No difference in corticosterone concentrations between Missouri three-toed box turtles living in an urban and a rural site

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Abstract: Baseline health data for species of conservation concern are important for understanding threats to the long-term viability of populations. One indication of health is physiological stress among individuals. Corticosterone (CORT) is frequently used to quantify stress in free-living reptile populations, as high values may be associated with reduced fitness. Herein, we describe and validate methods for quantifying blood CORT levels in three-toed box turtles (*Terrapene mexicana triunguis*). We subsequently use this information to evaluate stress levels in 2 populations of free-living three-toed box turtles in Missouri, USA. To our knowledge, this is the first quantification of CORT levels in the three-toed box turtle. In 2012 we collected blood samples from 11 three-toed box turtles in human care at the Saint Louis Zoological Park (zoo), St. Louis, Missouri for assay validation, and from 2012 to 2016 we collected 220 samples from 144 free-living three-toed box turtles at 2 sites, 1 urban and 1 rural. In the zoo turtles, mean CORT concentration was 0.71 ± 0.10 ng/mL. Following a handling experiment, CORT concentration increased to 3.14 ± 0.72 ng/mL (P = 0.011). Mean CORT levels between free-living turtles at the urban and rural sites did not differ (urban = 0.54 ± 0.08 ng/mL, rural = 0.37 ± 0.02 ng/mL, $F\ pr$ = 0.12). Sex did not influence CORT levels ($F\ pr$ = 0.29). These results suggest that the turtles living in the urban environment did not experience chronic elevated glucocorticoid production and supports urban parks as potential habitat for box turtles.

Key words: captivity, chelonian, conservation, corticosterone, glucocorticoid, health, stress, *Terrapene mexicana triunguis*, three-toed box turtles, urban parks

Human modifications to the earth have caused the planet to enter a newly defined geological epoch—the Anthropocene—characterized in part by elevated extinction rates compared to historic levels (Pimm et al. 1995, Crutzen and Stoermer 2000). Chelonians (tortoises and turtles) are among the most threatened of vertebrate taxa, with approximately half of the >325 species threatened with extinction (Rhodin et al. 2017). Certain characteristics of turtles, such as delayed sexual maturity and long generation times (Congdon et al. 1993), make them especially vulnerable to anthropogenic disturbances (Heppell 1998). Box turtles (*Terrapene* spp.) occur extensively throughout the Eastern and Midwestern United States and into Mexico (Dodd 2002, Martin et al. 2013). Population declines of these species are widespread due to a combination of threats faced by many reptiles, including habitat loss and fragmentation, unsustainable pet trade harvest, and increasing infectious and non-infectious diseases (Budischak et al. 2006, Rivas et al. 2014).

Stressors are commonly defined as stimuli in

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Figure 1. Three-toed box turtle (*Terrapene mexicana triunguis*) from cranial and dorsal views (*photos courtesy of B. Lamczyk*).

an animal's environment that induce a physiological response (Tokarz and Summers 2011, Atkinson et al. 2015). There are predictable stressors associated with daily and seasonal demands such as feeding, reproduction or migration, and unpredictable stressors that may be life threatening such as predation (Landys et al. 2006). Anthropogenic changes often present unpredictable and life-threatening events that may increase stress in wildlife (McLennan et al. 2019). Behavioral, neuroendocrine, biochemical, and physiological changes are used to evaluate stress in reptiles (Silvestre 2014). A measurable neuroendocrine and physiological indicator such as glucocorticoid production can help ascertain how an animal is reacting to its environment (Wikelski and Cooke 2006).

In response to stress, vertebrate endocrine systems release glucocorticoids (Romero 2004, Selman et al. 2012, Silvestre 2014, Taylor et al. 2014). In reptiles, the main glucocorticoid is corticosterone (CORT; Silvestre 2014). Normal physiological levels of glucocorticoids are necessary and beneficial for daily functions (Landys et al. 2006). Acute glucocorticoid increases in response to stressors are also beneficial by facilitating immediate survival (Baker et al. 2013, Atkinson et al. 2015). Additionally, positive relationships between CORT and reproduction have been described in some amphibian and reptile species in which moderately increased CORT levels have been found during their breeding season (Moore and Jessop 2003). However, if elevated glucocorticoid production is chronic or severe, it can be potentially detrimental in some instances because processes not necessary for immediate survival, like reproduction, are suppressed (Pride 2005, Landys et al. 2006, Refsnider et al. 2015). Chronic stressors may have accumulated costs that contribute to physiological dysfunction (Atkinson et al. 2015). For example, short, repeated exposure to exogenous glucocorticoid has been shown to reduce immune function in ocellated skinks (*Chalcides ocellatus*; Saad et al. 1987).

In wildlife, glucocorticoid production has been investigated with regard to anthropogenic disturbances, including the impact of vehicle use on free-living elk (*Cervus elaphus*; Millspaugh et al. 2001) and the impact of tourism on Galapagos marine iguanas (*Amblyrhynchus cristatus*; Romero and Wikelski 2002). Determining temporal patterns of glucocorticoid levels of animals living in human-modified landscapes may help to advance species conservation efforts and to inform management strategies (Wikelski and Cooke 2006, Romano et al. 2010). There is a paucity of data for turtles, including a native Missouri box turtle, the three-toed box turtle (*T. mexicana triunguis*; Missouri

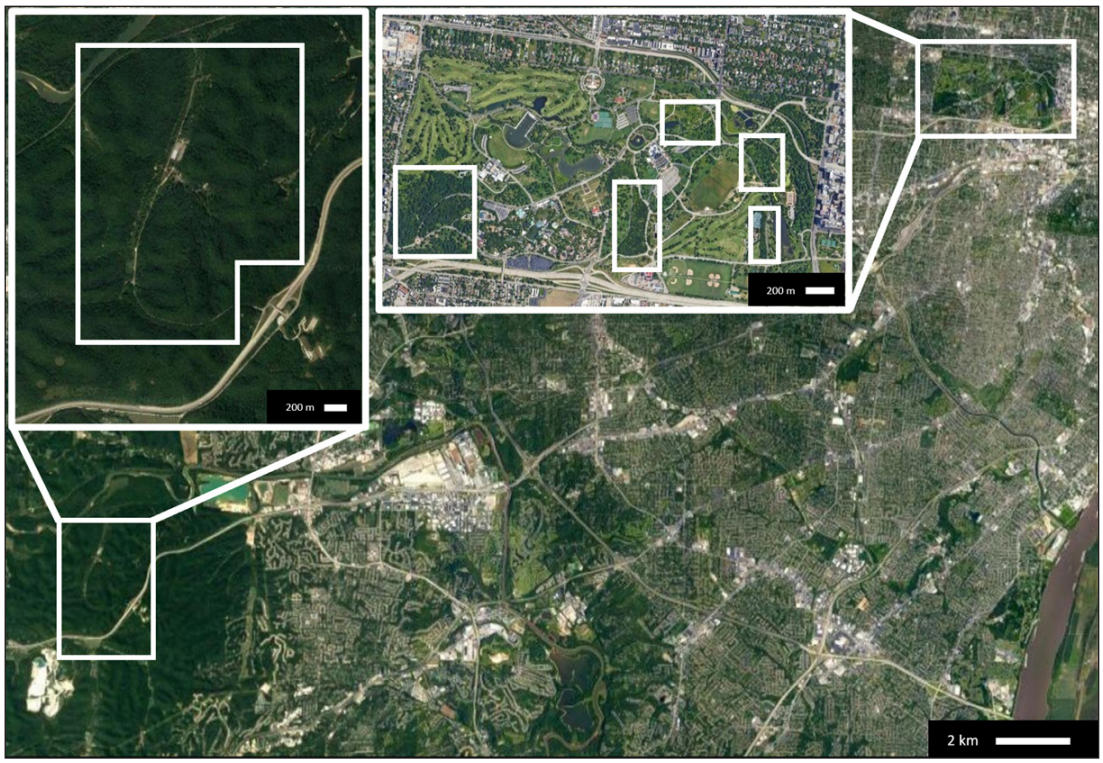

Figure 2. Reference map of the greater St. Louis, Missouri, USA area with Tyson Research Center highlighted (left) and Forest Park highlighted (right). Missouri three-toed box turtles (*Terrapene mexicana triunguis*) can be found within the fragmented ranges outlined within Forest Park bordered by streets and city buildings in addition to busy public hiking trails. Tyson Research Center has only 1 road crossing the property with no public access, and box turtles can be found across the entire area.

Department of Conservation 2008, Rhodin et al. 2017; Figure 1).

The goal of our study was to examine CORT production of three-toed box turtles living in 2 habitats, 1 rural and 1 urban, with varied levels of human impact. First, we validated a methodology for quantifying CORT in three-toed box turtle plasma, and then we used this technique to examine CORT production of the free-living three-toed box turtle populations. We hypothesized that box turtles living in the more disturbed urban environment would have higher levels of CORT compared to those in a less human-disturbed, rural environment.

Study area

Free-living three-toed box turtles were sampled at two sites. Forest Park (FP; urban) is a 556-ha park in St. Louis, Missouri (38°63'N, 90°28'W) that is a mosaic of land use, including recreational areas with fragmented forest and forest edge habitats suitable for native box turtles (Figure 1). Tyson Research Center (TRC; rural; 38°52'N, 90°56'W) is an 809-ha contiguous oak-hickory (*Quercus* spp., *Carya* spp.) forest and biological research station, in Eureka, Missouri, 20 km southwest of FP (Figure 2). This Ozark region of Missouri contains some of the largest and least impacted forests in the Midwest and may represent a refuge for oak-hickory forest biodiversity, including terrestrial turtles (Brookshire and Hauser 1993, Semlitsch et al. 2014).

Methods

Sample collection

We collected most of the blood samples from free-living three-toed box turtles from May to June, 2012–2016, with few samples collected in other months. Once encountered, we manually restrained the turtle and collected blood within 3 minutes. This period was based on previously published studies that reported CORT levels in chelonians can increase within 3 minutes of

handling (Mader 2006, Zachariah et al. 2009). We collected blood (<1% of body weight) from the subcarapacial sinus using a 3-ml syringe and heparinized, 25-g needle (Jacobson 1993, Hernandez-Divers and Cooper 2006). We placed the blood samples in lithium heparin-coated microtainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) and stored them on ice for up to 4 hours in the field. Each turtle was marked for identification with marginal scute notches if recaptured, as previously described by Palmer et al. (2016).

We centrifuged whole blood samples for 10 minutes at 12,000 rpm and stored 100 µL of plasma in Eppendorf tubes (Eppendorf AG, Hamburg, Germany) at -80°C until extraction and assay. All turtle handling procedures were approved by the Saint Louis Zoo Institutional Animal Care and Use Committee (IACUC #14-06), and handling of turtles in FP and TRC was permitted by the Missouri Department of Conservation. We used only samples from adult (carapace length >110 mm [Schwartz and Schwartz 1974]), sexed three-toed box turtles in the study.

Plasma hormone analysis

All CORT testing was performed at the Saint Louis Zoo Endocrinology Laboratory. We extracted plasma samples with absolute ethanol to precipitate proteins and lipids. Per sample, we added 100 µl of absolute ethanol to 100 µl of plasma, mixed using a vortex and incubated at 37°C for 30 minutes. Samples were then spun at 16,300 *g* for 10 minutes to remove the lipid fraction. The supernatant was transferred into a sterile cryotube (Nunc™ Thermo Scientific™, Waltham, Massachusetts, USA) and remaining pellet washed with additional 100 µl of absolute ethanol. The samples were spun at 16,300 *g* for an additional 10 minutes and supernatant added to achieve a dilution factor of 1:3 for each sample extract. Extracts were frozen at -80°C until assays were performed.

We measured CORT using a commercially available radioimmunoassay (RIA) kit (Double Antibody I-125 Corticosterone Kit, ICN MP Biomedicals). The standard curve ranged from 0.125–10.0 ng/ml. Detection limits of assay, with the dilution factor (1/3) of the samples, were 0.375 ng/ml and 30.0 ng/ml. We added a standard diluent to extracted plasma samples, and steroid-free box turtle plasma extract was added to standards and quality controls. Box turtle plasma extract was stripped of steroids using dextran-coated charcoal (DCC# 6241, Sigma Chemical, Saint Louis, Missouri). All samples were assayed in duplicate, and samples from the same turtle were run in the same assay. In total, we completed 3 CORT assays. The mean intra-assay variation of duplicate samples was 8.7%. The mean inter-assay variation of quality controls was 10.3%.

Biochemical assay validation

Extraction efficiency. To determine efficiency of extraction procedure, we added a known amount of CORT to plasma samples before extraction and measured after the extraction process. We pooled plasma from several turtles and divided into 6 aliquots. Exogenous CORT was added to 3 100-µl aliquots, and 3 others were set aside so that the amount of endogenous CORT in the pool could be determined. Exogenous CORT was also added to 3 100-µl aliquots of phospho-buffered saline. These samples served as controls from which recovery could be calculated. The 6 plasma samples were extracted as described above, and 200 µl of absolute ethanol was added to the control samples. The amount of endogenous CORT present in the sample pool was subtracted from the amount in the spiked samples and compared to the amount present in the control samples.

Recovery. We performed recovery experiments to verify that our extraction procedure removed any substances that could interfere with binding between the hormone and the antibody. Plasma extracts were prepared as described above, and a known amount of CORT was added to 3 extracts containing low values of CORT. This procedure was performed at 3 different dosage levels. Plasma extracts without exogenous CORT were also measured to determine the amount of endogenous CORT in the sample.

Parallelism. We performed parallelism experiments to ensure that the extracts maintained linearity under dilution. Three samples that contained high levels of CORT were diluted 1:2, 1:4, and 1:8 with steroid-free plasma extract. The CORT concentrations were measured using these dilutions, as well as the full-strength sample, using the RIA procedure described above.

Table 1. Mean plasma corticosterone (CORT) concentrations for three-toed box turtles (*Terrapene mexicana triunguis*) in human care at the Saint Louis Zoological Park, St. Louis, Missouri, USA, for both initial (within 3 minutes of handling) and post-stressor (after 20 minutes of handling). Samples collected in 2012. Results demonstrate that the assay methods described are capable of detecting changes in CORT in box turtles due to handling stress.

	Saint Louis Zoo three-toed box turtle ID										
	A	B	C	D	E	F	G	H	I	J	K
Initial CORT (ng/mL)	0.63	<0.38	0.80	1.07	1.45	0.51	0.55	0.46	0.44	0.48	1.05
Post-stressor CORT (ng/mL)	0.72	0.85	0.90	1.20	1.54	4.20	5.85	1.92	5.31	4.59	7.43
Increase (%)	15	126	13	12	5	722	959	320	1,117	862	608

Table 2. Sample and population data by site and sex within site for individual three-toed box turtles (*Terrapene mexicana triunguis*) and total number of samples collected. Between 2012 and 2016, some individuals were sampled multiple times accounting for the greater number of plasma corticosterone samples. FP = Forest Park; TRC = Tyson Research Center.

	Free-living three-toed box turtles	
	Individuals	Samples
FP	72	116
Male	37	53
Female	35	63
TRC	72	104
Male	32	48
Female	40	56
Total	144	220

Table 3. Mean corticosterone (CORT) concentration values for the free-living three-toed box turtle (*Terrapene mexicana triunguis*) populations based on site and sex, 2012–2016. The *F* statistic and *F pr* from generalized linear mixed-model analysis are reported. Results showed no significant difference from these effects. FP = Forest Park; TRC = Tyson Research Center.

	Free-living three-toed box turtles			
	Site		Sex	
	FP	TRC	Male	Female
CORT (ng/mL)	0.54	0.37	0.49	0.44
SE	0.08	0.02	0.09	0.03
F / *F pr*	2.46 / 0.12		1.11 / 0.29	

Biological assay validation

To demonstrate that the methods described can detect changes in plasma CORT related to stress, we collected samples from adult, sexed three-toed box turtles (n = 11) in human care at the Saint Louis Zoological Park (zoo), St. Louis, Missouri. Collection methods are described above. Following the first blood collection, turtles were held for 20 minutes during collection of morphometric data and visual health assessments, after which we collected a second blood sample of the same volume (<1% body weight total). The defined 20-minute handling time between samples was based on data that suggests in some reptile species, CORT may increase in 10–15 minutes following handling (Moore et al. 1991). All zoo box turtle blood samples were processed, stored, and quantified as described above for the free-living three-toed box turtle samples.

Statistical analyses

We analyzed data from free-living box turtles using a Generalized Linear Mixed Model using Genstat 17.0 (VSN International Ltd., Hemel Hempstead, United Kingdom). The CORT concentration was the dependent variable in all cases, with site and sex sampled as fixed effects, and turtle ID as a random effect to account for repeated sampling of some individuals.

Results

The efficiency of the extraction procedure was 93.6 ± 8.0 % (mean ± SE). Recovery experiment

results established that this extraction method removed any material that would interfere with the accuracy of the CORT assay. The addition of known amounts of CORT resulted in a mean recovery of 91.5 ± 5.1% (mean ± SE). The results of the parallelism study indicated that box turtle plasma extracts maintained linearity under dilution. Serial dilutions of box turtle plasma extract gave calculated observed/expected values of 103.7 ± 4.6 % (mean ± SE).

Initial CORT for the three-toed box turtles housed at the zoo had a mean of 0.71 ± 0.10 ng/mL (range 0.38–1.45), and post-stressor CORT mean was significantly higher at 3.14 ± 0.72 ng/mL (range 0.72–7.43; $P = 0.011$). The CORT values for 7 of the zoo box turtles increased by at least 100% following handling. Of those, CORT increased by at least 700% in 4 turtles with 1 that exhibited a 10-fold increase (Table 1).

Among free-living three-toed box turtles (Table 2), the concentration of CORT among TRC turtles had a mean of 0.37 ± 0.02 ng/mL (range 0.15–1.25) and FP mean was 0.54 ± 0.08 ng/mL (range 0.14–8.99) with 6 FP samples higher than the highest TRC CORT value observed. Site ($F_{pr} = 0.12$) and sex ($F_{pr} = 0.29$) had no significant effect on CORT concentrations (Table 3).

Discussion

For the free-living three-toed box turtle populations in our study, site had no significant effect on CORT concentration. These results suggested that the sample population of urban box turtles is not experiencing chronic physiological disturbances from stressors compared to those in the rural site. Additionally, our results demonstrated that the extraction and assay techniques described are valid methods for quantifying CORT concentrations in three-toed box turtle plasma. The differences in CORT seen between zoo-housed three-toed box turtle samples collected at 2 time points support the use of CORT as an indicator of stress in three-toed box turtle species, similar to other species of reptiles (Aguirre et al. 1995, Cash et al. 1997).

Although no significant differences in CORT were observed between FP and TRC, a higher range of CORT was observed in the FP turtles (TRC = 0.15–1.25 ng/mL; FP = 0.14–8.99 ng/mL). It is possible that occasional, acute episodes of stress may be more frequent among urban turtles from such events as park maintenance, mowing, human and pet encounters, and other disturbances associated with human presence. For example, a box turtle in FP found near workers using chainsaws to clear brush had a CORT level of 6.11 ng/mL. This high value may be attributed to the nearby noise and disturbance. This turtle could not be sexed and therefore was excluded from statistical analysis. Another FP turtle that met the criteria for statistical inclusion was found with an even higher CORT level of 8.99 ng/mL, but there were no obvious acute stressors determined at the time of sample collection. These 2 values are extremely elevated above other free-living turtles, as the next highest CORT concentration recorded was 2.29 ng/mL. The zoo-housed turtles, after being handled, also showed extremely elevated CORT samples averaging 3.14 ng/mL in response to acute stress, supporting that these 2 FP turtles may have experienced some acute stressor before sampling.

Chronically elevated concentrations of glucocorticoids are known to be immunosuppressive and detrimental to health across species (Sapolsky 2002). The lack of difference in mean CORT concentrations suggested turtles in the urban park did not experience heightened glucocorticoid production compared to turtles at the rural site. Urban parks may play an important role in protecting wildlife by including patches of semi-native vegetation in an otherwise urban environment (Ferguson et al. 2001). However, anthropogenic disturbances may present other challenges for box turtles in urban settings, including vehicle, lawn mower, and human encounters and harvesting for the pet trade (Gibbs and Shriver 2002, Nazdrowicz et al. 2008, Palmer et al. 2019). Annual survival of box turtles is higher in protected natural settings like TRC with less road and public access compared to human-disturbed environments such as FP with high traffic and public access (Nazdrowicz et al. 2008, Palmer et al. 2019). In Forest Park specifically, brumation failure, referred to as winter kill, was the leading cause of higher mortality of box turtles compared to TRC (Palmer et al. 2019). Ongoing research focused on box turtle hibernaculum and surface temperatures in FP and TRC will allow us to determine whether differences in microclimates may partially explain the higher box turtle mortality in the FP population.

Management implications

Stress is correlated with physiologic change, and studies on stressors for wildlife species can inform best practices for conservation. This study demonstrated that blood plasma CORT concentrations were not significantly different among three-toed box turtles in an urban and rural site, thus leading to reject the hypothesis that an urban, fragmented habitat like FP causes chronically increased stress in this species.

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