential mediators of the energetic shifts during a stressful event is glucocorticoid (GC). These are energy-mobilizing hormones and can be energetically costly to secrete, although it is often necessary to do so in stressful situations (Wingfield and Romero, 2001). Organisms must also allocate their limited energy to self-maintenance processes such as immune function and tissue and cellular repair (French et al., 2006, 2007). GCs have also been well documented on their effects on reproductive ability. Specifically, GCs suppress the hypothalamic–pituitary–gonadal axis and subsequently the release of gonadotropin-releasing hormone, which results in lower reproductive function (Whirledge and Cidlowski, 2010).

To examine whether garter snakes have a similar response to the natural toxin with which they have evolved (TTX) to an anthropogenic toxin with the same mechanism of action (INDOX), we designed two experiments. In the first experiment, we tested whether snakes respond to increasing doses of TTX absent from any other potential stressor (i.e., racing). In the second experiment, we gave a different set of snakes both TTX and INDOX to measure response. We predicted that snakes would have a minimal to no response to the increasing doses of TTX. Further, we hypothesized that exposure to the synthetic toxin INDOX would elicit a much higher stress response in snakes than the naturally occurring TTX to which they have evolved a resistance. This higher stress response would necessarily require trade-offs in

energetic allocation, with likely a decrease in self-maintenance energy and/or reproductive investment.

METHODS

Collection

Male garter snakes (*T. sirtalis*) were hand-collected in Cache County, UT in 2014 as they emerged from their hibernacula. Each individual was transported immediately to Utah State University and housed separately in glass aquaria (37.8 L) 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 on a 12 L:12 D cycle. Each snake was weighed to the nearest tenth of a gram and the snout-vent length was determined (distance from snout to cloaca). All procedures were approved by the Utah State University IACUC (Protocol #2299).

Experiment 1: Dose Response to TTX

Twenty snakes were injected in the coelomic cavity with a mass-adjusted dose of Ringer's solution. After 30 min, a blood sample was obtained via the caudal vein. This 30 min was selected because it is the time at which TTX has the maximal effect on snake motor function (Brodie and Brodie, '90; Brodie et al., 2002). Snakes fully recover from TTX injections after 24 hr (Brodie and Brodie, '90). After 48 hr, each snake was injected with 1 mass-adjusted mouse unit (MAMU, that is, the amount of TTX required to kill 1 g of mouse (Brodie and Brodie, '90)) and a blood sample was taken after 30 min. After another 48 hr, snakes were injected with 3 MAMU and bled after 30 min. Finally, the last injection of 5 MAMU was given after 48 hr and snakes were bled after 30 min. All samples were stored on ice for less than 1 hr and centrifuged at 2,400 rpm. The plasma was separated from the red blood cells and stored at −80°C until further processing.

Experiment 2: TTX versus INDOX Responses

Racing and Determining 50% Resistance. This bioassay was conducted as described by Brodie and Brodie ('90). Briefly, 24 hr 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, '99). To test maximal racing speed, each individual was removed from the aquaria and placed on a 3-m 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 0.5 m sections are measured using a digital timer and the fastest time is used. For the baseline measurement, this process was repeated twice, 4 hr 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, '90; Brodie et al., 2002). Therefore, by determining the dose at which the speed of the snake is reduced by 50% of the baseline, individual 50% resistance can be calculated.

Dosing with TTX and INDOX. The day after the baseline race, each snake was given an intracoelomic 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. Blood samples were taken and processed as described above. Two days later, the snakes were injected with 1 MAMU. This dose was selected relative to known resistance from previous investigations of this population (Brodie and Brodie, '90). After 30 min, snakes were raced and bled as described before.

Two days after TTX injections, all snakes were injected with mass-adjusted acetone solution because indoxacarb does not dissolve into Ringer's solution (control for TTX). There were no adverse effects of this injection. Four days after this control injection, snakes were injected with 0.167 mg indoxacarb/g snake (Indoxacarb [98.5% pure; ChemService, West Chester, PA, USA]). Dosage was calculated using previous data showing that this concentration reduced snake speed by approximately 50% (L. N. L., unpublished data). The concentration of INDOX in this study was not meant to be ecologically relevant, only elicit a similar behavioral response to TTX as a way to approximate comparable exposure. Snakes were raced and bled in the same manner as described for TTX.

Radioimmunoassay. Circulating corticosterone and testosterone levels were determined using a previously described protocol (Moore, '86; French et al., 2010, 2006). Samples were extracted using isooctane:ethyl acetate, dried, and resuspended in PBS buffer. Samples were assayed in duplicate and the mean of the two were used in analysis for both CORT (MP Biomedicals, Santa Ana, CA, Lot #3R3PB-19E) and TEST (Fitzgerald, Acton, MA, Lot #01916). 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 for each hormone. For CORT, intraassay variation was 12.1% and TEST intraassay variation was 15.3%.

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, USA) plus 4 nM L-glutamine (Sigma-Aldrich St. Louis, MO), and 10^5 CPU (colony producing unit) *Escherichia coli* (EPowerTM Microorganisms #0483E7, ATCC 8739; MicroBioLogics, St. Cloud, MN, USA), and agar broth on a 96-well microplate. We calculated the background absorbance using

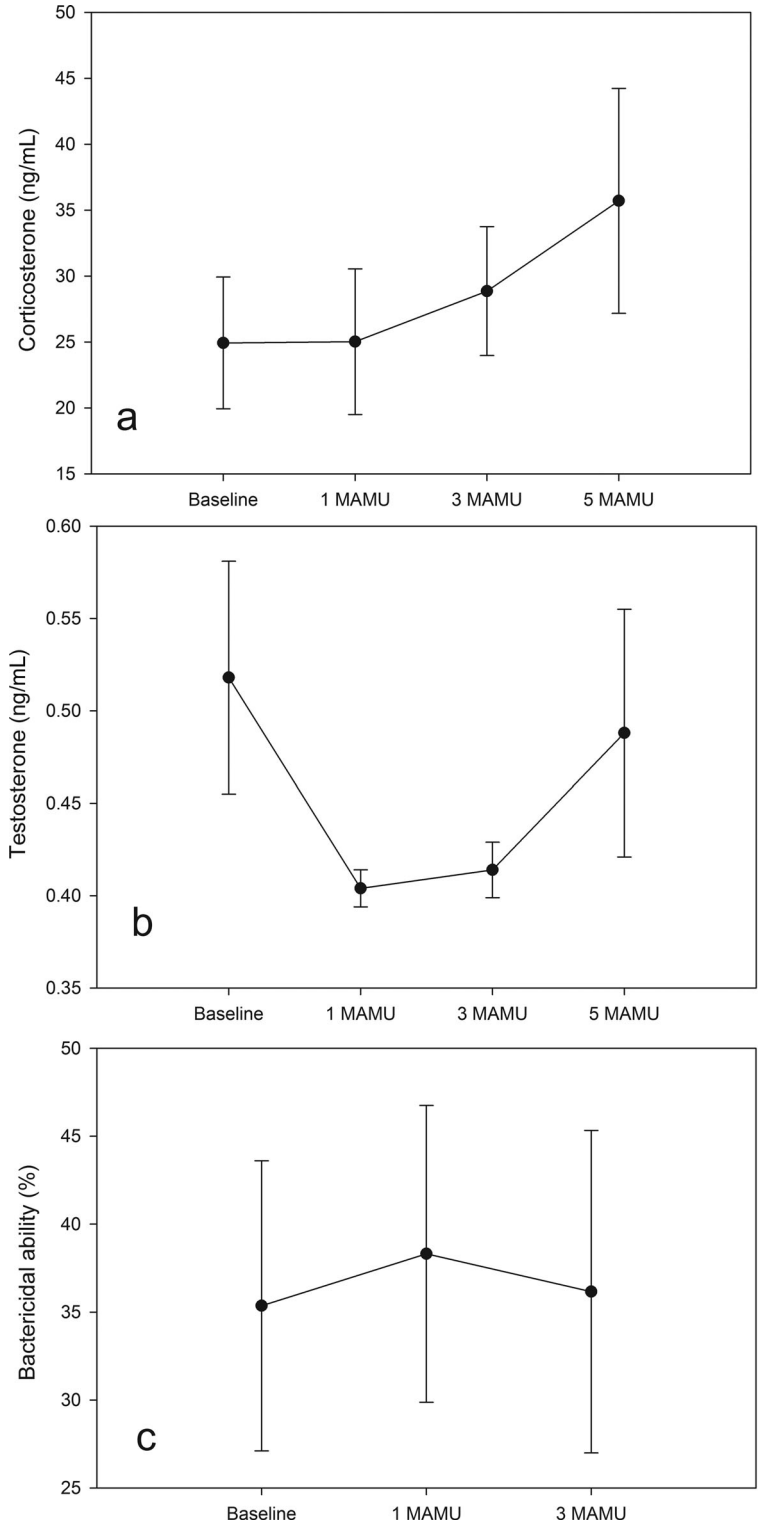

Figure 1. Physiological responses to increasing levels of TTX in common garter snakes (*Thamnophis sirtalis*). There was no differences in CORT (a), TEST (b), or BKA (c). Data are shown as raw values with 1 ± standard error.

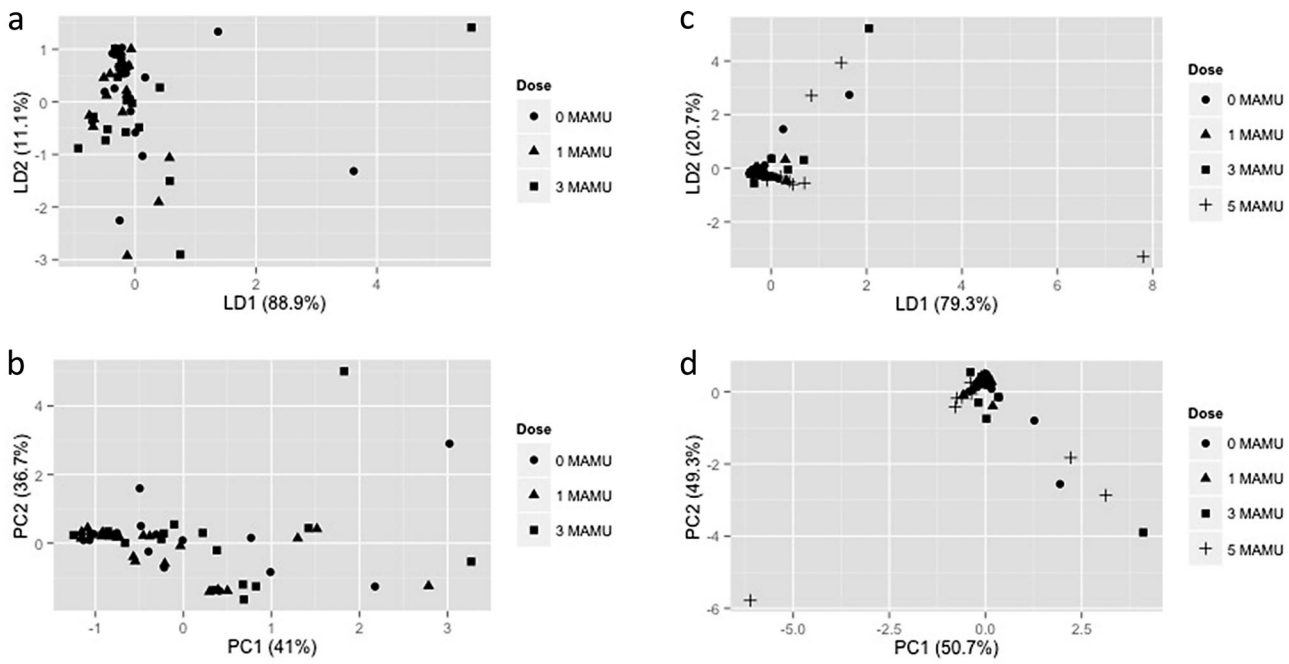

Figure 2. Visual representation models examining variance in TTX doses in common garter snakes (*Thamnophis sirtalis*) in bactericidal ability, TEST, and CORT (LDA: a, PCA: b) and TEST and CORT (LDA: c, PCA: d).

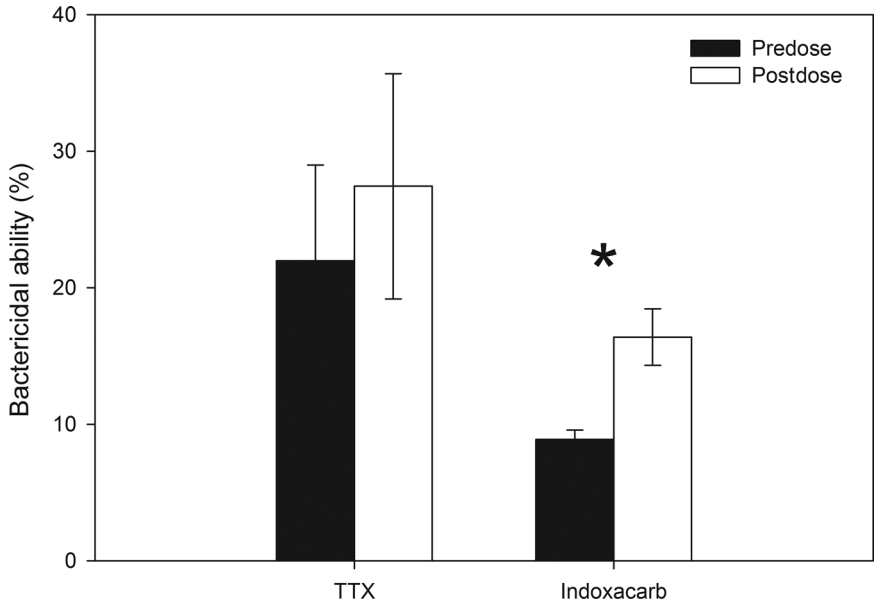

Figure 3. Bactericidal ability in common garter snakes (*Thamnophis sirtalis*) after exposure to TTX or INDOX. The predose indicates the vehicle injection only (Ringer's solution for TTX and acetone for INDOX). There is no difference between the vehicle injection and TTX, but bactericidal ability increased significantly between vehicle injection and indoxacarb. Error bars indicate $\pm$ 1 standard error, and the asterisk indicates significance ($\alpha = 0.05$).

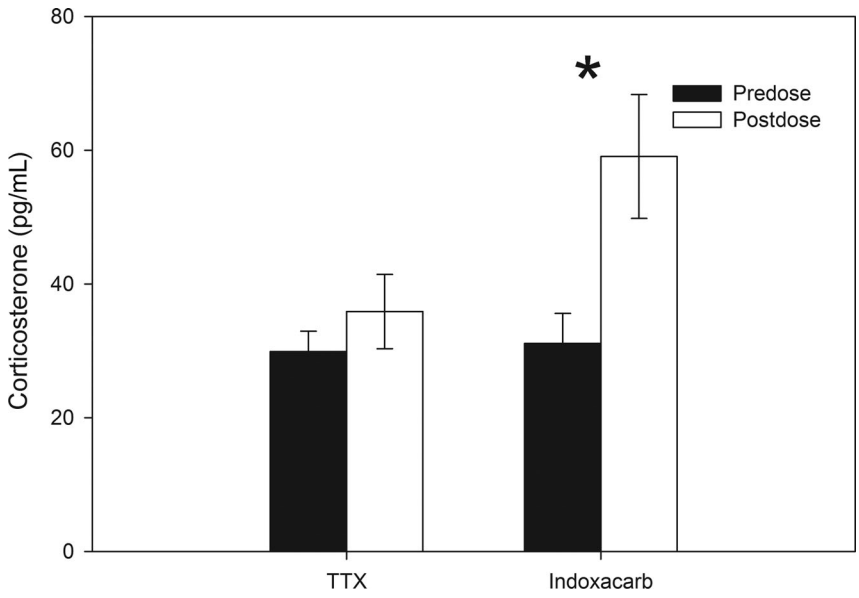

Figure 4. Corticosterone in common garter snakes (*Thamnophis sirtalis*) after exposure to TTX or INDOX. The predose indicates the vehicle injection only (Ringer's solution for TTX and acetone for INDOX). There is no difference between the vehicle injection and TTX, but corticosterone increased significantly between vehicle injection and indoxacarb. Error bars indicate ± 1 standard error, and the asterisk indicates significance ($\alpha = 0.05$).

BioRad xMark microplate reader. After a 12-hr 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 to the positive controls. Negative controls (containing media only) were also run to ensure contamination was absent. Interassay variation between plates was 1.1%.

Statistics. For experiment 1, we examined CORT, TEST, and BKA as explanatory variables and dose as the independent variable. We $\log_{10}$-transformed CORT to meet assumption of normality and homoscadicity and conducted an analysis of variance. For both TEST and BKA, we were unable to transform the data to meet assumptions of normality and therefore these tests were completed using the Wilcoxon test. We also examined the CORT response between the first TTX injection for experiment 1 (1 MAMU, snakes did not race) and the first TTX injection for experiment 2 (1 MAMU, snakes did race). CORT had to be $\log_{10}$-transformed, and we analyzed the data using a *t*-test. These analyses were conducted in JMP 11.0 (SAS Institute, Inc., 2013). We also conducted a linear discriminate analysis (LDA) and a principle component analysis (PCA) to examine the multiple metrics over the three dosages (bactericidal ability, CORT, and TEST) and over the four dosages (CORT and TEST; Scarpino et al., 2014). These analyses were performed in R version 3.2.3 using the "MASS" package (Venables and Ripley, 2002).

In experiment 2, because the vehicle was different for the two chemicals (Ringer's solution for TTX and acetone for indoxacarb), we analyzed the response from baseline (vehicle injection) to sodium-channel blocker (i.e. saline compared to TTX only and acetone compared to INDOX only). We conducted a *t*-test on $\log_{10}$-transformed CORT for TTX data to meet the assumptions of normality. We could not transform TEST or BKA data, therefore nonparametric Wilcoxon tests to compare the baseline and post-TTX. For the indoxacarb data, both CORT and BKA were $\log_{10}$-transformed and *t*-tests were conducted comparing baseline and postindoxacarb data. The indoxacarb TEST could not be transformed and therefore a Wilcoxon test was performed. These analyses were performed in JMP 11.0 (SAS Institute, Inc., 2013).

RESULTS

Experiment 1: Dose Response of TTX

There was no relationship between injection dose and any of the variables (Fig. 1; $\log_{10}$ CORT $= F_{(3,77)} = 1.12$, $P = 0.35$; TEST $= X^2 = 5.49$, $P = 0.14$, df $= 3$; BKA $= X^2 = 0.37$, $P = 0.83$, df $= 2$). We were only able to measure BKA for baseline, 1 MAMU, and 3 MAMU (not 5 MAMU) due to limits on

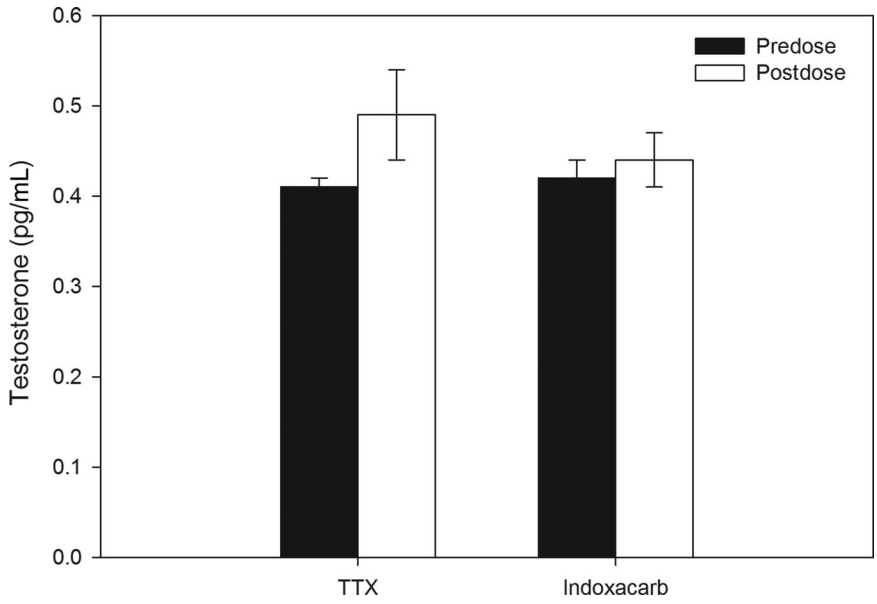

Figure 5. Testosterone concentrations in common garter snakes (*Thamnophis sirtalis*) after exposure to TTX or INDOX. The predose indicates the vehicle injection only (Ringer's solution for TTX and acetone for INDOX). There were no differences between the vehicle and chemical injections. Error bars indicate ± 1 standard error.

plasma volume. Both the LDA and PCA revealed no clear distinction between the dosages (Fig. 2).

When comparing the CORT response for the 1 MAMU doses for the snakes that ran versus the snakes that did not run, we found that there was no difference ($t = 1.39$, $P = 0.17$).

Experiment 2: TTX and INDOX

There was no difference between baseline and post-TTX BKA (Fig. 3; $X^2 = 0.079$, $P = 0.79$, df = 1), CORT (Fig. 4; $t = 0.61$, $P = 0.55$, df = 30.77), or TEST (Fig. 5; $X^2 = 0.039$, $P = 0.84$, df = 1).

There was a difference between baseline and post-INDOX BKA (Fig. 3; $t = 3.32$, $P = 0.0024$, df = 29.93) and CORT (Fig. 4; $t = 2.62$, $P = 0.013$, df = 36.84). There was, however, no difference in TEST (Fig. 5; $X^2 = 0.23$, $P = 0.63$, df = 1).

DISCUSSION

This research indicates that being exposed to anthropogenic chemicals may elicit a greater response than exposure to chemicals with which an organism has an evolutionary history, even when the chemicals have a similar mechanism of action. In our first experiment, we showed that increasing doses of TTX did not elicit a significant physiological response among the parameters we measured. When examining the anthropogenic chemical INDOX in comparison with TTX, we were unable to directly compare the TTX and INDOX exposures. However, the increase from the baseline (vehicle) in INDOX treatment for both CORT and BKA was significant but not in TTX treatment. Given the highly toxic nature of TTX, it is surprising that there is virtually no hormonal or immune response against any of the received injections. This work highlights the need for determining how different chemicals affect the same model organism.

An increase in CORT in response to a chemical stressor is not unusual and has been documented in a variety of species and for many chemicals (Sanders et al., '74; Hopkins et al., '97; Franceschini et al., 2008; Adams et al., 2009). However, organisms do not always respond with an elevated CORT response. This lack of response may be associated with a depression of the HPA axis due to chronic stress (Rich and Romero, 2005; Dhabhar, 2009), lack of perception of a threat (Cockrem, 2007; Neuman-Lee et al., 2015), or blocking of the HPA axis by the action of chemical (Ilan and Yaron, '80; Gendron et al., '97). Further, it seems that these responses may be dependent upon context-dependent factors such as age, sex, and reproductive status (Lattin et al., 2012; Crespi et al., 2013; Bechshoft et al., 2015). While we cannot rule out that TTX itself blocks action by the HPA axis, the mechanism of action does not appear to interfere with this process (Narahashi, 2001). Further, a recent study showed that females (but not males) elevated their CORT in response to exposure to TTX, which indicates that TTX itself likely does not block the process (Neuman-Lee et al., in press).

It was surprising, however, that these snakes had no response to TTX, but mounted a strong response to INDOX given the similar mechanism of action. However, this could be explained by

two nonmutually exclusive reasons. First, the molecular structure of INDOX is very different from TTX (McCann et al., 2001; Narahashi, 2001) and thus the snake's body likely processes the toxin in a different manner. Unfortunately, there is limited reptilian toxicokinetic data for TTX (Williams et al., 2012) and none for INDOX to either substantiate or refute this possibility. Second, the evolutionary history that snakes share with TTX exposure (through eating *T. granulosa*) may reduce responses to TTX exposure. While these snakes tested were naïve to TTX and do not coexist with *T. granulosa*, they still have some resistance to TTX (Feldman et al., 2009). Other evolutionary adaptations, such as adaptations to the HPA axis, immune function, or reproduction have not yet been examined and therefore cannot be ruled out as a possible reason for the lack of response to TTX.

The innate immune response also followed the same pattern as CORT. We saw a marked increase in bactericidal ability after INDOX exposure, while there was no change after TTX exposure. This is likely due to the corresponding elevation in CORT. During many acute stressors, the secretion of CORT is correlated with an increase in immune activity (Dhabhar and McEwen, '97; Dhabhar, 2009). While not a universal pattern, this relationship is thought to arise as a protective mechanism (Sapolsky et al., 2000; Dhabhar et al., 2012) and has been seen in previous studies of acute stress exposure (Dhabhar and McEwen, '97; Martin, 2009).

Neither TTX nor INDOX appeared to affect TEST secretion. There is a possibility that because TEST levels were already low, there is no appreciable action that can either block or increase TEST secretion. However, while research on other populations of *T. sirtalis* has indicated that TEST remains low during the spring and through mating (Moore et al., '67), long-term data on this population in Utah indicate that many snakes do have higher circulating TEST during the spring (L. N. L., unpublished data). We therefore hypothesize that neither INDOX nor TTX at these levels has an impact on TEST. Snakes from another population also responded similarly to varying levels of TTX by not elevating or decreasing their circulating TEST concentrations (Neuman-Lee et al., in press).

Because there are no ecological data on the levels of INDOX in the environment, it is not possible to determine if the amount that was given is ecologically relevant. Therefore, these results should not be interpreted as a challenge, which wild snakes would necessarily encounter. However, with the increasing amounts of chemicals, as well as other anthropogenic pressures, understanding how reptiles respond to chemicals will allow the regulatory community to better manage populations. As a taxonomic group, reptiles remain imperiled and often overlooked in ecotoxicological studies. Snakes, in particular, face a new potentially devastating threat of the infectious snake fungal disease (Allender et al., 2011; Sutherland et al., 2014), and any challenge that alters their immunity and physiology puts them at increased risk. Therefore, determining as much about their physiology and response to anthropogenic factors is critical.

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