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Stress and Body Composition of Juvenile Alligator Snapping Turtles (Macrochelys temminckii)

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STRESS AND BODY COMPOSITION OF JUVENILE ALLIGATOR SNAPPING TURTLES (*MACROCHELYS TEMMINCKII*)

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Master of Science, Biology

By

Brandon Tappmeyer

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ABSTRACT

The alligator snapping turtle (Macrochelys temminckii), is a species of conservation concern that is the subject of multiple head-start and reintroduction efforts across its range. In captive propagation programs, producing offspring that are in optimal physiological condition maximizes the likelihood of success after release. The purpose of my study was to compare stress and body composition between one free-ranging reintroduced population and two captive populations. The two captive populations were both housed in southern Oklahoma, but one group was reared indoors whereas the other inhabited outdoor ponds at a national fish hatchery. I used circulating glucocorticoid (corticosterone) concentrations as an indicator of stress level and dual-energy X-ray absorptiometry (DXA) to estimate body composition. Corticosterone concentrations did not differ between captive and free-ranging populations, possibly suggesting that corticosterone is a poor predictor of stress in this species or under chronically stressful conditions. DXA accurately and precisely estimated fat mass (FM), lean tissue mass (LTM), and bone mineral mass (BMM). The captive-outdoor and free-ranging populations exhibited greater BMM than the captive-indoor population. However, both captive populations exhibited higher FM and lower LTM than turtles in the free-ranging population. A body condition index calculated by regressing log-transformed mass on length did not correlate with FM or BMM, but did correlate significantly with LTM.

KEYWORDS: *Macrochelys temminckii,* BCI, dual energy X-ray absorptiometry, stress, corticosterone, stress response

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OVERVIEW

Captive propagation, head-starting, and reintroductions have become important conservation tools in recent decades as more populations are extirpated and more species pushed toward the brink of extinction. The relative utility of these approaches varies among taxa, but even for species that are well-suited to these conservation tactics, it is critical that released individuals be in good condition behaviorally and physiologically. Captive rearing comes with risks, including invoking behavioral syndromes that become disadvantageous in the wild, and malnutrition that can exert long-term effects on growth and reproduction.

For my thesis, I focused on the effects of captive propagation and rearing on stress levels and body composition of alligator snapping turtles (*Macrochelys temminckii*). Captive propagation and reintroduction efforts were initiated by the U.S. Fish and Wildlife Service in 2002 and 2008, respectively. To better inform these efforts, I compared stress levels—based on circulating corticosterone concentrations—and body composition using non-invasive dual-energy X-ray absorptiometry (DXA).

The first chapter of my thesis introduced the applicability of DXA to juvenile *M. temminckii* and several metrics that are commonly used to characterize the general health of organisms. These include the body composition parameters fat mass, lean tissue mass, and bone mineral density, as well as a whole-organism body condition index that is calculated from a mass-length regression and is typically interpreted to indicate individuals' fat stores relative to other individuals of the same species or in the same population. The assumption of the index's ability to indicate relative fat stores was not supported.

The second chapter of my thesis compared stress levels of captive and free-ranging (but previously captive) alligator snapping turtles. Additionally, I tested the hypothesis that, among free-ranging alligator snapping turtles, high corticosterone levels correlate with slower growth rates. The results from my research will inform husbandry and reintroduction protocols for alligator snapping turtles, and may be more broadly informative to head-start/reintroduction efforts across a broad array a taxa by identifying some of the pitfalls associated with captive rearing and how animals adjust physiologically after release.

VARIATION IN BODY COMPOSITION OF FREE-RANGING AND CAPTIVE ALLIGATOR SNAPPING TURTLES (*MACROCHELYS TEMMICKII*)

Abstract

Body composition of animals may be affected by many factors, including age, sex, reproductive stage, diet, and environment. The body composition of animals reared in captivity frequently differs from that of wild conspecifics due to differences in diet and environmental conditions, and for animals that are reared for reintroduction these differences can measurably impact post-release survival and fitness. I used dual-energy X-ray absorptiometry (DXA), a non-invasive method of quantifying fat mass, lean tissue mass, bone mineral density, and bone mineral mass, to compare body composition of captive alligator snapping turtles (*Macrochelys temminckii*) housed both indoors and outdoors in a head-start program to free-ranging individuals. I used gravimetric and chemical analytical methods to validate DXA values. Finally, I compared DXA-derived body components to body condition index (BCI) values calculated from the residuals of mass:length regressions. After adjusting for variation in body size, bone mineral mass differed among the three groups (Outdoor Pond > Free-ranging > Indoor-reared). The free-ranging population exhibited lower fat mass and higher lean tissue mass than either captive population. BCI values correlated most strongly with lean tissue mass, suggesting that variation in BCI better represents variation in morphology than fat or bone mass. Our results suggest that captive alligator snapping turtles likely benefit from readily available food that permits them to store more fat, but may suffer reduced bone development as a result of growing indoors without access to sunlight.

Introduction

Captive propagation and head-starting have become critically important to the survival of a diverse array of taxa that have experienced population declines, including plants such as orchids (Stewart 2008), invertebrates such as freshwater mussels (Neves 2004), and vertebrates such as black-footed ferrets (*Mustela nigripes*; Miller et al. 1994), California condors (*Gymnogyps californianus*; Walters et al. 2010), and Burmese star tortoises (*Geochelone platynotan*; Platt et al. 2011). Propagating animals in captivity has several benefits over relying solely on *in situ* recruitment: it reduces losses to predation by allowing animals to grow to a size that minimizes risk from smaller, gape-limited predators, and can provide optimal nutrition

during early life stage when resource acquisition is often most challenging. For some species, however, head-starting presents drawbacks that can have adverse long-term consequences by acclimatizing individuals to conditions that deviate significantly from what they will experience after release into the wild (Osborne and Seddon 2011). For example, captive animals are often reared in conditions that fail to mimic natural environmental heterogeneity (e.g., unnaturally consistent temperatures, humidity, light cycles, topography), fail to provide opportunities to learn predator avoidance, foraging, and social skills, and may not offer exposure to critical abiotic factors such as solar heat, ultraviolet light, and natural substrates on which good health may depend (Snyder et al. 1996). Additionally, unnatural housing conditions and crowding may induce chronic stress levels that impact activity, immune function, hormone cycles, and growth (Knoder 1959; O'Brien and Evermann 1988; Thorne and Williams 1988; Elsey et al. 1990; Snyder et al. 1996).

Head-start efforts may induce obesity and/or osteoporosis as a result of various effects of captivity. Obesity may result from reduced activity and readily available, nutritionally rich forage (Schwitzer and Kaumanns 2001). In turn, obese individuals may be susceptible to health risks including lethargy, decreased muscle tone, and compromised pulmonary and cardiac performance (Schwitzer and Kaumanns 2001). Osteoporosis is also a common concern for captive animals (Anderson and Capen 1976; Turner et al. 2001; Smith et al. 2009). Calcium regulation is complex and may be impacted by low dietary calcium availability or low absorption rates due to insufficient exposure to ultra-violet light for vitamin D synthesis (Elsey 2006). Additionally, chronic stress can decrease bone density by affecting the activity of osteoblasts and osteoclasts (Frye 1991, Kiecolt-Glaser et al. 2003). Whereas adult animals with historically normal bone density may survive long periods of calcium anhomeostasis before exhibiting

clinical signs of osteoporosis, young animals often succumb much more quickly without previously accrued mineral reserves to rely upon.

A simple and oft-applied method for assessing the overall condition of animals is to calculate a body condition index (BCI) based on individuals' length and mass. A variety of approaches have been used to calculate such indices (Jakob et al. 1996, Stevenson and Woods 2006), but all assume that variation among individuals reflects differences in density or volume that, in turn, correspond with differences in lipid stores. Although the validity of BCIs is often assumed without validation, studies have demonstrated a correlation between a BCI score and lipid stores in some taxa (Secor and Nagy 2003; Johnson et al. 2009). On the other hand, in some instance BCIs clearly do not correlate with lipid stores (Stone et al. 2010; Warner et al. 2016). Fortunately, several other non-invasive—though arguably less convenient—methods for assessing lipids and other body composition variables are available. Non-invasive methods are often critical because they can be applied to longitudinal studies that require repeated measurements of individuals, as well as to studies of rare or endangered species where sacrificing individuals for analysis is counterproductive (Speakman 2001; Secor and Nagy 2003; Stone et al. 2010). Noninvasive methods such as total body electrical conductivity (TOBEC), isotope dilution, lipid-soluble gas absorption, and bioelectrical impedance analysis (BIA) are appropriate for repeated measurements of individuals (Speakman 2001; Johnson 2009). However, their applicability is sometimes taxonomically limited, and results may be imprecise or—worse—inaccurate (Speakman 2001; Secor and Nagy 2003; Stone et al. 2010).

Although there are some limitations to its application, dual-energy X-ray absorptiometry (DXA) demonstrably generates reliable body composition data for a variety of taxa (Swanpalmer et al. 1998; Bertin et al. 2000; Speakman 2001; Secor and Nagy 2003; Stone et al. 2012).

However, the utility of DXA for determining body composition of Emydid turtles is limited because a contiguous bony shell prevents DXA from accurately comparing tissues of different density (Stone and Turner 2012). Although at least two other studies have used DXA to assess one or more components of body composition of turtles (Fledelius et al. 2005, Gramanzini et al. 2013), only one has validated this method for accurately and precisely determining bone mineralization, total body water content, and lean tissue mass (Stone et al. 2010). In light of the problem that a contiguous bony shell presents, it is perhaps surprising that DXA reliably estimated bone mineral content, lean tissue mass, and body mass of red-eared slider turtles (*Trachemys scripta elegans*); only estimates of fat mass proved inaccurate (Stone et al. 2010). However, the accuracy of DXA for species of turtle with reduced shell morphology has not been determined. The same is true for turtles of many species that exhibit expansive fontanelles during early life stages, resulting in regions that, in a DXA scan, would likely not be occluded by bone.

Alligator snapping turtles (*Macrochelys temminckii* [Troost in Harlan, 1835]) are native to the southeastern United States, and evidence indicates that populations have declined precipitously in many areas (Ernst and Lovich 2009; IUCN 2017). This was demonstrably true in Oklahoma by the 1990s, when extensive population surveys detected just a single apparently healthy population remaining in the state (Riedle et al. 2005). Captive propagation and head-start efforts were initiated in 2000 to help restore the species to waterways that it had historically occupied (Riedle et al. 2008; Oklahoma 2015). By 2012, the program included more than 20 female brood stock and produced 375–600 eggs annually (Thompson 2013). Hatchlings produced in the program are reared for 2–8 yr prior to being released into natural systems. This time is variably split between indoor enclosures and outdoor ponds, and all individuals spend at least 1 yr outdoors prior to release to gain exposure to natural light and temperature cycles, and

to practice foraging under semi-natural conditions. Curiously, what were previously assumed to be optimal rearing conditions in captivity were shown to produce slower growth than was observed among individuals post-release (Anthony et al. 2015). Furthermore, it is unclear to what extent *M. temminckii* rely on UV exposure to synthesize vitamin D (some fraction could also be derived from animal tissue in their diet). While growing indoors, turtles in this program do not receive exposure to natural light, and therefore may not have the capacity to regulate calcium absorption to optimize bone development. For these reasons, I elected to compare body composition among indoor captive, outdoor captive, and reintroduced free-ranging *M. temminckii*.

In this study, I validated the accuracy of DXA for assessing the body composition of juvenile *M. temminckii*, and then used this approach to determine the effects of captivity on body composition. I compared fat mass (FM), lean tissue mass (LTM), and bone mineralization—including bone mineral density (BMD) and bone mineral mass (BMM)—of free-ranging *M. temminckii* to those of captive turtles housed both indoors and in outdoor ponds. I also compared a commonly used BCI (residuals calculated from a $\log_{10}$mass:$\log_{10}$length regression) to the estimates of body components generated by DXA to assess the utility of the BCI for generating inferences about the body composition of my focal taxon.

Methods

This research was conducted with approval from the Missouri State University Institutional Animal Care and Use Committee (protocol 17-030) and Oklahoma Department of Wildlife Conservation (Scientific Collectors Permit 6764).

Study Animals. Captive Study System. I used juvenile *M. temminckii* that were hatched and reared in captivity in a head-start program at Tishomingo National Fish Hatchery (NFH) in southeastern Oklahoma. In this program, turtles were reared in cohort-specific groups, and released in rivers and oxbows after 2–8 yr. While in captivity, turtles were reared indoors for 1–2 yr and then were maintained in outdoor ponds for at least 1 yr prior to release to give exposure to seasonal cycles, natural light, and semi-natural foraging conditions. All individuals were implanted with a passive integrated transponder (PIT) tag prior to release to ensure long-term identification.

Riverine Study System. The free-ranging *M. temminckii* used in my study originated from the head-start program at Tishomingo NFH and had been released into the Caney River in northern Oklahoma at least 2 yr prior to the inception of my study. Headwaters of the Caney River are located in the Central Mixed-Grass Prairie Ecoregion in central Kansas. It flows southeast into the Flint Hills Prairie and Cross Timbers Ecoregions in Oklahoma where it ultimately joins the Verdigris River (Woods et al. 2005). My study site was located in the Flint Hills Prairie Ecoregion and consisted of 8.0 km of the Caney River and 4.8 km of a shallow tributary, Pond Creek. The lower reach of the study site was located just above a dam and reservoir (Hulah Lake), which are used for downstream flood control. As such, the study site experienced frequent and prolonged flood events. Potential predators of juvenile *M. temminckii* that are common at this site include, but are likely not limited to, raccoons (*Procyon lotor*), river otters (*Lontra canadiensis*), and great blue herons (*Ardea herodias*). There are several historical records of *M. temminckii* occurring in the Caney River, but the species was extirpated from the system by the mid to late 20th century (Riedle et al. 2005). However, the U.S. Fish and Wildlife Service has conducted reintroductions that started in 2008 (Anthony et al., 2014).

I obtained DXA data from 31 indoor-housed, 26 outdoor-housed, and 13 free-ranging *M. temminckii*.

DXA Validation. Eleven turtles that were determined to be unfit for reintroduction were obtained from Tishomingo NFH for validation of DXA-generated body composition values. The width of the DXA scanning field was 190 mm; therefore, no turtles with carapace widths that exceeded this limit were included in the study. Each turtle was DXA scanned (model QDR-4500A, Hologic®, Marlborough, MA) and then euthanized and frozen for later determination of: 1) lean tissue mass (LTM), 2) fat mass (FM), and 3) bone mineral mass (BMM). DXA also generated an estimate of bone mineral density (BMD), which is calculated as a ratio of BMM over the scanned surface area of a turtle, expressed in mm^2. To validate DXA results, body composition of each of the turtles was determined using gravimetric and chemical analytical methods (Reynolds and Kunz 2001). The turtles were weighed and measured prior to dissection. Fat bodies were removed and placed in drying trays. The mass of the wet fat bodies and the rest of the wet body were recorded and then the trays were desiccated in a 60°C drying oven. Trays were reweighed daily until mass stabilized, and the difference between wet and dry mass was inferred to represent total body water, which is included in DXA calculations as a component of LTM. The desiccated body—absent fat bodies—was then milled into a homogeneous powder (Wiley Mill, Thomas Scientific, Swedesboro, New Jersey, USA).

I measured FM using methods described by Stone et al. (2010). I analyzed 8 g aliquots of homogenate in triplicate. Each subsample was weighed and placed into a 50 mL plastic conical centrifuge tube. I added 35 mL of chloroform:methanol (2:1 v/v) solution to each tube and then vortexed the tubes for 5 min. After suspended particles settled, I removed the liquid upper fraction and added 8 mL of 0.73% sodium chloride solution. I vortexed each tube 2 min, and

then transferred the contents of each tube to a filter paper and rinsed the tube onto the filter paper. I used a vacuum filtration pump to remove the aqueous solution, and then placed the samples into a drying oven at 60°C for 24 hours to complete the drying process. I reweighed the subsamples, calculated the average percent of mass lost across triplicate samples, and extrapolated that value to the entirety of the homogenous mixture. Fat mass was estimated by summing the mass of extracted fat and of desiccated fat bodies that were separated during dissection.

To measure BMM, four replicate aliquots, each weighing 2 g, were ashed in a muffle furnace to remove organic material. To prevent samples from igniting, I used a step-wise temperature regime to ash the aliquots. Samples were initially incinerated at 300 °C for 1 hour; the temperature was then increased in 100 °C increments hourly until reaching 600 °C, which was then maintained for 5 hours. The remaining mineral fraction was then weighed, and the result was extrapolated to the total dried body mass to calculate BMM.

Finally, lean tissue mass (LTM) was calculated by subtracting BMM and FM from total wet mass.

Calculating Body Condition Index. I calculated a BCI as the residuals of a linear regression of $\log_{10}$-transformed mass on $\log_{10}$-transformed carapace length. This BCI is applicable to all individuals in this population. In this system, animals with positive BCI values are presumed to have greater length-corrected density or volume—or both—than animals with zero or negative BCI values. A common assumption of this method is that larger values indicate greater size-corrected FM, while lower values indicate lower FM. It is important to note that this method of calculating a BCI is different than the methods employed in two other studies of

alligator snapping turtles because of the allometric relationship between length and mass (Moore et al. 2013; Trauth et al. 2016).

Statistical Analysis. Because of the allometric relationships between the body composition variables measured and mass, I $\log_{10}$-transformed all data prior to analysis to improve the distribution and reduce heteroscedasticity. I used an analysis of covariance (ANCOVA), with mass included as a covariate, to correct for variation in body size among the three populations that I compared. I used regressions to characterize the relationships between DXA-generated values of BMM, FM and LTM and those values determined from direct tissue composition analyses, as well as to BCI values. Unless otherwise noted, values are reported as mean ± 1 SE.

Results

Validation of DXA Estimates. X-ray images that were generated with each DXA scan of a turtle, as well as examination of prepared skeletons, confirmed that the carapace of juvenile *M. temminckii* includes large fontanelles, leaving room to grow and, importantly to this study, providing spaces between mineralized bones that are predicted to improve the accuracy of body composition estimates (Stone et al. 2010) (Figure 1). Specimens that were used for validation were between 3 and 9 years of age (Table 1) and were 222–1577 g. The size of the turtles correlated with age ($F_{1,9}$ = 37.9124, P = 0.0002, R^2 = 0.8082). Regressions of DXA-generated values with chemically-derived BMM, FM, and LTM were all high (R^2 = 0.9581–0.9995), indicating high precision (Figure 2). The raw estimates of DXA for LTM and FM were close to the chemically derived values while the raw estimate for BMM was less accurate (Table 2). However, DXA consistently and predictably underestimated BMM. Thus, accuracy of raw DXA

estimates was improved by adjusting values for slope and intercept to be closer to the true values (Table 2).

Body Composition among Populations. The size ranges of turtles in each of the three groups overlapped, but the free-ranging turtles were larger, on average, than those from either of the captive groups (straight midline carapace lengths: indoors = 97 ± 7 mm, outdoors = 106 ± 2 mm, free-ranging = 193 ± 7 mm; $F_{2,67} = 79.62$, $P < 0.01$). There was a strong positive relationship between size and BMM (Figure 3A). However, BMM varied among populations even after adjusting for variation in body size (Outdoor Pond > Free-ranging > Indoors; $F_{2,65} = 41.61$, $P < 0.0001$; Figure 3B), suggesting population differences in bone development.

Like BMM, FM correlated positively with size (Figure 3C). However, after adjusting for body size, the free ranging population had lower FM than the captive populations ($F_{2,65} = 34.41$, $P < 0.0001$; Figure 3D). LTM showed the strongest relationship with mass (Figure 3E), and after correcting for body size the free-ranging population showed significantly higher LTM than did the similar captive populations ($F_{2,65} = 43.38$, $P < 0.0001$; Figure 3F).

Body Condition Index Comparisons. There was a strong regression between log-transformed mass and length ($R^2 = 0.990$; Figure 4), resulting in low variation in residual values (BCI values) that did not differ among groups ($F_{2,66} = 0.9045$, $P = 0.4097$). BCI values were not predicted by BMM ($R^2 = 0.0280$), weakly correlated with FM ($R^2 = 0.1651$), and exhibited a stronger correlation with LTM ($R^2 = 0.8036$) (Table 2, Figure 5).

Discussion

Validation of DXA Values. Whereas previous research determined that DXA worked well to estimate BMM and LTM, but not FM of turtles (Stone et al. 2010), I found that all three

body composition variables could be accurately determined for juvenile alligator snapping turtles, thereby providing a powerful tool for noninvasively assessing the health status of an imperiled species (Figure 6). DXA draws inferences about body composition by comparing each pixel at one of the two frequencies of each scan with its neighboring pixels, as well as with the same pixel of the scan at the second frequency to determine body composition. The apparently conflicting results of the two studies are likely due to differences in the species and life stage of study subjects. With a nearly contiguous shell of ossified bone, the apparent density of *T. scripta* was insufficiently variable in DXA scans to accurately estimate FM (Stone et al. 2010), whereas the large fontanelles of juvenile *M. temminckii* provided sufficient heterogeneity to make accurate calculations. If DXA is broadly applicable to all turtle species with reduced shells or large fontanelles, then its utility for other turtle species would be easily identified. Nonetheless, I propose that additional validation studies should be performed to verify the predicted pattern between DXA accuracy and morphology. The issue of a nearly contiguous outer layer of bone may be unique to chelonians; DXA has been shown to accurately infer body composition for a range of other vertebrate taxa (Rose et al. 1998; Nagy and Clair 2000; Secor and Nagy 2003; Korine et al. 2004).

Variation in Body Composition among Populations. Perhaps unsurprisingly, free-ranging *M. temminckii* tended to have lower FM, and therefore proportionally greater LTM than those in captivity. Turtles in captivity, whether reared indoors or outdoors, received food pellets that require minimal effort to acquire, consume, or digest. With such low activity requirements associated with foraging, captive turtles likely were able to store more of the calories that they consumed. This energy could alternatively be allocated to growth, but my data indicate that this was not the case. In fact, it has been demonstrated that *M. temminckii* that are produced and

head-started at Tishomingo NFH frequently accelerate their growth after release. The reason for this counterintuitive pattern remain unclear.

Turtles living outdoors, whether in captivity or free-ranging, exhibited higher BMM than conspecifics reared indoors. The most parsimonious explanation for this pattern is that *M. temminckii* synthesize at least some vitamin D_3 endogenously, a process that is initiated in the skin and requires ultra-violet B (UV-B) light exposure. Vitamin D_3, in turn, plays a key role in absorption of dietary calcium in the small intestine. Although indoor turtles received ambient natural light from windows, no measurable UV-B reached animals in their enclosures. Although the difference was modest, the outdoor-reared population had higher BMM than free-ranging turtles. One explanation for this difference is that captive outdoor turtles both received abundant UV-B and had access to readily available, nutrient-rich forage. In comparison, the free-ranging population likely had comparable access to sunlight but may have subsisted on forage that was less rich in calcium. It is somewhat surprising that UV-B exposure may be important for *M. temminckii* because it is a species that is conspicuous among aquatic turtles for not basking (Carr et al. 2011). Furthermore, the species frequently inhabits turbid aquatic systems where very little UV-B light penetrates the surface. Laboratory tests of vitamin D_3 synthesis by *M. temminckii* are warranted. It is conceivable that, like nocturnal geckos, *M. temminckii* possess the ability to synthesize vitamin D_3 with exceptionally low UV-B exposure (Carmen et al. 2000). Finally, the fact that outdoor-reared turtles exhibited significantly higher BMM than indoor-reared turtles indicates that bone mass is dynamic and responds relatively quickly to changing conditions.

While differences in populations' exposure to UV-B provide an intuitive explanation for the patterns that I observed in BMM, effects of diet and stress should also be considered. Leopard tortoises (*Geochelone pardalis*) reared on a low calcium diet had far lower BMM than

those with extra calcium in their diets (Fledelius et al. 2005), highlighting the importance of calcium availability in the environment. In my study system the indoor-reared population is routinely fed a pelleted fish-based diet. The outdoor-reared population has access to a more natural forage in the form of live fish, macroinvertebrates, aquatic plants and detritus, and their diet is supplemented by fish carrion, as well. However, despite the rich and diverse diet that is available to the outdoor-reared turtles, it is not clear what is actually consumed. The same is true of the free-ranging *M. temminckii* in my study; diet studies have identified what they eat, but have been unable to convincingly determine relative quantities of different dietary components (East and Ligon 2013). Alternatively, chronic stress is known to trigger a reduction in the rate of bone mineralization. This can lead to weak or brittle bones or can induce the onset of osteoporosis (Kiecolt-Glaseret al. 2003). Indoor-reared *M. temminckii* at Tishomingo NFH typically are reared at higher densities than they experience in outdoor ponds or after release; it is conceivable that crowding results in chronic stress that, in turn, may result in reduced bone density. The strong relationship between LTM and wet body mass is likely due to the fact that LTM comprises up to 93% of the total mass of the specimens.

Body Condition Index Comparisons. For *M. temminckii*, the assumption that a mass:length-based BCI is useful for inferring relative FM had mixed support. BCI was not predicted by BMM. Although significant, only about 16% of the differences in BCI were explained by differences in FM, while 80% were explained by differences in LTM. This suggested that BCI represents variation in morphology (e.g. volume) of this species because LTM represents the majority of the mass of the animal. Whereas for some species an increase in volume results primarily from added FM, turtles are mostly constrained within a rigid shell. Thus, relatively 'high-domed' individuals will likely receive a higher BCI score than a 'low-

domed' individual of the same length, regardless of fat stores. An additional concern regarding the utility of BCIs in this species is the extraordinarily tight correlation of mass on length. With so little variation among individuals, even slight errors in measurement or fluctuations in mass may dictate the BCI score an individual receives.

Conclusion. DXA was determined to be both accurate and precise in determining the body composition of juvenile *M. temminckii*. While DXA fails to accurately infer FM in some other chelonians, the technology appears to work well for species with reduced plastrons, or juveniles with fontanelles. Importantly, DXA-derived BMM and LTM are consistently accurate across studies and turtle species. BCI correlated only weakly with FM, the metric with which it is most commonly associated, but correlated strongly with LTM. Thus, while a BCI is non-invasive and simple to calculate, its utility for inferring ecologically relevant body composition values appears to be limited. When used, BCIs should be interpreted with caution.

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Table 1. Midline carapace length, wet and dry body mass, and total body water of 11 juvenile alligator snapping turtles (*Macrochelys temminckii*) used to validate DXA-generated body composition values. Also reported are comparisons between gravimetrically-derived and DXA bone mineral mass, fat mass, and lean tissue mass. Proportions of wet mass are reported as mean±s.d.%.

	Body mass (g)		Total body water content (g)	Bone mineral mass (g)		Fat mass (g)		Lean tissue mass (g)	
Carapace length (cm)	wet	dry		ash	DXA	lipids	DXA	lean tissue	DXA
120	497.9	150.7	355.9	21.7	35.8	74.3	50.5	462.0	441.6
110	334.6	105.5	237.0	13.5	23.5	42.2	43.5	317.7	296.9
182	1486.0	439.3	1074.9	68.0	105.8	142.7	166.6	1366.3	1332.1
132	613.1	180.7	449.0	26.9	45.0	87.5	67.1	592.1	548.8
158	1115.4	326.3	821.0	47.2	85.0	124.3	119.2	1046.4	1020.4
176	1334.3	376.8	991.9	53.8	97.2	177.6	147.9	1246.9	1208.2
153	906.5	273.3	670.1	40.2	64.8	145.0	108.1	836.9	836.6
105	307.5	96.5	225.3	15.8	28.2	34.9	18.6	287.5	287.3
91	193.7	61.2	142.7	8.8	15.7	30.2	19.5	183.0	179.5
98	253.1	76.4	188.4	12.2	22.2	25.5	16.0	235.1	235.8
129	523.3	151.4	392.0	22.3	36.6	52.2	43.5	497.9	494.8
Mean	687.8	203.4	504.4	30.0	50.9	85.1	72.8	642.9	625.6
Percent of wet body mass		30.0±0.01%	73.3±0.01%	4.4±0.3%		12.6±2.3%		93.8±1.4%	
Range		28.2–31.6%	70.9–74.9%	4.0–5.1%		9.6–15.6%		92.0–96.6%	

Table 2: Regression statistics of validation experiment and BCI evaluation experiment.

Test	$Y= ax+y_0$	R^2	*F*	df	*P*
BMM vs. DXA BMM	DXA BMM=0.4877 BMM+4.6410	0.9782	449	2,10	<0.0001
FM vs. DXA FM	DXA FM=1.057FM+ 0.9788	0.9581	229	2,10	<0.0001
LTM vs. DXA LTM	DXA LTM=1.0103 LTM+9.227	0.9995	20341	2,10	<0.0001
DXA BMM vs. BCI	BCI=-0.2038DXA BMM-0.0008	0.0280	1.96	2,68	0.1662
DXA FM vs. BCI	BCI=0.1269DXA FM-0.0008	0.1651	13.25	1,67	0.0005
DXA LTM vs. BCI	BCI=0.8746DXA LTM-0.0005	0.8036	270	2,66	<0.0001

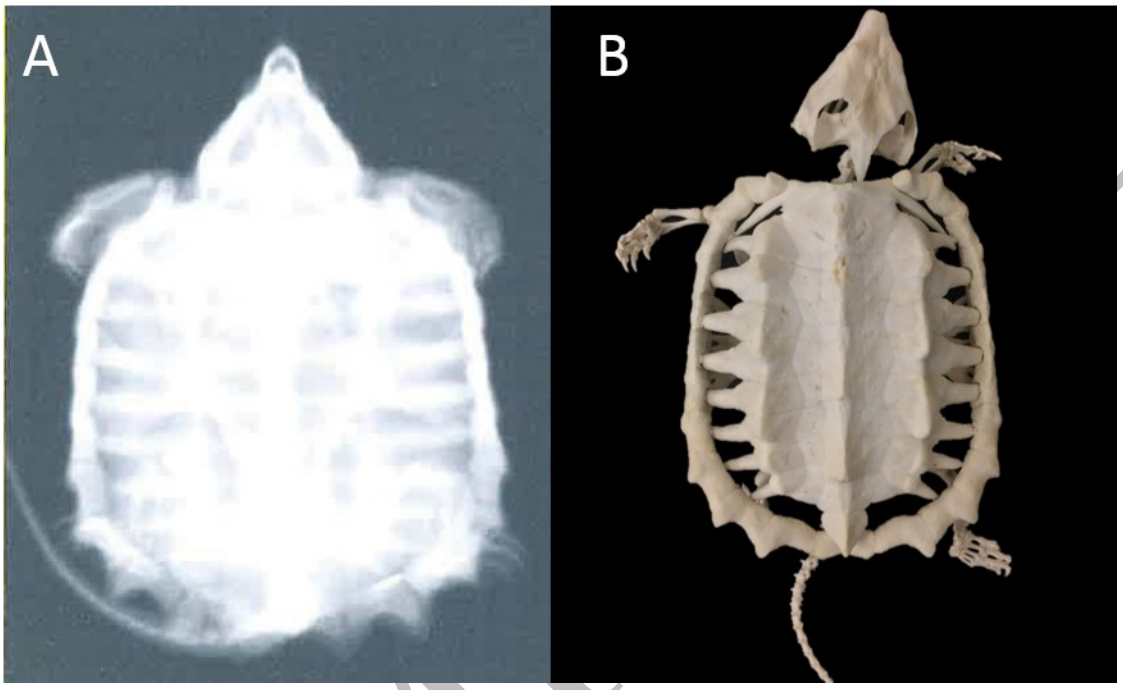


Figure 1: A) DXA image scan of a juvenile alligator snapping turtle and B) photograph of a juvenile alligator snapping turtle skeleton. The large fontanelles and reduced plastron result in areas of the scan that are not dominated by bone, an important factor for accurately estimating bone mineral mass.

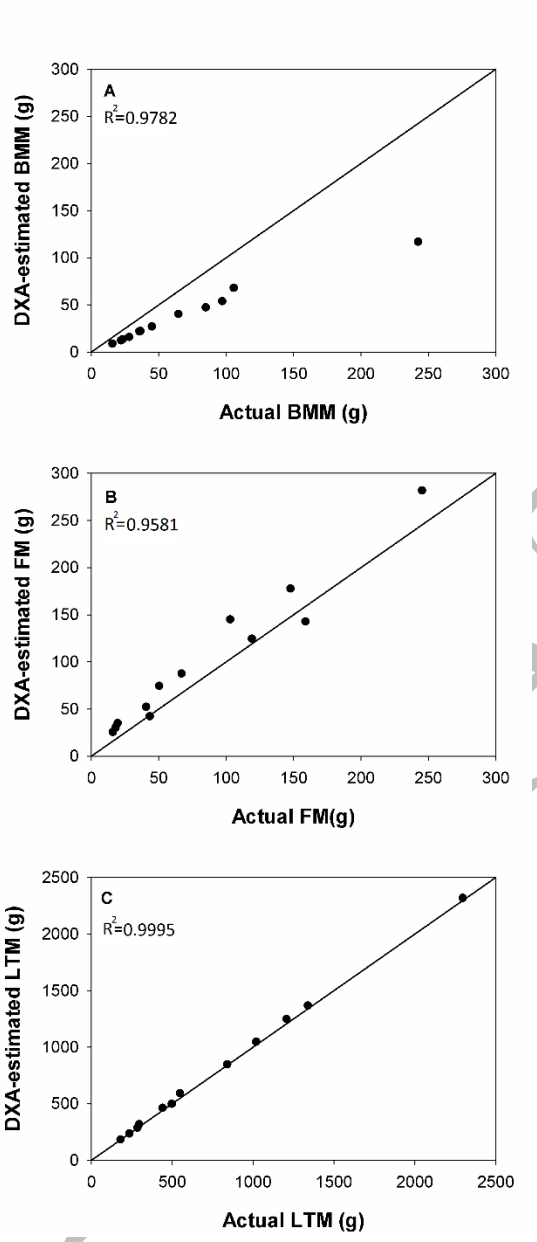


Figure 2: Comparisons of dual-energy X-ray absorptiometry estimates of A) bone mineral mass (BMM), B) fat mass (FM), and C) lean tissue mass (LTM) regressed upon direct measurements of the same components.

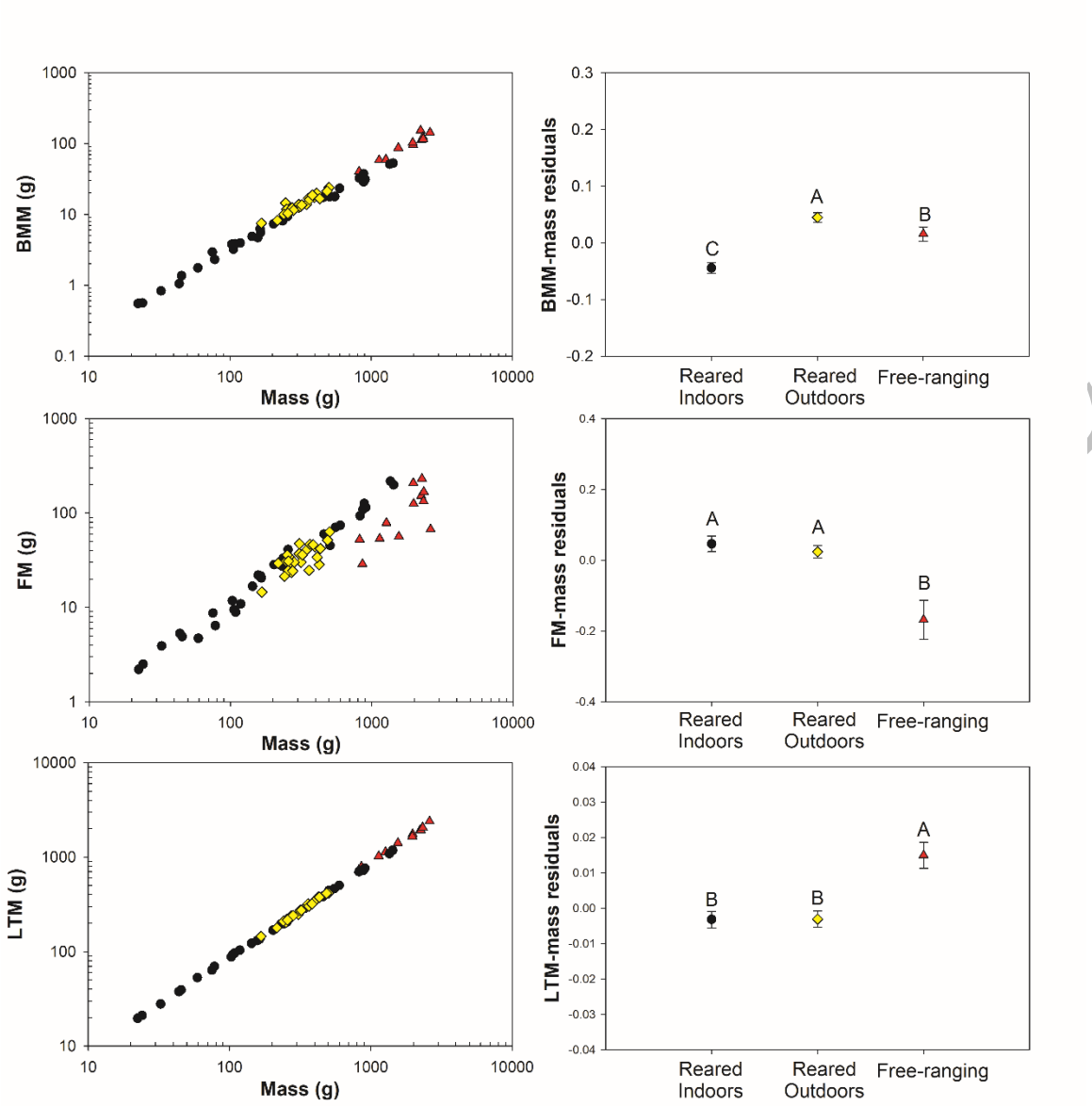


Figure 3: Bone mineral mass (BMM), fat mass (FM), and lean tissue mass (LTM) regressed on body mass of indoor-reared (closed circles), outdoor-reared (yellow diamonds), and reintroduced (free-ranging) (red triangles) populations of juvenile alligator snapping turtles. A) Regression of BMM on mass; B) mean estimates of BMM; C) fat mass regressed on mass; D) mean estimate of FM; E) lean tissue mass regressed on mass; F) mean estimates of LTM. Letters above symbols indicate significant differences among treatment groups.

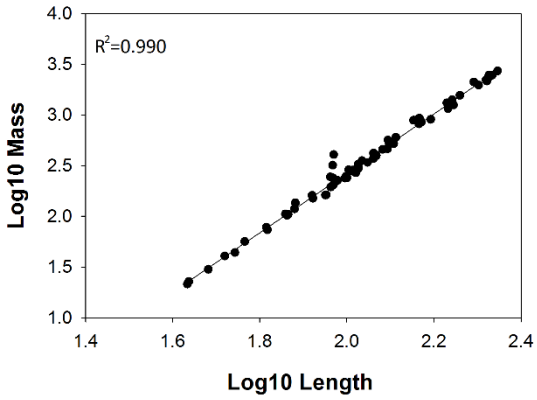


Figure 4: Regression of log-transformed mass onto log-transformed length. BCI values were calculated as the residuals from this regression

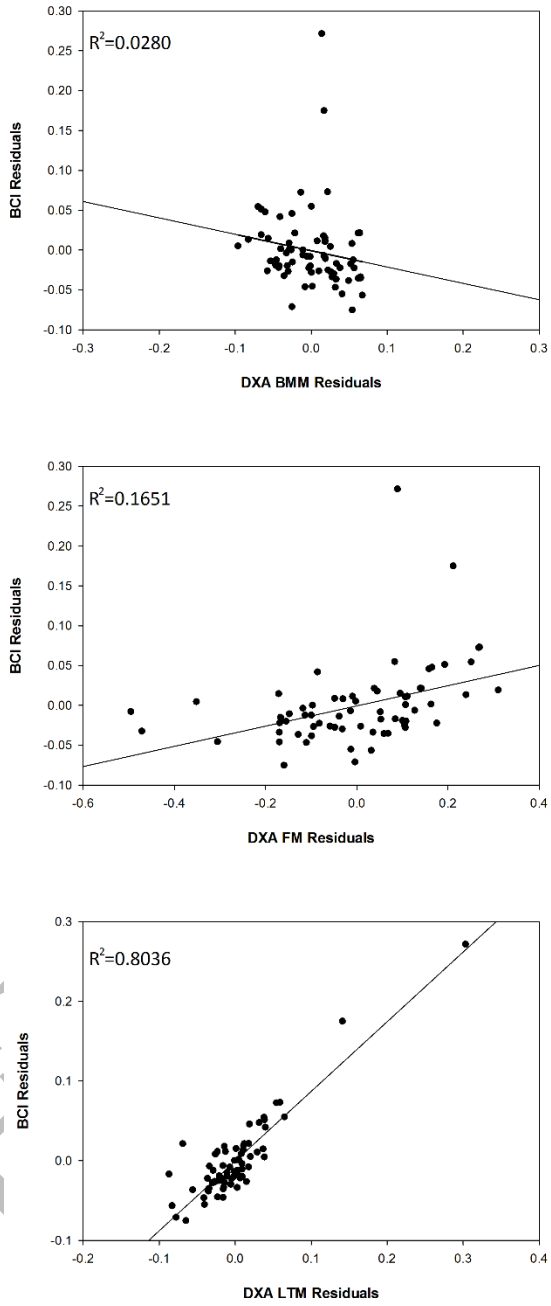
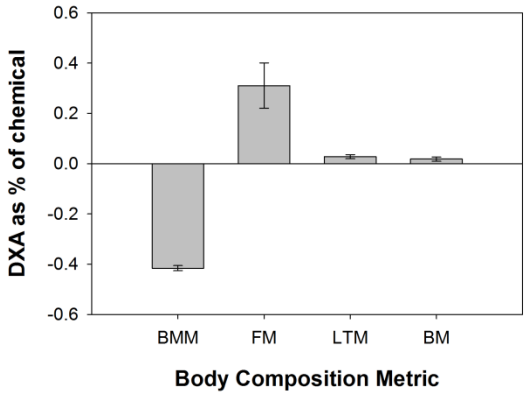


Figure 5: Regression between body condition index (BCI) residuals and DXA-estimated residuals. BCI values correlated strongly with DXA-estimated lean tissue mass but not lipid content or bone mineral density.



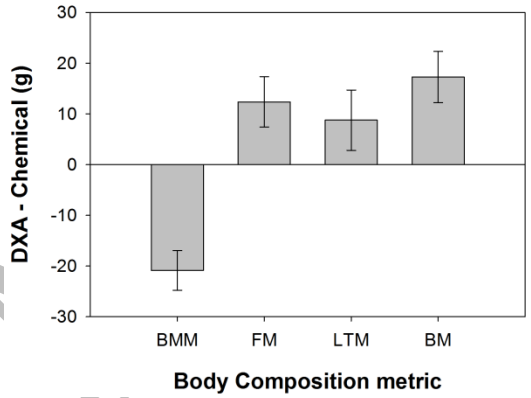


Figure 6: Mean difference between DXA and chemical estimates of BMM, FM, LTM, and body mass (BM).

CIRCULATING CORTICOSTERONE DOES NOT AFFECT GROWTH RATES OF HEAD-STARTED ALLIGATOR SNAPPING TURTLES (*MACROCHELYS TEMMINCKII*)

Abstract

Acute stress responses are generally characterized as beneficial to maintaining homeostasis of animals, while chronic stress is often maladaptive. Although animals reared in captivity often benefit from enhanced nutritional resources and safety from predators relative to their wild counterparts, the captive environment may nonetheless induce chronic stress due to frequent disturbance, crowding, or absence of key environmental resources. Chronic stress can generate deleterious results, including compromised immunity, reduced growth rates, and psychoses. A head-start program in Oklahoma breeds alligator snapping turtles *(Macrochelys temminckii*) for reintroduction in parts of the species' range where populations have been extirpated. Surprisingly, growth rates tend to increase dramatically following release into the wild, despite food being readily available in captivity. I conducted an experiment to determine whether or not population-level differences in stress could explain the source of this pattern. I compared the circulating plasma corticosterone levels of 25 free-ranging/reintroduced and 25 size-matched captive juvenile *M. temminckii* to compare stress. Overall, corticosterone levels were equally low between groups. Among free-ranging individuals, corticosterone levels did not correlate with handling time, body size, growth rates, or time since release into the wild. These results suggest one of two things: stress does not differ between captive and free-ranging alligator snapping turtles and therefore does not explain the previously reported acceleration in growth after release, or else corticosterone is not a reliable indicator of acute or chronic stress in this species.

Introduction

Stress, defined as an organism's response to a physiological challenge, is often closely linked to the concentration of corticosterone and other glucocorticoids in the blood of vertebrates (Aguirre et al. 1995, Gregory et al. 1996, Kitaysky et al. 2003). Glucocorticoids are secreted by the adrenocortical tissue through the hypothalamic-pituitary-adrenal (HPA) axis (Cash et al. 1997, Romero 2004). The dominant glucocorticoid varies among taxa, with cortisol dominating among fish and some mammals, and corticosterone dominating among birds, other reptiles, amphibians, and most rodents (Romero 2004). Many studies have shown how a variety of

environmental stressors correlate with elevated levels of these hormones (Dickens et al. 2010; Honarvar et al. 2011; Cockrem 2013). Studies have shown that acute elevated concentrations of circulating stress hormones can be triggered by capture and handling (Gregory et al. 1996, Cash et al. 1997), while chronically elevated concentrations can be caused by such factors as prolonged captivity (Moore et al. 1991) and high population density (Elsey et al. 1990). In addition, diseases and malnutrition may also precipitate chronically elevated corticosterone (Aguirre et al. 1995, Romero and Wikelski 2002, Cote et al. 2005).

Stressors may cause an increase in glucocorticoid concentrations that can precipitate a variety of effects. While some effects can be beneficial, such as increasing metabolic rate to meet increased energy demands (Love et al. 2003; DuRant et al. 2008), increasing locomotor capacity for better foraging, or enhanced dispersal (Huey et al., 1984; Bennett and Huey, 1990; de Fraipont et al., 2000; Clobert et al., 2000, Cote et al. 2005), others can be negative. The negative effects of increasing corticosterone concentration come from chronically elevated corticosterone concentrations. Some of these effects are an inhibition of normal expression of reproductive hormones (Dunlap and Schall, 1995; Mahmoud and Licht, 1997; Nijagal and Yajurvedi, 1999, Moore and Jessop 2000), immunosuppression (Aguirre et al. 1995, Dunlap and Schall 1995, Morici et al. 1997), lipidogenesis (Gray et al. 1990, Harvey et al. 1984), inhibited growth (Morici et al., 1997), and osteoporosis (Abdollahi et al. 2005). Interestingly, elevated metabolic rates may be of particular concern for reptiles, as approximately 80% of their normal energy budget is dedicated to maintenance while only 20% is available for new tissue development (Congdon et al. 1982, 2001). If maintenance costs increase but energy acquisition does not, it could leave a smaller portion of the total energy budget available for new tissue growth if the stressor becomes chronic (DuRant et al. 2008).

The alligator snapping turtle (*Macrochelys temminckii*), is a large aquatic turtle that is endemic to rivers flowing into the Gulf of Mexico (Ernst and Lovich 2009; Conant and Collins 1998; Riedle et al. 2005; Pritchard 2006). It is highly aquatic and females only leave the water to lay eggs (Holcomb and Carr 2013). Limited previous research has been conducted on the endocrinology of *M. temminckii* (Chaffin et al. 2008; Teare 2010), where low basal corticosterone concentrations were reported, similar to levels reported for other chelonian species (Cash et al. 1997, Keiver et al. 1992).

Although results have been variable, some past studies have found that introduced *M. temminckii* can grow at much faster rates than do captive individuals, even when controlling for age and size (Anthony et al. 2015, Dreslik et al. 2017). Environmental stressors likely differ between captive and wild populations, especially with respect to crowding and microenvironmental features. Therefore, I hypothesized that the comparatively suppressed growth rates of captive individuals may be due to a glucocorticoid-mediated stress response. I used two populations of *M. temminckii* to test my hypothesis: one was a captive population that consisted of juveniles being reared for reintroduction, and the other was composed of free-ranging—but previously head-started—individuals inhabiting a river in northern Oklahoma. I predicted that free-ranging individuals would exhibit lower corticosterone levels than conspecifics maintained in captivity, and that among free-ranging individuals circulating corticosterone levels would correlate negatively with growth rates.

Methods

This research was conducted with approval from the Missouri State University Institutional Animal Care and Use Committee (protocol 17-030) and Oklahoma Department of Wildlife Conservation (Scientific Collectors Permit 6764).

Study Populations. Free-ranging alligator snapping turtles that I used in my research were collected from the Caney River in northern Oklahoma. Headwaters of the Caney River are located in the Central Mixed-Grass Prairie Ecoregion in central Kansas (U.S. EPA Level III Ecoregions; www.epa.gov/eco-research/ecoregions). It flows southeast into the Flint Hills Prairie and Cross Timbers ecoregions in Oklahoma where it ultimately joins the Verdigris River. My study site was located in the Flint Hills Prairie Ecoregion and consisted of 8 km of the Caney River and 5 km of a tributary, Pond Creek. The study site is upstream of a reservoir (Hulah Lake). This impoundment is used by the U.S. Army Corps of Engineers for downstream flood control, and as such, increases the duration and frequency of upstream flood events. Potential predators of *M. temminckii*, such as raccoons (*Proycon lotor*), river otters (*Lontra canadiensis*), and great blue herons (*Ardea herodias*), are common at this site. Reintroductions of *M. temminckii* have occurred at this site since 2008 (Anthony et al. 2015). The individuals included in my study were released 14 days–15 months prior to being recaptured for the purposes of this study.

I compared the free-ranging turtles to captive *M. temminckii* that were hatched and reared at Tishomingo National Fish Hatchery, located in southeastern Oklahoma. This captive population is part of a head-start program and was the source for all of the *M. temminckii* that had been introduced at the Caney River. Turtles there are raised at variable densities in indoor tanks (ca. 44 turtles/m^2) and outdoor ponds (ca. 0.42 turtles/ m^2) with other members of the same cohort.

Sampling methods. Effects of trapping and handling on circulating corticosterone concentrations are well documented in many species (Dufty and Belthoff 1997; Lance and Elsey 1999; Mathies et al. 2001; Lance et al. 2004; Dickens et al. 2010). Therefore, all turtles used in

my study were hand-ca0ptured rather than trapped to eliminate trap-induced stress responses, and blood samples were taken within 30 minutes of initiating pursuit, a period that is within the time before corticosterone spikes in adult *M. temminckii* (D. Thompson, unpublished data). Additionally, no blood samples were obtained from free-ranging *M. temminckii* for at least two weeks following the initial introduction of juvenile *M. temminckii* to allow time for acclimatization and recovery from potentially stress-elevating transport and release.

To facilitate hand-capturing free-ranging *M. temminckii*, I located radio transmittered 2–5-year-old individuals from a boat before entering the water to search by hand. To assess the effects of acute stress associated with pursuit of capture of individuals, I recorded the time from entering the water to noodle for a turtle until a blood draw was initiated. All turtles were measured and weighed before being released, and these measurements, along with comparable measurements obtained at the time of turtles' release or else at the time of the most recent recapture, were used to calculate growth rates, expressed as the change in straight midline carapace length ($\mu m \cdot mm^{-1} \cdot d^{-1}$).

A similar approach to obtaining blood samples was used to sample captive turtles at Tishomingo National Fish Hatchery; however, those housed indoors could be processed much more quickly because search and pursuit times were negligible. Those in outdoor ponds were captured by hand without the aid of radio telemetry, but search time was reduced relative to that of free-ranging turtles because turtles occurred at higher density and individuals were selected indiscriminately rather than targeting specific individuals. For each free-ranging *M. temminckii* from which a blood sample was obtained, a sample was collected from a captive individual that was similar in age and size to facilitate making paired comparisons and avoid potentially confounding effects of season, age, and body size. I compared corticosterone levels of captive

and free-ranging turtles using a 2-sample t-test, and effects of handling time were tested using linear regression.

Blood samples were drawn using 21-gauge needles and transferred to 1.5-mL microcentrifuge tubes. The samples were stored on ice until they were centrifuged, usually within 4 hours. The plasma fraction was then aspirated, placed into a new 1.5-mL tube, and frozen. Corticosterone measurements were conducted using radioimmunoassay following procedures described in Love et al. (2017).

Statistics. I used linear regression analyses to test for effects of time since reintroduction and search and handling time on circulating corticosterone concentrations. I also used linear regressions to test for effects of turtle size on circulating corticosterone, and to test the effects of circulating corticosterone on growth rates of free-ranging turtles. Finally, I used a 2-sample t-test assuming unequal variances to compare circulating corticosterone concentrations of free-ranging and captive *M. temminckii*. Except where noted, all values are reported as mean ± 1 SE. All conclusions are based on a Type I error rate of 0.05.

Results

I collected plasma samples from 25 free-ranging and 25 captive *M. temminckii*. Sampling from the captive population included animals housed both indoors and in outdoor ponds, which was necessary to closely size-match captive individuals to those obtained from the free-ranging population. The time elapsed from the release of free-ranging *M. temminckii* to the time that plasma samples were obtained for corticosterone analyses ranged 15–439 days. The time between initial release and recapture did not have a significant impact on the circulating corticosterone concentrations ($F_{1,23} = 0.2434$, $r^2 = 0.0105$, $P = 0.6264$). Additionally, search and

handling time for free-ranging turtles was highly variable for free-ranging turtles (mean = 8.3 ± 1.5 minutes (range = 0.3–30 minutes) and consistently negligible for captive animals (<1 minute). Despite the wide variability, search and handling time did not affect plasma corticosterone concentrations among free-ranging *M. temminckii* ($F_{1,23} = 0.469$, $r^2 = 0.02$, $P = 0.500$) (Figure 1) and corticosterone concentrations did not differ between captive and free-ranging *M. temminckii* (captive population mean = 2.92 ± 0.66 ng/mL, free-ranging population = 3.79 ± 0.82 ng/mL; $t = 1.14$, df = 48, $P = 0.264$).

The *M. temminckii* used in my study varied in body size (straight mid-line carapace length: mean = 105.87 ± 3.72 mm, range = 165–74 mm). However, there was not a significant effect of body size on circulating corticosterone among either captive or free-ranging populations (captive turtles: $F_{1,23} = 1.386$, $r^2 = 0.0568$, $P = 0.251$; free-ranging turtles: $F_{1,23} = 1.416$, $r^2 = 0.0661$, $P = 0.248$; Figure 2). Additionally, among free-ranging turtles, corticosterone concentrations were not predicted by growth rates ($F_{1,23} = 0.938$, $r^2 = 0.04$, $P = 0.343$; Figure 3).

Discussion

I found no evidence to support the prediction that captive *M. temminckii* are exposed to greater stressors than free-ranging conspecifics or, for that matter, of a corticosterone-mediated acute stress response among either captive or free-ranging juveniles. A simple explanation for these results is that the turtles in both groups were not physiologically stressed. Despite the comparatively crowded conditions for both indoor and outdoor captive-reared turtles, the absence of elevated corticosterone levels could indicate that access to food and shelter were adequate to meet the basic physiological needs of the animals, and social interactions within groups are not sufficiently antagonistic to trigger a stress response.

The conditions experienced by free-ranging *M. temminckii* are less readily observable, but forced proximity to conspecifics is not likely an issue as it is in captivity. Furthermore, the generally high growth rates that are achieved by reintroduced *M. temminckii* suggest that food resources are not a limiting factor. These observations suggest that free-ranging *M. temminckii* might face fewer stressors than captive conspecifics, yet these differences were not reflected in circulating corticosterone.

In addition to failing to exhibit differences between captive and free-ranging populations, circulating corticosterone concentrations also failed to differ with respect to factors that I tested within free-ranging animals. Search and handling time associated with capturing animals to obtain plasma samples from free-ranging *M. temminckii* varied widely, and yet I found no evidence of an acute stress response. The lack of a relationship contrasts with observations in other turtle species such as the red-eared slider (*Trachemys scripta*) that exhibited elevated corticosterone within 11–25 minutes of capture (Cash et al. 1997) and the loggerhead sea turtle (*Caretta caretta*) that also exhibited elevated corticosterone within 10–30 minutes of capture (Gregory et al 1996). This difference could reflect taxonomic differences in the response to handling or could reflect ontogenetic differences; Cash et al. (1997) sampled adult *T. scripta* whereas I limited my investigation to juvenile *M. temminckii*. Alternatively, it is possible that, like juvenile American alligators (*Alligator mississippiensis*), the adrenal response in juvenile *M. temminckii* is short-lived even when a perceived stressor such as handling persists (Morici et al. 1997). Finally, it is possible that the series of events that I perceived to be potentially stressful—from the time that I entered the water to pursue a turtle until it was captured, and blood was drawn—did not actually correspond with the events that might trigger an adrenal response. In nearly every case, the pursuit of a turtle lasted far longer than post-capture processing. Perhaps

corticosterone secretion is only triggered upon capture rather than during the pursuit phase. While I do not have the means of testing this hypothesis, this pattern would coincide with certain aspects of the species' typical behavior; during pursuit *M. temminckii* remain evasive but notably non-aggressive, whereas upon capture individuals quickly transition to aggressive attempts to bite (Ligon, pers. comm.)

Some events and circumstances that would generally be predicted to induce stress do not upregulate glucocorticoid secrection in all taxa. The stress response can be variable in different species due to differences in physiological and psychological responses to environmental or physiological stressors (Tyrrell and Cree 1997; Dickens et al. 2010; Cockrem 2013). There is precedent for ontogenetic changes in corticosterone expression among other taxa, and it may be that juvenile *M. temminckii* simply do not exhibit a stress response that is comparable to that of adults. For instance, a few avian studies have indicated that hatchlings do not exhibit an adrenal response to stress, possibly because the lives of young animals are so often filled with a barrage of environmental stressors that any corticosterone-mediated response would likely remain a chronic response, the results of which are often maladaptive (Kitaysky et al. 2003; Love et al. 2003; Wada et al. 2007; Schoech et al. 2011). Furthermore, when they do begin to exhibit a stress response, it is typically expressed in behavioral responses such as increased begging (Wada and Bruner 2008), increased locomotor activity (Astheimer et al., 1992), or increased aggression toward nest mates (Kitaysky et al. 2003).

Ontogenetically delayed stress responses are hypothesized to be an adaptation to the deleterious effects of chronically high corticosterone concentrations (Love et al. 2003). Reduced growth (Morici et al. 1997), neuron damage (Howard and Benjamins 1975), alteration of cognitive development (Kitaysky et al. 2003), and alteration of the HPA axis (Seckl 2001) are a

few of the long-term effects that may be avoided by suppressing the stress response during early life stages (Love et al. 2003). Similarly, some species of reptiles have been shown to exhibit little to no known stress response to a variety of stressors (Greenberg and Wingfield 1987; Gregory et al. 1996; Valverde et al. 1999).

Other indicators of physiological stress, such as heterophyl:lymphocyte ratios or oxidation rates, may be more suitable than corticosterone as biomarkers of chronic stress (Mayne 2003; Goessling et al. 2015).

Basal corticosterone concentration has been shown to negatively correlate with growth in a variety of taxa (Elsey et al. 1990; Morici et al. 1997; Glennemeier and Denver 2002), yet I failed to detect a similar pattern among free-ranging *M. temminckii*. This adds further support to the conclusion that turtles in my study generally did not exhibit high or chronic stress, but also fails to explain the distinctly divergent growth rates that have been reported in captive versus free-ranging turtles in the same study system in which I conducted my research (Anthony et al. 2015, Dreslik et al. 2017) Further study will be necessary to identify the source or sources of these patterns, which could include varying foraging schedules, different nutritional resources, or different activity levels that result in dramatically different energy budgets. Future research should investigate these possibilities and identify ways to increase growth rates while turtles are maintained in captivity.

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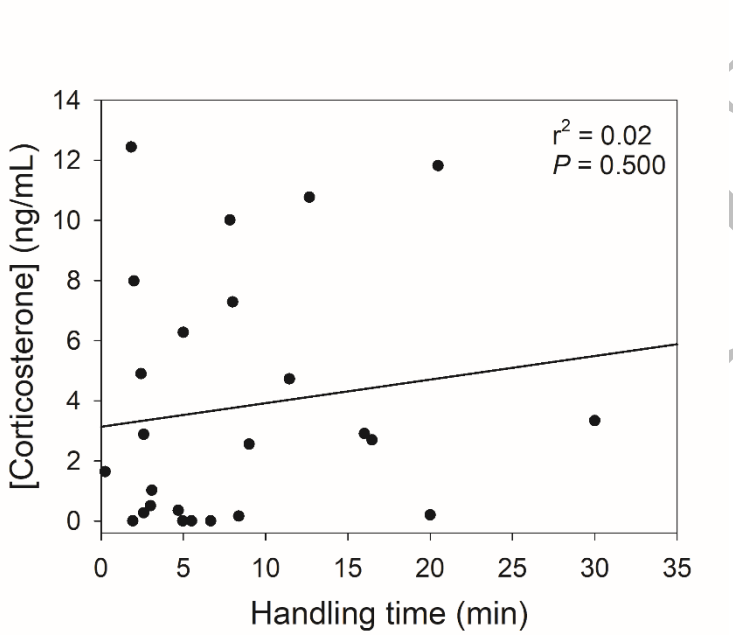

Figure 1: Relationship between search and handling time and corticosterone concentration among free-ranging *M. temminckii*. Despite prolonged search and handling time for some individuals, there was no evidence of acute stress-induced elevated corticosterone.

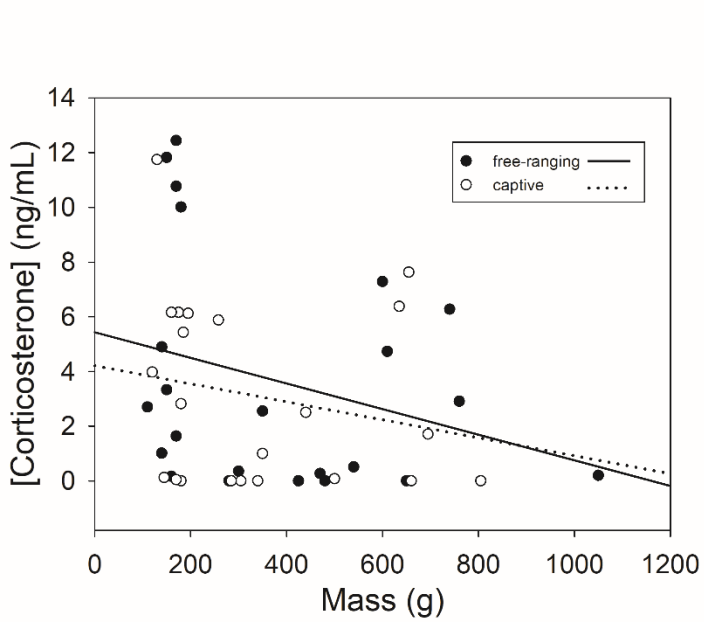

Figure 2: Regression of corticosterone concentration on body size of free-ranging and captive *M. temminckii*. The relationship was nonsignificant in both populations (free-ranging turtles: r^2 = 0.08, P = 0.178; captive turtles: r^2 = 0.04, P = 0.344).

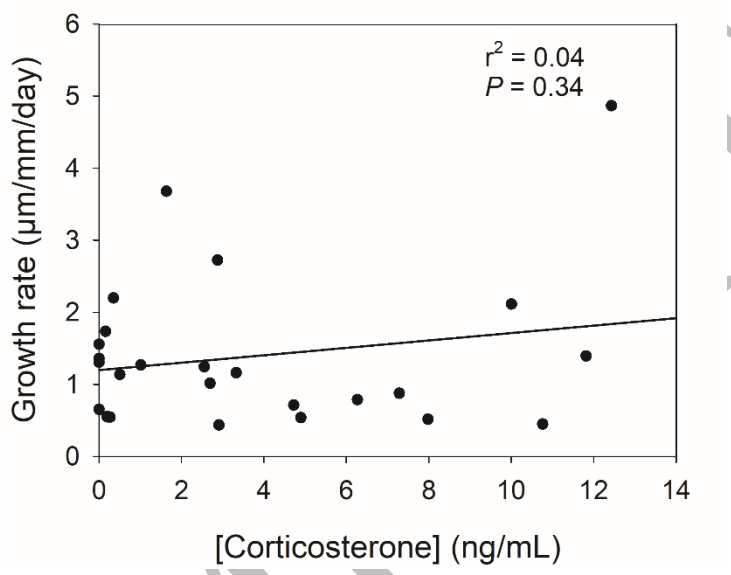

Figure 3: Relationship between growth rates and circulating corticosterone levels of free-ranging *M. temminckii*.

SUMMARY

DXA proved to be both accurate and precise in determining the body composition of juvenile *M. temminckii*. While DXA fails to accurately infer FM in some other chelonians, the technology appears to work well for species with reduced plastrons, or juveniles with fontanelles. Importantly, DXA-derived BMM and LTM are consistently accurate across studies and turtle species. BCI correlated only weakly with FM, the metric with which it is most commonly associated, but correlated strongly with LTM. Thus, while a BCI is non-invasive and simple to calculate, its utility for inferring ecologically relevant body composition values appears to be extremely limited. When used, BCIs should be interpreted with caution.

Endocrinology of turtles is a difficult field to study. While adult *M. temminckii* may exhibit traditional and expected stress responses, juveniles did not. Without any significant relationships, the observed differences in growth rates is likely due to other environmental factors. One such factor may be the timing of feeding. If fed during the day in captivity, for instance, the food could degrade and nutritional value could decline. Further research into this aspect is warranted.

When piecing together the complete set of changes *M. temminckii* undergoes in each part of the reintroduction effort, it becomes clear that although the dramatic increases in growth rate are evident, they are not caused by a decrease in stress, but are accompanied with lower FM, higher LTM, but average BMM. While captivity has its benefit for reintroduction, it also is coupled with several negative factors. All factors must be considered when proposing various methods for reintroduction.