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Exposure to the Herbicide Atrazine Nonlinearly Affects Tadpole Corticosterone Levels

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ABSTRACT.—**To determine whether the herbicide, atrazine, affects the stress hormone corticosterone, we exposed *Osteopilus septentrionalis* (Cuban Treefrog) tadpoles to four concentrations of atrazine and two controls (water and acetone) for three time durations (4, 28, and 100 h). Atrazine concentration, but not exposure duration, had significant nonlinear effects on whole-body corticosterone. Relative to controls, intermediate concentrations of atrazine (10.2 and 50.6 µg/L) tended to lower corticosterone, whereas the lowest (0.1 µg/L) and highest atrazine concentrations (102 µg/L) elevated corticosterone. These results indicate that atrazine exposure might dysregulate corticosterone, a hormone integral to vertebrate immunity, neurogenesis, and health.**

Amphibians are important biological indicators of environmental health and are one of the most threatened vertebrate taxa on the planet (Stuart et al., 2004). Agrochemical pollution is a major concern for amphibians because many breed in and use waterways associated with pesticide use (e.g., near agriculture fields and orchards; see Boone et al., 2008; Johan et al., 2009), regularly exposing them to pesticides during development (Rohr et al., 2004). Additionally, some pesticides can persist in the environment, such as atrazine, one of the most commonly used pesticides in the United States that has a half-life on the order of months (deNoyelles et al., 1989; Solomon et al., 1996). Therefore, amphibians near agricultural areas may experience chronic exposure to some pesticides during development.

Although exposure to pesticides has been linked to mortality of non–target organisms (Rohr et al., 2008a; McMahon et al., 2012), sublethal effects (Rohr et al,. 2006; Rohr et al., 2013), such as hormonal dysregulation (i.e., abnormal or impaired regulation; Larson et al., 1998; Rohr et al,. 2003; Hayes et al,. 2006; Rohr and McCoy, 2010) and altered disease risk (Rohr et al., 2008b), appear to be more common than direct mortality. For example, exposure to many pesticides can alter levels (increase or decrease) of circulating glucocorticoid stress hormones in vertebrates, such as corticosterone (CORT), the glucocorticoid in amphibians (Larson et al., 1998; Goulet and Hontela, 2003; Hayes et al., 2006; McMahon et al., 2011). The effects of glucocorticoids on organismal health often depend on whether stressor exposure is acute or chronic (Sapolsky et al., 2000): short-term CORT elevations can be protective (Davis et al., 2008), whereas long-term can cause immune suppression, muscle atrophy, and reduced neurogenesis (Martin, 2009). Thus, chronically elevated CORT is often detrimental, but too little CORT can also compromise health (Wingfield and Sapolsky, 2003). More important, if glucocorticoid dysregulation occurs during development, the hypothalamic–pituitary–adrenal (HPA) regulatory axis for CORT, as well as immune responses (Plotsky and Meaney, 1993; Matthews, 2002; Belden and Kiesecker, 2005; Glaser and Kiecolt-Glaser, 2005; Martin et al., 2010), and many other traits, can be enduringly compromised (Martin, 2009). Therefore, any dysregulation of CORT, whether a reduction or an increase, could affect organismal health (Woods and Wilson, 2014).

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Exposure to the herbicide, atrazine (chemical class triazine), can dysregulate CORT in amphibians (Larson et al., 1998; Goulet and Hontela, 2003; Hayes et al., 2006; Hernández et al., 2014). For example, exposure to a mixture of pesticides, including atrazine, caused a fourfold increase in CORT in *Xenopus laevis* (Hayes et al., 2006) and atrazine-modulated CORT in *Ambystoma tigrinum* (Larson et al., 1998) and *Rhinella marina* (Hernández et al., 2014). We do not know how general the effects of atrazine are in terms of pesticide concentration, however, nor how the duration of atrazine exposure affects CORT. To address these gaps in the literature, we exposed *Osteopilus septentrionalis* (Cuban Treefrog) tadpoles to naturally relevant concentrations of atrazine for different durations of times and quantified levels of CORT thereafter.

MATERIALS AND METHODS

Experimental Design.—*Osteopilus septentrionalis* eggs were collected from pesticide-free, outdoor wading pools (1.5 m diameter, 30 cm deep) at the University of South Florida (USF) Botanical Gardens (28°03.537′N 082°25.410′W). Tadpoles were housed individually in 500-mL mason jars filled with 300 mL of artificial spring water (ASW; Cohen et al., 1980) until all tadpoles reached Gosner stage 25 (Gosner, 1960). The jars were randomly dosed with 1 mL of a stock of one of four atrazine (technical grade, purity >98%) concentrations (final water concentrations: 0.1, 10.2, 50.6, or 102 µg/L atrazine; $N = 6, 8, 6, 6$, respectively; Table 1) or one of two controls (water or acetone solvent (500 ng/L acetone) for atrazine; $N = 16$/control treatment). The atrazine stock concentration was 106 µg/L, verified by ELISA (Abraxis, Inc., Warminster, PA) and diluted in series to create working concentrations; nominal concentrations are reported throughout the manuscript. The highest atrazine concentration tested (102 µg/L) was the Estimated Environmental Concentration (EEC) according to GENEEC software, v. 2 (U.S. Environmental Protection Agency). We chose the EEC as our highest concentration to be sure that all concentrations used in the study were naturally relevant and because the effects of atrazine at the EEC may impact pesticide regulations and usage. The four atrazine concentrations and two controls were crossed with three exposure durations: 4, 28, and 100 h (for sample sizes, see Table 1). We expected short-term exposure to atrazine to dysregulate CORT less than longer-term exposure. Thus, we had the largest samples sizes in the 4-h exposure treatment because we expected

TABLE 1. Sample sizes for tadpoles exposed to different concentrations of atrazine for different durations of time.

Atrazine concentration (μg/L)	Duration (h)			Overall sample size/ concentration
	4	28	100	
0 (water)	8	4	4	16
0 (acetone)	8	4	4	16
0.1	3	1	2	6
10.2	3	2	3	8
56	3	1	2	6
102	3	2	1	6

the smallest effect sizes there; other differences in sample size were caused by mortality that occurred before euthanasia.

Corticosterone Extraction and Measurement.—We followed the methods described in McMahon et al. (2011) to extract and quantify CORT in tadpoles. After atrazine exposure, tadpoles were euthanized by placing them directly into a beaker of 10% benzocaine sitting in dry ice. This process immediately euthanatized and flash froze the animals with a handling time of less than 5 sec. Each tadpole was weighed (± 0.0001 g) and homogenized (Power Gen 125 homogenizer, Thermo-Fisher Scientific, Inc., Waltham, MA). Whole tadpole homogenates were extracted twice with a 7:3 ethyl ether-petroleum ether cocktail (4 mL per 1 mL homogenate). The supernatant was removed from the water fraction by freezing the water and decanting the ether cocktail that was collected in a glass vial (13 × 100 mm) and dried under a pure nitrogen stream at 40°C. The petroleum ether helped separate the fats into a monolayer allowing us to collect the steroid layer effectively, and the freezing cycle helped to precipitate out excess fat and other unwanted compounds. To determine extraction efficiency, we added 20uL of diluted 250 μCi tritiated CORT stock solution (PerkinElmer NET-399) equivalent to a decay rate of 2,000 cpm, to each sample prior to extraction. Following sample reconstitution (see below), we used a scintillation counter to measure the amount of tritiated steroid remaining in each reconstituted sample. We then compared this value to reference sample readings of nonextracted 20uL working solutions of tritiated CORT (i.e., 2,000 cpm) to determine the percent of CORT that was lost or bound by lipids during the extraction process. Recoveries varied among individual tadpoles, but recoveries were consistent among replicates of the same tadpole homogenate. Mean (± SE) extraction efficiency was 48 ± 0.03%, and CORT results were adjusted based on individual extraction efficiencies.

We used a CORT EIA kit to quantify hormone levels extracted from each sample (Assay Designs: cat. number 900-097, Ann Arbor, MI). Immediately before running the assay, dried samples were reconstituted in 500 μL of Assay Buffer 15, mixed with 12.8 μL of steroid displacement buffer, and vortexed vigorously. This reconstituted sample extract (50 μL) was then added to wells (along with enzyme conjugate and other reagents) and incubated for 1 h. Next, the plate was washed three times, and then the last reagents were added to elicit colorimetric reactions for visualization. Plates were read at 405 nm with a 96-well ELISA plate reader (Bio-Tek, Winooski, VT). Samples were run in duplicate following the manufacturer's instructions for the EIA kit, and standard curves for each run of the assay ($N = 2$) spanned a 32–20,000 page range. Interassay variation was 11.6%, and intra-assay variation was 7.6%.

Data Analysis.—To identify the best predictors of $\log_{10}$-transformed whole body CORT levels, we compared six statistical models (with least trimmed squares): 1) the intercept only; 2) atrazine duration only ($\log_{10}$ transformed); 3) atrazine concentration only ($\log_{10}$ [conc. +1] transformed); 4) duration + concentration; 5) duration + concentration + concentration^2; 6) duration + concentration + concentration^2 + concentration^3. Model selection and averaging was conducted using the *model.sel* function in the "MuMIn" package of R statistical software (R Development Core Team, 2010). Once the best model was identified, ANOVA tables were generated using the "car" package of R. We used the information theoretic approach for the statistics, because it allowed us to use model selection to choose the most appropriate model for the data. We did not calculate a $LOEC_{50}$, because there were clear nonlinear responses, which would violate the assumptions of LOEC calculations.

RESULTS

There was never a difference between the two control treatments (water and solvent) for any of the end points analyzed ($P > 0.05$); thus, we pooled them into one control treatment for all subsequent analyses. There was no effect of treatment (chemical, chemical concentration, or exposure duration) on tadpole stage or weight ($P > 0.05$ for all factors).

Model selection and averaging revealed the third order polynomial model had the largest likelihood and weight (69.4%) and a ΔAICc of 3.46 from the next model (Table 2). A significant third order model (atrazine as a categorical variable: $F_{4,21} = 4.414$, $P = 0.0096$; log concentration^3: $F_{1,31} = 7.666$, $P = 0.0094$) indicated that whole body CORT levels were nonlinearly affected by atrazine concentration (Fig. 1). Indeed, relative to control tadpoles, tadpoles exposed to the two intermediate concentrations of atrazine tended to have lower CORT levels and tadpoles exposed to the lowest and highest atrazine concentrations tended to have higher CORT levels (Fig. 1).

In contrast to concentration, whole body CORT was not significantly affected by atrazine exposure duration ($F_{2,21} =$

TABLE 2. Results of model selection and model averaging for a series of statistical models that considered the effects of $\log_{10}$ atrazine exposure duration and linear, quadratic, and cubic effects of $\log_{10}$ atrazine concentration (plus 1 because of zero concentration) on $\log_{10}$ whole body corticosterone levels in *Osteopilus septentrionalis* tadpoles.

Model no.	Intercept	Duration	Concentration (conc.)	Conc.^{2}	Conc.^{3}	df	Log likelihood	AICc	ΔAICc	Model weight
6	1.977	0.09783	2.579	−4.267	1.522	6	−15.859	46.1	0	0.694
1	2.054	–	–	–	–	2	−22.634	49.6	3.46	0.123
5	2.017	0.09922	−0.7845	0.408	–	5	−19.43	50.5	4.41	0.077
2	1.97	0.07024	–	–	–	3	−22.412	51.5	5.34	0.048
3	2.078	–	−0.03208	–	–	3	−22.549	51.7	5.61	0.042
4	1.994	0.07058	−0.03248	–	–	4	−22.323	53.7	7.61	0.015

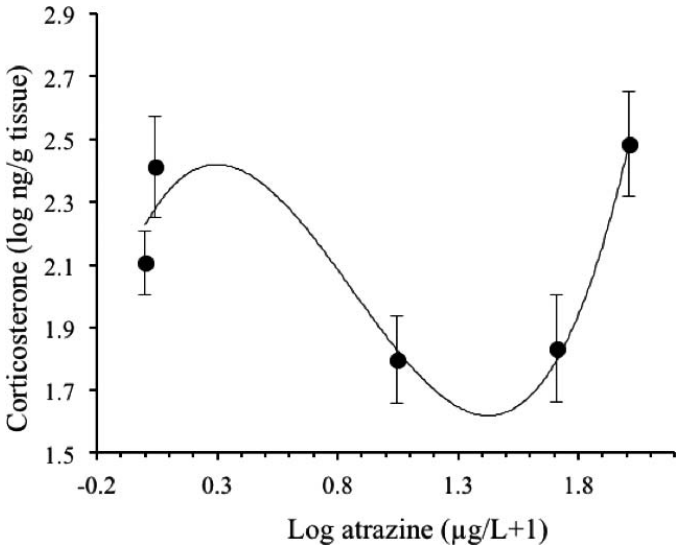

FIG. 1. Effect of atrazine concentration on corticosterone (CORT) per gram of *Osteopilus septentrionalis* tissue. There was no effect of atrazine exposure duration; therefore, means (± SE) were averaged across the exposure durations (4, 28, and 100 h). Also shown is the significant third-order polynomial function for the relationship between $\log_{10}$ atrazine concentration and $\log_{10}$ CORT, adjusted for the effect of exposure duration (see text for statistics).

0.618, $P = 0.549$; duration × atrazine: $F_{8,21} = 1.451$, $P = 0.234$). Given our sample size and a correlation coefficient between duration and CORT of 0.217, we only had 34% power to detect an effect of exposure duration. Detection of a significant effect of exposure duration with a power of 80% would have required an approximate 4.5-fold increase in our sample size.

DISCUSSION

The early toxicologist Paracelsus emphasized that dose makes the poison; however, many modern dose-response studies are discovering this to be an over simplification, because nonlinear dose responses have been repeatedly reported (Alker et al,. 2001; Rohr et al., 2006; McMahon et al., 2011, 2013; Vandenberg et al.; 2012). To add to this growing list, we discovered that naturally occurring concentrations of atrazine nonlinearly affected CORT levels in *O. septentrionalis* tadpoles; low and high levels of atrazine seemed to elevate CORT. This nonlinear pattern can manifest through multiple mechanisms, which we did not investigate here but may include the body responding differently to different contaminant concentrations (Welshons et al., 2003; Vandenberg et al., 2012) and dysregulation of negative feedback systems, hormone receptors, and hormone production (Cyr and Romero, 2009).

Support for a nonlinear effect of atrazine exposure on CORT also comes from the work of Larson et al. (1998), where *A. tigrinum* larvae exposed to 75µg/L atrazine had reduced CORT compared to those exposed to either 0 and 250µg/L of atrazine, very similar to the patterns we observed in *O. septentrionalis*. Atrazine also has shown a nonlinear dose response relationship with other hormones, such as testosterone (Vandenberg et al., 2012). Other pesticides also have affected CORT nonlinearly. For example, exposure to naturally relevant concentrations of the fungicide chlorothalonil induced a nonlinear effect on CORT in *O. septentrionalis* tadpoles similar as we observed for atrazine (McMahon et al., 2011).

Elevated CORT does not typically have an immediate lethal impact on tadpoles. Indeed, we did not see any tadpole mortality. However, elevated CORT exposure can cause detrimental immunomodulation. For example, in *Xenopus laevis* (African Clawed Frogs) mitogen-stimulated proliferation of lymphocytes was reduced by elevated endogenous glucocorticoids (Rollins-Smith and Blair, 1993). Therefore, given that we show atrazine dysregulates CORT, we were not surprised that one of the most consistent effects of atrazine exposure on amphibians and fish is altered immunity and elevated infection prevalence and burden (Rohr and McCoy, 2010). Additionally, sustained or early-life exposure to elevated glucocorticoids can permanently alter the HPA axis (Plotsky and Meaney, 1993; Glaser and Kiecolt-Glaser, 2005). The HPA axis helps control the neuroendocrine system, regulating not only stress responses like CORT expression and immune function (Silverman et al., 2005) but also normal body processes, like digestion and protein transport across tissue (Plotsky and Meaney, 1993; Matthews, 2002; Belden and Kiesecker, 2005; Glaser and Kiecolt-Glaser, 2005; Denver, 2009; Martin, 2009; Martin et al., 2010). More specifically, in amphibians, corticosteroids interact with the thyroid hormone to mediate amphibian metamorphosis, and changes in hormone levels can influence timing of and size at metamorphosis (Denver, 2009), which can influence adult fitness (Denver, 2009; Rohr and Palmer, 2013).

We decided to measure whole body CORT in this experiment because it is the most relevant metric of predicted CORT interference by atrazine as it encapsulates all circulating and adrenal reservoirs of corticosterone. We had to euthanize the tadpoles and due to these experimental design restrictions, we could not quantify how atrazine-altered CORT levels would impact long-term fitness. More research is needed to understand the long-term fitness impacts of atrazine and abnormally induced CORT responses (but see Kaiser et al., 2015). Hence, this work emphasizes the need to further study the effects of naturally occurring concentrations of pesticides on the physiology of changed CORT responses impacting amphibian health and population dynamics.

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