Effects of radiotransmitters on fecal glucocorticoid metabolite levels of three-toed box turtles in captivity

Chadwick D. Rittenhouse, Joshua J. Millspaugh, Brian E. Washburn, and Michael W. Hubbard

Abstract The increased use of radiotelemetry for studying movement, resource selection, and population demographics in reptiles necessitates closer examination of the assumption that radiotransmitter attachment does not bias study results. We determined the effects of radiotransmitter attachment on fecal glucocorticoid metabolite levels of wild three-toed box turtles (*Terrapene carolina triunguis*) in captivity. During May 2002 we captured 11 adult three-toed box turtles in central Missouri. We housed turtles in individual pens in a semi-natural outdoor setting. We radiotagged 6 turtles, and the remaining 5 turtles served as controls. We captured and handled all turtles similarly during treatments. We collected feces daily prior to attachment (14 June–05 July 2002), while transmitters were attached (06 July–02 August 2002), and after transmitters were removed (03 August–24 August 2002). We conducted a standard assay validation and found that the assay accurately and precisely quantified fecal glucocorticoid metabolites of box turtles. We did not find a significant effect of radiotransmitter attachment on fecal glucocorticoid metabolite levels of three-toed box turtles ($F_{1,9}=0.404$, $P=0.541$). Fecal glucocorticoid metabolite levels of control and treatment turtles increased significantly during the study ($F_{2,166}=7.874$, $P=0.001$), but there was no treatment:period interaction ($F_{2,166}=0.856$, $P=0.427$). Additionally, we did not find a significant relationship between glucocorticoid metabolite levels and time in captivity ($r^2=0.01$, $F_{1,179}=2.89$, $P=0.091$) or maximum daily temperature ($r^2<0.01$, $F_{1,179}=0.301$, $P=0.584$). Our results suggested that radiotransmitter attachment did not significantly increase fecal glucocorticoid metabolite levels in adult three-toed box turtles; however, we conducted our study in captivity and sample sizes were small. Thus, more research is needed to assess potential effects of radiotransmitters on turtles in the wild. We believe this study is the first to validate the use of fecal glucocorticoid metabolite measures for reptiles, which might prove useful in other research studies.

Key words fecal glucocorticoids, stress, *Terrapene carolina triunguis*, three-toed box turtles, transmitter

Radiotelemetry is a powerful tool with diverse applications for both terrestrial and aquatic turtles. Recent reptile studies involving telemetry include determining daily or seasonal movements (Haxton and Berrill 2001), home-range size (Morrow et al. 2001), resource use (Compton et al. 2002, Rittenhouse 2003), and demographic rates such as reproduction (Converse et al. 2002) and survival (Hellgren et al. 2000).

Although telemetry studies provide valuable information that may be difficult to obtain otherwise, several assumptions are made when using

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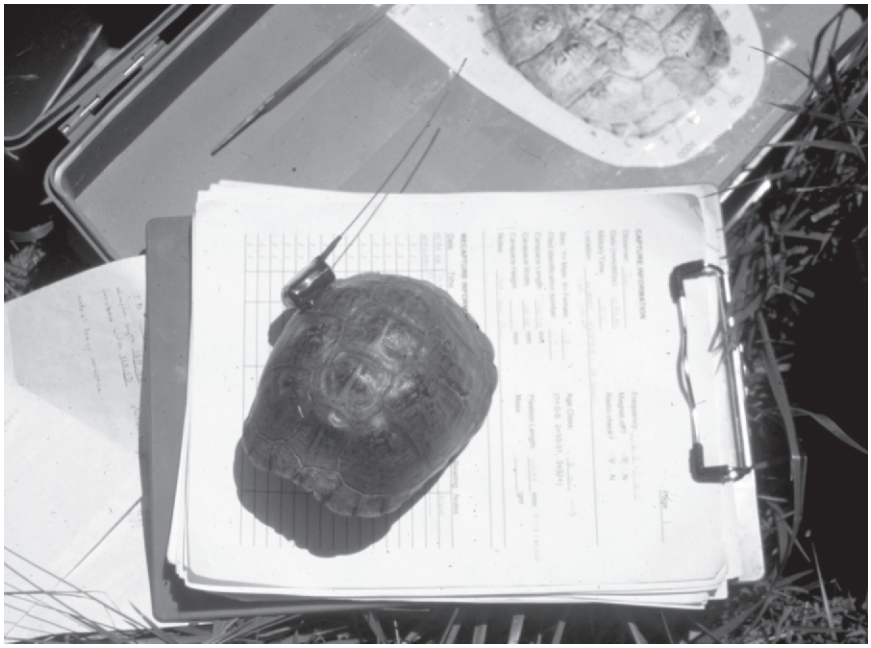
radiotelemetry on free-ranging animals. Most importantly, we assume that radiotagged animals behave in a manner similar to non-instrumented animals (White and Garrott 1990). Studies on avian taxa have shown that radiotagging may alter behavior (Pietz et al. 1993), body condition (Greenwood and Sargeant 1973), reproduction (Rotella et al. 1993, Paquette et al. 1997), or survivorship (Gammonley and Kelley 1994). Radiotagged mammals might have reduced activity levels (Mikesic and Drickamer 1992) and survivorship (Swenson et al. 1999). While the effects of radiotransmitter attachment have been documented on avifauna and mammals (see Withey et al. 2001 for review), few studies have examined effects on turtles (but see Boarman et al. 1998 for review).



Three-toed box turtle with radiotransmitter. Photo by J. Strong.

In addition to the effects from radiotransmitter attachment such as those described above, animals might exhibit more subtle physiological responses (Schulz et al. 1998, 2001; Suedkamp Wells et al. 2003). In particular, radiotransmitter attachment might result in increased glucocorticoid secretion (i.e., stress hormones; Suedkamp Wells et al. 2003). Chronic elevation of glucocorticoids might reduce resistance to disease, survival, and reproductive output (Dunlap and Schall 1995, Wingfield 1988). Thus, monitoring glucocorticoids can serve as a useful index of an animal's physiological response to various disturbances (Wingfield et al. 1982, Millspaugh et al. 2001). Glucocorticoid secretion might be monitored through invasive (e.g., in blood; Cree et al. 2000, Moore et al. 2001) or non-invasive techniques (e.g., fecal or saliva glucocorticoid measurements; Wasser et al. 2000; Millspaugh et al. 2001, 2002). Non-invasive techniques, including fecal-based studies, allow sample collection with minimal disturbance to the study animal (Wasser et al. 2000, Millspaugh et al. 2001) and might provide a more accurate assessment of stress without the bias of capture-induced or disturbance-induced increases in glucocorticoids (Harper and Austad 2000). In addition, fecal glucocorticoid metabolite analyses reflect an integrated level of circulating glucocorticoids over time, rather than a point sample, and therefore may provide a more accurate assessment of long-term stress (Harper and Austad 2000).

Fecal glucocorticoid metabolite assays have been developed for a variety of birds (Wasser et al. 1997) and mammals (Wasser et al. 2000) but not reptiles. These procedures have been used to assess the effects of various disturbances on animals (e.g., Wasser et al. 1997, Millspaugh et al. 2001), including effects of handling and radiocollaring (Creel et al. 1997, Suedkamp Wells et al. 2003). The purpose of this study was to examine the physiological effects (i.e., changes in glucocorticoid secretion) of radiotransmitter attachment on wild three-toed box turtles (*Terrapene carolina triunguis*) held in captivity.

Study area and methods

Experimental design

All research was conducted at the University of Missouri's Thomas S. Baskett Wildlife Research and Education Center (Baskett) located near Ashland, Missouri (38°45′N, 92°12′W). The 890-ha area was approximately 75% forested, with oak (*Quercus* spp.), hickory (*Carya* spp.), and maple (*Acer* spp.)

predominant. Nonforested areas were maintained as old-field habitat with periodic mowing and prescribed burns. Mean annual temperature was 12.8°C; January and July mean temperatures were –1.0°C and 25.6°C, respectively. Mean annual precipitation was 940 mm.

We captured 11 adult three-toed box turtles (n = 3 males and n = 8 females) opportunistically and during systematic searches of the study area from May 26–29, 2002. Immediately after capture, we transported turtles less than 5 km to the main research facility. We housed turtles individually in pens consisting of a lumber frame covered with fiberglass screening. Individual pens were approximately 1.5 m (L) × 0.60 m (W) × 0.75 m (H), with the box bottom consisting of 6.35-mm hardware cloth. We provided water ad libitum throughout the experiment. We fed turtles earthworms and crickets every 2–3 days. We collected all feces daily when available. Fecal samples consisted of all feces within pens since the previous collection; average time between collections was 1.24 ± 0.46 days. During each collection we removed feces from the pen, transferred them to Ziploc bags, and froze each sample at –20°C.

Following a 2-week acclimation period, we collected pre-attachment fecal samples daily from 14 June–5 July 2002 (pretreatment period). On 6 July 2002, we handled all turtles and assigned treatments. We randomized the treatment (control or transmitter) assigned to each turtle. We attached model R2020 reptile glue-on transmitters (Advanced Telemetry Systems, Inc., Isanti, Minn.) using quick-set epoxy to half (n = 6) of the turtles. Weight of transmitters (12.5 g) plus epoxy totaled <25 g, constituting 3–5% of turtle body weight. We attached transmitters to the posterior of the carapace to avoid increasing carapace height or width. To control for possible effects of capture and handling, we handled all turtles for approximately 15 minutes whether or not a transmitter was attached. From 6 July–2 August 2002, we collected all feces daily when available (treatment period). On 3 August 2002, we recaptured all turtles, removed transmitters, and obtained a final body weight. We continued collecting feces from 4 August–24 August 2002 (post-treatment period). On 25 August 2002, we transported all turtles to their original capture sites and released them.

Fecal glucocorticoid analyses

We placed frozen fecal samples (n = 181 total) in a lyophilizer (Freeze-dry Specialties, Inc., Osseo, Minn.) for 24 hours. Once they were freeze-dried, we ground feces and sifted samples individually through a stainless-steel mesh. To prevent cross-contamination of fecal samples, we removed any remaining fecal material from the mesh with a brush between each sample and visually inspected the mesh to ensure all residue was removed. We extracted fecal glucocorticoid metabolites from box turtle feces using a modification of Schwarzenberger et al. (1991). We placed dried feces (~0.2 g) in a test tube with 2.0 mL of 90% methanol and vortexed them at high speed in a multi-tube vortexer for 30 minutes. We then centrifuged samples at 500 *g* for 20 minutes and saved the supernatant and stored it at –84°C until assayed.

We used corticosterone I^{125} radioimmunoassay (RIA) kits (ICN #07-120103, ICN Biomedicals, Costa Mesa, Calif.) to quantify box turtle fecal glucocorticoid metabolite concentrations. This assay likely does not quantify all of the fecal glucocorticoid metabolites (e.g., conjugated metabolites). Studies with mammals suggest that only a small proportion of metabolites are conjugated in the species examined, and although not known for reptiles, we suspect the same might be true. We analyzed fecal samples in 3 assays, with all the samples from the same individual included within an assay. We followed the ICN protocol for the corticosterone I^{125} RIA, except that we halved the volume of all reagents (Wasser et al. 2000).

Our assay validation included an assessment of parallelism, recovery of exogenous analyte, intra- and interassay precision, and assay sensitivity (Jeffcoate 1981, Grotjan and Keel 1996, O'Fegan 2000) to confirm that the assay accurately and precisely measured glucocorticoid metabolites in turtle feces. We conducted parallelism and recovery of exogenous corticosterone validation assays on 2 pooled fecal extract samples (low and high; each pool consisted of feces from five individuals). Parallelism ensures the assay maintains linearity under dilution, and recovery of exogenous corticosterone verifies accurate measurement throughout the working range of the assay (Jeffcoate 1981). We conducted the recovery of exogenous corticosterone validations by mixing corticosterone standards (range = 0.50–1.25 ng/mL) in a 50:50 mixture with the low-pool and high-pool turtle fecal extracts. After vortexing, we assayed these "recovery samples" using our standard assay procedure.

We randomly selected 3 turtle fecal samples and analyzed them in each assay; interassay variation was calculated from these samples. We calculated intra-assay variation by averaging the CVs of replicate tubes from 20 randomly chosen samples.

Data analysis

We compared fecal glucocorticoid metabolite levels among periods between control and radio-tagged turtles with a two-way repeated-measures ANOVA using a mixed-effects model. Both treatment (between-subject) and period (within-subjects) factors were considered fixed effects. Because we had multiple fecal samples per individual turtle per period and expected individual turtles to vary independently, we treated repeated observations on turtles as random effects. We did not include sex as a factor in our analyses because we had too few turtles to do so. Thus, we pooled sexes in the following models. We tested for a possible increase in fecal glucocorticoid metabolite levels over the period of the study due to time in captivity using linear regression. Additionally, because we housed turtles in semi-natural conditions (e.g. outside in the shade, but without leaf litter), the turtles may not have experienced microclimate conditions that exist in the wild. For poikilotherms, microclimate conditions are important to metabolic functions and potentially stress. Thus, we used linear regression to determine whether air temperature was related to fecal glucocorticoid metabolite levels. We fit linear mixed-effects models by maximum likelihood using the function "lme" from library section "nlme" (Venables and Ripley 2002) in program R (Ihaka and Gentleman 1996). Given our limited sample sizes, we recommend caution when interpreting statistical results.

Results

Serial dilutions (1:2 up to 1:128) of box turtle fecal extracts yielded displacement curves that were parallel (all $P > 0.18$) to the corticosterone standard curve (Figure 1). Mean recovery of added exogenous corticosterone (range = 0.5–1.25 ng/mL) was 93.1% (SE = 3.88, $n = 12$). Acceptable recovery of exogenous corticosterone (within 90–110%) and demonstration of parallelism suggested no sample matrix effects (Jeffcoate 1981, Grotjan and Keel 1996, O'Fegan 2000). Assay sensitivity was 1.25 ng/g. The manufacturer's reported cross-reactivity of the antisera was 100% with corticosterone and <1% for other steroids. Inter-assay variation for 3 assays was 10.4% and average intra-assay variation was 2.1%.

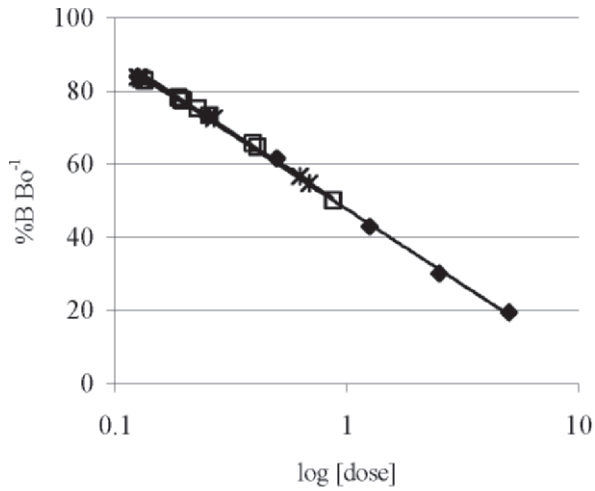


Figure 1. Parallelism of fecal glucocorticoid metabolite results for fecal extracts from box turtles captured at the University of Missouri Thomas S. Baskett Wildlife Research and Education Center, Ashland, Missouri, June–August 2002. Curves of percent binding of I^{125} tracer (%B Bo^{-1}) versus serially diluted (log-transformed doses of 1:2 to 1:128) low-pool (n = 2) and high-pool (n = 2) fecal extracts from wild box turtles in captivity were parallel (test of equal slopes, all $P > 0.18$) to corticosterone standard curves (log-transformed doses of 0.125 to 5.0 ng mL^{-1}). Corticosterone standard curve points are represented by diamonds, points from serially diluted low pool fecal extracts are represented by squares, and points from serially diluted high pool fecal extracts are represented by stars.

We collected and analyzed an average of 16.5 fecal samples (SE = 5.5) from each individual box turtle during the 10-week study period. Radiotransmitter attachment did not affect fecal glucocorticoid metabolite levels of three-toed box turtles ($F_{1,9} = 0.404$, $P = 0.541$; Figure 2). Fecal glucocorticoid metabolite levels of both control and treatment turtles increased significantly during the treatment period ($F_{2,166} = 7.874$, $P = 0.001$; Figure 2), but there was no treatment:period interaction ($F_{2,166} = 0.856$, $P = 0.427$). Thus we did not detect a difference between control and treatment turtles before, during, or after radiotransmitter attachment. The estimate of residual variance was 36.989 and variance of random effects between groups (turtles) was 9.699, indicating more variation in fecal glucocorticoid metabolite levels within individual turtles than among turtles. We did not find a significant relationship between time in captivity ($r^2 = 0.01$, $F_{1,179} = 2.89$, $P = 0.091$) and fecal glucocorticoid metabolite levels. Additionally, we did not find a significant relationship between maximum daily

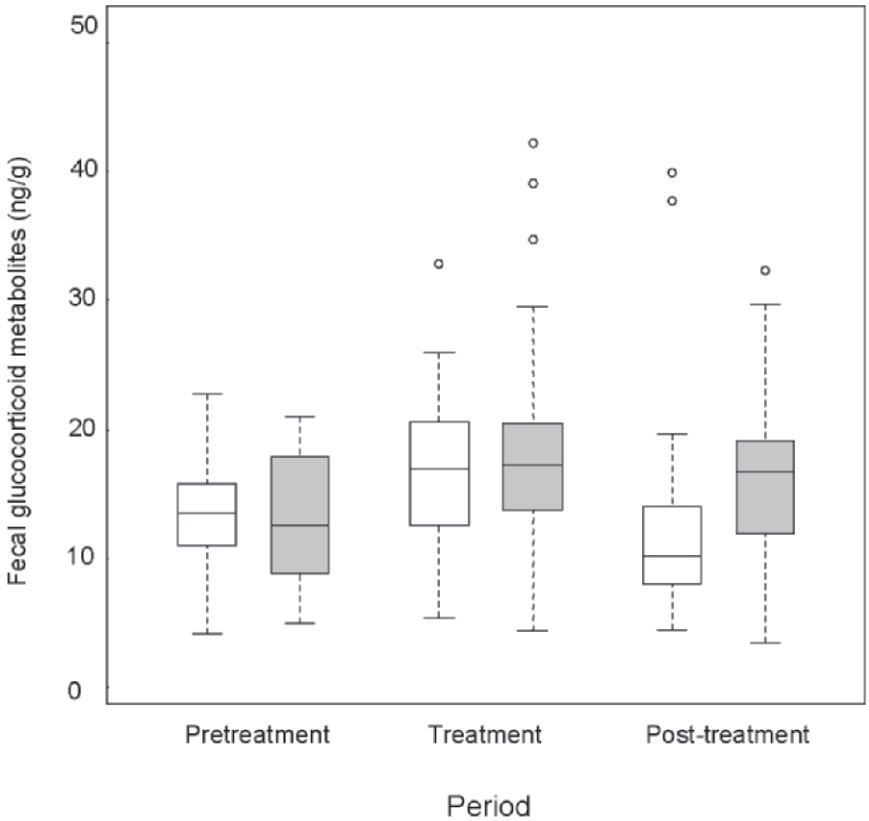


Figure 2. Fecal glucocorticoid metabolites (ng/g; box-and-whisker plot) of captive three-toed box turtles in control (open box) and transmitter (shaded box) groups during pretreatment, treatment, and post-treatment periods at the University of Missouri Thomas S. Baskett Wildlife Research and Education Center, Ashland, Missouri, June–August 2002. Outliers are marked with open circles. No significant difference was detected between control and treatment groups ($F_{1,9}$ = 0.404, P = 0.541).

temperature over a range of 20.6–37.2°C (r^2<0.01, $F_{1,179}$=0.301, P=0584) and fecal glucocorticoid metabolite levels of control or treatment turtles.

Discussion

Results of our assay validation and the variability we observed in fecal glucocorticoid metabolites indicates we can reliably detect and monitor changes in adrenocortical activity in adult three-toed box turtles using fecal glucocorticoid analysis. The performance characteristics (parallelism, recovery of exogenous corticosterone, intra- and interassay precision, and assay sensitivity) of this assay verify that it is accurate, precise, demonstrates linearity under dilution, and has an appropriate range of sensitivity. These results corroborate other studies (e.g., Wasser et al. 2000, Millspaugh et al. 2002) that demonstrate the ability to assess glucocorticoid levels from feces of wild animals. To our knowledge, this study is the first to validate the use of fecal glucocorticoid analyses for stress assessment in reptiles.

Fecal glucocorticoid metabolite analysis offers one method to assess physiological responses of turtles to radiotransmitters and other potential stressors. Because of its non-invasiveness, this procedure might prove useful in other studies that evaluate the stress response of turtles to environmental or human-induced disturbances. Although collection of fecal material from free-ranging turtles might be challenging, fecal collections have occurred for many wildlife species, including turtles. Creel et al. (1997) and Millspaugh et al. (2001) used a combination of radiotelemetry and visual observation to collect fecal samples from free-ranging radiocollared African wild dogs and elk, respectively. Josseaume (2002) used a fecal collector, which consisted of a wire-mesh framework and plastic casing attached to the carapace and the plastron, to study digestive responses in forest tortoises. In combination with visual observation or thread-trailing techniques (Breder 1927, Schwartz and Schwartz 1974, Claussen et al. 1997), the fecal collector might be a useful way to collect feces from individual, free-ranging turtles. Such a collection system could also help ensure that samples are fresh (Washburn and Millspaugh 2002) and from known individuals.

We did not detect a difference in fecal glucocorticoid metabolite levels between control and transmitter turtles during our study. This result is important because it suggested that radiotransmitter attachment did not significantly increase stress hormone secretion in adult three-toed box turtles. However, fecal glucocorticoid metabolite levels for control and transmitter turtles increased during the

treatment period and then declined during the post-treatment period. We considered 2 factors, temperature and time in captivity, which alone or in combination may have served as additional stressors to the turtles. However, these factors were not related to fecal glucocorticoid metabolite levels.

In addition to the possible physiological effects of radiotransmitter attachment, animals might have altered movement or behavior patterns due to the radiotransmitter. As a general rule, radiotransmitter mass should constitute ≤3% of total body mass for birds (Withey et al. 2001) and ≤10% total body mass for amphibians (Richards et al. 1994). Although no guidelines exist for turtles, previous research on extrinsic mass loads on eastern (*Terrapene carolina carolina*) and ornate (*Terrapene ornata ornata*) box turtles suggests that loads less than 50% of body mass may not affect locomotive ability (Marvin and Lutterschmidt 1997, Wren et al. 1998). Certainly, keeping radiotransmitters small may reduce behavioral changes owed to the radiotransmitter itself, but if the greater concern is change in behavior from handling during radiotransmitter attachment, then this issue needs to be addressed. Additionally, the question is not about the ability to move but rather how behaviors related to movement ecology may change as a result of radiotelemetry and handling. We suggest conducting joint behavioral–physiological studies as one way to evaluate these effects.

The increased use of radiotelemetry for studying movement, resource selection, and population demographics in reptiles necessitates closer examination of the assumption that radiotransmitter attachment does not bias study results. Our results suggested that radiotransmitter attachment did not significantly increase fecal glucocorticoid metabolite levels in adult three-toed box turtles; however, our study was conducted in captivity and sample sizes were small. Thus more research is needed to assess potential effects of radiotransmitters on turtles in the wild.

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