EFFECTS OF SEASON, SEX, AND AGE ON THE CALCIUM PHYSIOLOGY AND BONE DYNAMICS OF TURTLES

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PREFACE

The chapters contained within this dissertation were written as manuscripts that will be or have been submitted for publication in peer-reviewed journals.

The style and format of each chapter have been written to the requirements of the respective journals. Repetition in some sections, such as figure legends, was intentional and conforms to both journal and OSU requirements.

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PRELUDE

Chapter I of this dissertation evaluates the use of dual-energy x-ray absorptiometry (DXA) technology in predicting body composition of turtles. The ability to measure bone density in turtles could be useful for a variety of nutritional, physiological, and developmental research applications. Of particular interest to me was the evaluation of seasonal change in bone density. Until relatively recently, the quantification of bone density was challenging, costly, or was limited to destructive studies. Many studies, including longitudinal studies, do not lend themselves too well to destructive techniques, primarily because they require the use of large sample sizes. In my belief, turtle populations could not sustain the harvest required for such studies. Destructive studies also are not feasible for research involving repeated measures of individuals. Therefore, I elected to incorporate the validation of DXA as a means of predicting bone density into my dissertation because of its potential importance to turtle research and longitudinal studies of bone.

Chapters II and III evaluate the temporal, species-specific, and sex-related effects on bone density and plasma biochemistry in turtles. The impetus for these projects was based on the findings of Richard Kazmaier and colleagues (Hellgren et al. 2000). In their study of Texas tortoise (*Gopherus berlandieri*) demography, they found that females had a higher incidence of perforations in the carapace and they suggested that the presence of such perforations might be a result of a calcium deficiency imposed by the combined

effects of a diet high in oxalates, loss associated with the production of eggs, and the naturally thin bones of this species. Therefore, production of offspring might impose physiological demands on the mother, whereby the "decision" to produce (or not) offspring might have implications for her survival and ability to invest in future offspring. Understanding the potential tradeoffs associated with reproduction and bone density in *G. berlandieri* and other species of turtles might provide useful insight into understanding the role that calcium plays in parental investment. More specifically, the relative investment of calcium to yolk and eggshell, both of which can be utilized by the developing embryo, may also influence bone reserves because of the rate of calcium needed for these two investments (Simkiss, 1967).

In chapters II and III, I partially address the possible tradeoffs between bone density and maternal calcium investment by documenting seasonal changes in calcium physiology in two species of turtles, *G. berlandieri* and the red-eared slider, *Trachemys scripta*. *Gopherus berlandieri* was selected for inclusion in this study because of the findings discussed above. The findings from our study (chapter II) suggest significant changes in calcium physiology that are associated with reproduction in female tortoises. These physiological changes might have important implications for the maintenance of maternal bone density in *G. berlandieri*. Unfortunately, due to the listing of *G. berlandieri* as a state-threatened reptile in Texas, I considered it an undesirable candidate for the bone-density portions of this study, because mortality was required for prior validation (chapter I). *Trachemys scripta* was selected to fill this role because it is an excellent model species of turtle biology due to its abundance, wide distribution, and well-studied life-history (e.g. Gibbons, 1990). These findings (chapter III) suggest a

different pattern. In the case of *T. scripta*, I did not document the reproductive influences on calcium physiology associated solely with the production of the egg. I suggest that this disparity is in part due to differing maternal investment of calcium between *G. berlandieri* and *T. scripta*; however, much research is still needed before we can fully support such observations.

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CHAPTER I

DUAL-ENERGY X-RAY ABSORPTIOMETRY (DXA) AS A NON-INVASIVE TOOL FOR THE PREDICTION OF BONE DENSITY AND BODY COMPOSITION OF TURTLES

Abstract

Dual-energy x-ray absorptiometry (DXA) is a frequently used non-invasive method for the determination of bone density and body composition. Although the applicability for use in mammalian studies is well established, its use in other taxonomic groups has received little attention. We evaluated the accuracy of DXA in determining bone density and body composition in turtles. Although DXA estimates of body composition were different from those obtained through chemical analysis, we developed predictive equations that can compensate for differences in estimates. Our data indicate that DXA precisely estimates bone mineral content, soft tissue mass, and body mass. However, due to current limitations in DXA technology, it does not effectively distinguish fat from lean tissue mass in chelonians. DXA's poor ability to estimate lean/fat mass is likely due to the relatively broad distribution of bone in the body of turtles and could prevent its application to other organisms with similar body plans (e.g., armadillos [*Dasypus spp.*]). Despite this limitation, DXA can effectively estimate bone mass and might be useful for studies of bone dynamics or in identifying metabolic bone disorders in turtles.

Introduction

Knowledge of animal body composition is important to studies of energetics, reproduction, and life history (Congdon et al. 1982; Secor and Nagy 2003) because it allows researchers to estimate body condition, parental energy investment, and productivity throughout an individual's life. Additionally, quantifying body composition is important for clinical studies of nutrition and metabolic disorders (Elowsson et al. 1998). The use of non-destructive techniques to determine body composition is essential for many studies, including those involving mark-recapture and endangered species (Secor and Nagy 2003). With growing concern for the status of many free-ranging species and the increased availability of technology for non-invasive procedures, destructive techniques will become increasingly unpopular.

A need for non-destructive techniques in chelonian research is becoming more apparent. Currently 80 chelonian species are listed as extinct, extinct in the wild, endangered, or critically endangered (IUCN 2007). As long-lived vertebrates with delayed sexual maturity, some turtle populations may be especially prone to the negative effects of anthropogenic harvest (e.g., collection for food, pet trade, research) especially when adult survivorship is reduced (Congdon et al. 1993; Heppell et al. 1995). Therefore, the development of techniques that could reduce the permanent harvest of adult turtles for scientific purposes could help to reduce impacts on turtle populations. Potentially, dual-energy x-ray absorptiometry (DXA) could provide this service.

DXA was originally developed as a non-invasive tool that predicts bone density and risk of osteoporosis in humans. The physical principles of DXA technology have

allowed expansion of its uses to the quantification of body composition in humans and other mammals. Recent applications to snakes and lizards have shown promise for the use of this technique in reptiles (Secor and Nagy 2003; Zotti et al. 2004). However, the morphology of some taxa, in particular chelonians, may preclude effective determination of body composition with DXA without prior validation. Prediction of body composition analysis, particularly lean tissue and fat mass, may be complicated in chelonians by the bony encasement of internal organs. The estimation of lean tissue and fat mass is complicated when a large proportion of the scanning area contains bone (Jebb 1997). Moreover, turtles have a higher proportion of bone relative to body mass than most other animal species (Iverson 1984), which, along with their unique morphology, makes them of great interest for bone density research. The development of techniques to assess bone density in turtles is not only relevant to taxon-specific research, but could have broader basic applications in nutrition, ecology, and physiology, as well as practical applications in veterinary research and practice.

We examined the precision and accuracy of DXA in predicting bone density and body composition of turtles. Our aim was to develop predictive models that can be used to assess body composition from DXA measurements. These models would be available for researchers to monitor body composition of turtles in settings where destructive techniques are not feasible (e.g., clinical practice, mark-recapture studies). We collected DXA estimates of body composition on 25 male red-eared sliders (*Trachemys scripta*) and then compared these values to estimates determined later by chemical analysis of dried carcasses. Secondarily, this study compared the effects of three different techniques of immobilization – anesthesia, cooling, and euthanasia – on DXA body-composition

estimates because the accuracy of DXA results is known to be influenced by the movement of test subjects during scanning (Engelke et al. 1995).

Materials and Methods

Animal Housing and Use

We obtained an in-house transfer of 25 male *T. scripta* from a previous study conducted at Oklahoma State University (Ligon, Gregory, Kazmaier, and Lovern, unpublished data). Subjects were originally wild-caught from two populations in eastern Oklahoma and southern Texas. *Trachemys scripta* was chosen for this study because of its accessibility, wide distribution, abundance, and well-studied life-history (e.g., Gibbons, 1990). Subjects ranged in straight carapace length (SCL) from 123.9 to 222.8 mm (mean ± 1 SD = 159.3 ± 25.7 mm), in greatest width from 102.9 to 168.0 mm (127.4 ± 16.0 mm), and in mass from 260 to 1525 g (610 ± 281 g). Turtles were housed individually in plastic storage containers partially filled with water and were fasted for one week prior to DXA scanning to ensure evacuation of gut contents. This research was approved by OSU IACUC (permit AS0413).

DXA Estimation of Body Composition

To determine the effects of anesthesia, euthanasia, and cooling on DXA estimates, each individual was scanned using all three immobilization techniques. Scanning was

performed on a Hologic® QDR-4500A fan-beam scanner equipped with a small-animal software program. Prior to scanning, the densitometer was quality-checked daily using Hologic® calibration models (anthropomorphic spine phantom and small-step phantom). Calibration procedures followed those provided by the manufacturer. Body mass was determined for all individuals prior to scanning. Each individual was scanned 4 times per day for 3 consecutive days. During scanning, individuals were positioned with the plastron inferior and the cranial end facing the laser-alignment crosshair. The anterior end was placed 1 cm behind the crosshair, with the individual's midline directly in the middle of the scanning area. Each day, turtles were scanned twice, repositioned, and then scanned twice more. During the first two days of scanning, individuals were randomly placed in either the "anesthetized" or "cooled" condition. Anesthetized individuals received a 0.1 mg/kg medetomidine-5.0 mg/kg ketamine combination (administered IM) for immobilization during scanning followed by 0.5 mg/kg atipamezole (IM) for recovery (Greer et al. 2001). Cooled individuals were placed in a 4˚C incubator for a minimum of 5 hours prior to scanning and transported on ice. Individuals that were anesthetized on the first day were cooled on the second day and vice versa. On the third day of scanning, all turtles were euthanized with an overdose of sodium pentobarbital (60-100 mg/kg IP) and scanning was repeated as above. Following the three scanning days, individuals were frozen for subsequent chemical analysis of body composition.

Non-DXA Estimation of Body Composition

We estimated the following indices of body composition for each turtle: bone mineral content (BMC), fat mass (FM), bone-free lean tissue mass (LTM), fat-free tissue mass (FFM = LTM + BMC or BM - FM), total body water mass (WM), and body mass (BM). Individuals were thawed, dissected to remove fat bodies for easier analysis, and then dried to constant mass at 60˚C. Body mass was measured after drying (BM_{dry}) to a constant mass and then used to estimate water mass by subtracting it from wet body mass (BM_{wet}) determined prior to scanning. Carcasses (fat bodies excluded) were then ground and homogenized in a Wiley mill for determination of fat, lean tissue, and bone content. Fat mass was estimated by adding the dried mass of fat-bodies to total body lipid mass. Total body lipid mass was estimated by determining the average percent lipid content of two 2-g subsamples of the ground carcass, and then multiplying by dried body mass. The lipid content of carcass subsamples was determined by the Folch method (Folch et al. 1957). To estimate bone mass, four 1-g subsamples of the ground carcass were ashed in a muffle furnace at 600˚C for a minimum of 8 hours. The mean percent mineral content of the 4 samples was used to estimate total body bone mass by multiplying by dried body mass. Lean tissue mass was determined by subtraction.

Statistics

We related body mass and straight carapace length to each body composition parameter (determined chemically) using simple least-squares regression to develop models that

could be used to predict body composition from standard morphometrics. Regression was performed on $\log_{10}$-transformed data, but is presented in original scale by back-transforming regression coefficients. To examine the precision of DXA, intraindividual coefficients of variation (CV) were calculated from two scans where the subject was not repositioned, from two scans before and after the subject was repositioned, and also from all four scans for each type of body composition. To examine the effects of immobilization technique on DXA output parameters, we performed repeated-measures ANOVA for each DXA parameter. Subject was analyzed as a blocking variable. When significant differences among treatment levels were detected, Tukey multiple-comparisons were used to examine where differences existed. Prior to analysis, all variables were tested and deemed significantly non-normal (Anderson-Darling Test $P<0.001$). Therefore, we $\log_{10}$-transformed all masses prior to analysis to satisfy this assumption. All results involving transformed data are presented in the untransformed scale using back-transformed means and asymmetric 95% confidence limits.

We selected anesthesia as the preferred method of immobilization and all further validation analyses were performed using DXA data of anesthetized turtles. Anesthesia was selected as the most desirable method of immobilization because it reduced differences between DXA and chemical estimates of body composition. Mean ± 1SD differences between DXA and chemical estimates for combined fat mass, lean tissue mass, and bone mineral content were 160.0 ± 94.3, 168.8 ± 101.6, and 185.4 ± 139.8 g for anesthesia, cooling, and euthanasia, respectively. Additionally, anesthesia generally resulted in more precise measurements compared to cooling (see results).

We performed simple and multiple least-squares regression analysis to develop models predicting chemical body composition (dependent variable) from DXA estimates (independent variable) on anesthetized turtles. We employed a best subsets regression procedure to select the variable(s) most useful in creating the predictive models. After generating predictive models, we employed a jackknife cross-validation procedure described by Secor and Nagy (2003). All values are given as mean ± 1SD. The level of statistical significance was set at $P<0.05$. Statistical analyses were performed using Minitab version 13.1.

Results

Based on non-DXA estimates, water composed 69.46 ± 3.25% of total body mass. Body composition of wet body mass was composed of 84.82 ± 1.85% lean tissue, 13.83 ± 1.58% ash, and 1.35 ± 1.06% fat mass. Lean tissue mass, ash mass, and fat mass composed 50.29 ± 2.95, 45.48 ± 4.78, and 4.23 ± 2.84%, respectively, of dried body mass. All least-squares regressions between morphometrics and body composition estimates (determined chemically) resulted in non-zero slopes ($P \leq 0.002$; Table 1). All models had strong relationships between morphometric and body composition variables ($r^2 \geq 0.966$), with the exception of models predicting fat mass, which showed a poor relationship between variables ($r^2 \leq 0.361$; Table 1).

Precision of DXA

Mean intraindividual CV was calculated for 4 DXA parameters using all three immobilization techniques (Table 2). Mean intraindividual CV for 4 combined scans was greater than 53.7% for fat mass, but less than 2.0 and 6.6% for bone mineral content and lean tissue mass, respectively. CV tended to be highest for cooled individuals and lowest for euthanized individuals when examining bone mineral content, bone mineral density, and fat mass; however, for lean tissue mass there was less precision for euthanized than anesthetized or cooled individuals (Table 2).

Effects of Immobilization Technique

Immobilization technique significantly influenced the use of DXA to determine bone mineral content (repeated-measures ANOVA; $F_{2,48} = 12.07$; $P < 0.001$), bone mineral density ($F_{2,48} = 25.51$; $P < 0.001$), fat mass ($F_{2,48} = 16.26$; $P < 0.001$), lean tissue mass ($F_{2,48} = 15.44$; $P < 0.001$), and body mass ($F_{2,48} = 4.99$; $P = 0.011$). The effect of immobilization method was not consistent among the DXA parameters analyzed (Fig. 1).

Evaluation of DXA Accuracy

DXA estimates of body composition explained a large proportion of the variation in chemical estimates for bone mass ($r^2 > 0.986$) and lean tissue mass ($r^2 > 0.964$) regardless of the method used to immobilize individuals during DXA scanning (Fig. 2A

& C; Table 3); however DXA estimates were poorly related to chemical estimates for fat mass ($r^2 < 0.261$; Fig. 2B). DXA estimates were significantly different than chemical estimates for ash mass (paired t-test; $t_{24} = 112.95$; $P < 0.001$), lean tissue mass ($t_{24} = 7.71$; $P < 0.001$), body mass ($t_{24} = 4.42$; $P < 0.001$), and fat mass ($t_{24} = 4.46$; $P < 0.001$). DXA underestimated bone mineral content, but overestimated fat mass, lean tissue mass, and body mass regardless of the immobilization method used (Fig. 3).

Discussion

The goal of developing new techniques to quantify observations is to provide users with advantages not afforded by previous methodologies. The application of non-destructive techniques, although non-invasive and therefore desirable, often sacrifices accuracy and precision. The utility of any technique is dependent on its ability to produce accurate and, more importantly, precise measurements. Accuracy is less important because predictive regression equations can be developed to correct for any biases inherent to the technique.

We assessed the precision and accuracy of DXA in predicting bone density and body composition of turtles using our subset of “still” measurements for anesthetized subjects. Overall, the precision that we documented for measuring tissue components was relatively high and similar to that found in other studies of non-human vertebrates (Table 4). The most notable exception is fat mass, where our study had the highest reported intraindividual variability of the studies examined. Additionally, fat mass was the least precisely estimated component of body composition for all of the studies examined.

Although the precision of DXA fat mass estimates are discouraging, the similar degree of precision in estimating other indices of body composition in turtles is promising. The similarity of precision compared to that of other species, in particular humans and rodents, for which the software was originally designed, is promising for the continued application of DXA in research involving chelonians.

Influence of Immobilization

Use of DXA requires that subjects remain motionless during the entire scanning process. Subject movement during scanning significantly and unpredictably influences the accuracy and precision of body composition estimates (Koo et al. 1995; Cawkwell 1998). A goal of this study was to determine which of the immobilization techniques resulted in the most precise and accurate measurements while at the same time reducing negative consequences associated with immobilization. Negative consequences associated with immobilization include cost, ease of use, recovery rate, and potential for harm of test subject. Ignoring precision and accuracy, cooling is the most desirable method of immobilization because it eliminates the cost of narcotics associated with anesthesia/euthanasia, is easy to perform, results in virtually no mortality if cooling is monitored, and has quick recovery rates. Unfortunately, cooling was not as an effective method. We found that cooled individuals were more likely to move during scanning (personal observation) and could account for the lower precision of body composition estimates. Anesthesia effectively produces immobilization if a sufficient dose is given for induction; however, anesthesia can be less predictable in reptiles, producing occasional

long recovery periods, variable induction dosages, and increased mortality compared to mammalian species (Bennett 1998; Read 2004). Despite these limitations, anesthesia produced more precise and accurate estimates of body composition than cooling. Euthanasia, although required in this study for comparisons, is the least desirable method of immobilization for DXA because it necessarily defeats the purpose of using non-destructive techniques. Although the influence of immobilization technique produced significant differences in estimates and noticeable variation in precision, each technique regressed well with chemical estimates and therefore each is generally acceptable if predictive equations are produced.

Limitations of DXA in the Prediction of Fat and Lean Soft Tissue Mass

The results of this study suggest that accurate prediction of fat mass and, to a lesser degree lean tissue mass, using DXA is questionable in chelonians. The morphology of chelonians precludes the ability to effectively determine fat mass due to the method that DXA utilizes to estimate fat mass. Fat and bone-free lean tissue mass can be distinguished and are calculated from the ratio of attenuation from the low and high-energy beams when calculations are performed on non-bone areas; however, when calculations are performed where lean tissue, fat, and bone overlap, the calculations of fat and lean tissue are indirect, leading to less reliable estimates (Jebb 1997). Fat and lean tissue mass estimates are less accurate when a large portion of the pixel area contains bone, such as in the thoracic region and brain in humans (Jebb 1997). The scanning of chelonians results in nearly 100% of the scanning pixels containing bone, depending on

whether the appendages are extended beyond the margins of the carapace. Therefore, DXA cannot use non-bone-containing neighbor pixels to calculate the proportion of fat to be used for the majority of pixels that contain bone. Thus the unique morphology of turtles most likely prevents the use of current DXA technology to accurately estimate fat mass. Our data corroborate this view, particularly when compared to a non-chelonian reptile: Secor and Nagy (2003) found a relatively low CV when using DXA to measure FM in snakes (9.2% versus 28.5-97.0% in this study). Because current DXA technology precludes the use of this tool for the prediction of fat mass in turtles, chelonian research with primary interests in obtaining fat estimates will have to use other methods. Although its effectiveness is debated, the establishment of triple or multiple-energy x-ray absorptiometry may solve this issue in turtles by using a three-compartment system rather than the two-compartment approach of DXA (Swanpalmer et al. 1998). Another potential approach that may help overcome this deficiency could be to position the individuals such that the x-ray beams will pass craniocaudal. This approach would reduce the total scanning area that contains bone and therefore might allow for a more accurate estimate of fat and lean tissue components.

Future Research

Future studies should examine the variation among scanners. We developed models linking body composition and DXA estimates; however, the utility of these models for users of other DXA brand scanners is unknown. Significant effects of DXA manufacturers, hardware, and software have been documented (Tothill et al. 1994a;

Tothill et al. 1994b; Jebb 1997; Tothill and Hannan 2000). Validation of our predictive models on other brands of DXA scanners is needed before they can be applied to research using other brands. We also suspect that there might be interspecies variation and therefore that the use of these predictive equations, developed for *T. scripta*, with species differing drastically from the general turtle morphology (e.g., soft-shelled turtles) would be problematic.

In conclusion, DXA could potentially be used for a variety of evolutionary, ecological, nutritional, physiological, and diagnostic applications in animals. However, a poor ability to predict fat mass in turtles severely limits some of its application to energetic and nutrition studies until advances in technology overcome the difficulties of distinguishing soft tissues in species containing a high proportion of bone. Although the use of DXA in turtles is limited by soft tissue, DXA is still effective at measuring bone content and density, and therefore would prove useful for studies of bone dynamics in turtles. The technique could be used in the identification of metabolic bone disease in a clinical setting. The ability to monitor an individual's bone density over a lifetime could provide a wealth of information on the long-term dietary impacts on bone density. The potential applications of DXA in scientific research are many; however, continued validation is required before DXA can be put into practical use for chelonian research.

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Table 1: Results of least-squares regressions of water mass (WM), fat-free tissue mass (FFM), lean tissue mass (LTM), fat mass (FM), and ash mass (AM), determined chemically, against body mass (BM) and straight carapace length (SCL) in male *Trachemys scripta* (N = 25).

Regression Coefficients	F	*P* Value	r^2
$WM = 0.863(BM)^{0.965}$	2730.7	< 0.001	0.992
$FFM = 0.993(BM)^{0.999}$	50475.2	<0.001	1.000
$LTM = 0.982(BM)^{0.977}$	16040.4	<0.001	0.999
$FM = 0.005(BM)^{1.125}$	13.0	0.001	0.361
$AM = 0.063(BM)^{1.125}$	1038.1	<0.001	0.978
$WM = 2.662*10^{-4}(SCL)^{2.799}$	652.89	<0.001	0.966
$FFM = 2.193*10^{-4}(SCL)^{2.907}$	1168.33	<0.001	0.981
$LTM = 2.686*10^{-4}(SCL)^{2.837}$	935.18	<0.001	0.976
$FM = 3.404*10^{-7}(SCL)^{3.284}$	12.73	0.002	0.356
$AM = 4.155*10^{-6}(SCL)^{3.302}$	943.00	<0.001	0.976

Note. All mass units are in grams and length in millimeters. Regressions were conducted on transformed data and equations were back-transformed.

Table 2. Mean intraindividual coefficients of variation (%) of DXA scans for the three methods of immobilization and four tissue components in male *Trachemys scripta* (N = 25).

Body Composition Type	Anesthesia			Cooling			Euthanasia		
	Movement	Still	Combined	Movement	Still	Combined	Movement	Still	Combined
BMC	1.71±1.36	1.00±0.82	1.63±0.89	2.05±1.65	1.96±1.67	2.00±1.18	1.49±1.05	0.74±0.57	1.36±0.75
	(0.27-6.13)	(0.78-3.17)	(0.71-4.65)	(0.05-6.15)	(0.00-7.41)	(0.43-5.09)	(0.16-4.43)	(0.00-2.12)	(0.19-3.06)
BMD	1.81±1.49	0.97±0.73	1.59±1.24	1.54±1.52	1.53±1.40	1.63±1.04	1.34±1.47	0.70±0.49	1.23±1.02
	(0.25-6.94)	(0.06-3.27)	(0.39-5.58)	(0.00-5.00)	(0.15-6.08)	(0.29-4.09)	(0.10-5.56)	(0.00-1.78)	(0.35-4.20)
FM	52.11±42.48	28.54±24.16	78.89±63.85	65.7±58.65	77.38±62.39	97.03±60.75	51.58±45.64	46.0±51.51	53.72±41.93
	(2.40-141.42)	(0.29-69.74)	(17.70-200.00)	(8.61-141.40)	(0.24-141.42)	(10.03-200.00)	(0.95-141.42)	(0.14-141.42)	(9.39-200.00)
LTM	2.01±3.00	1.05±1.45	3.40±6.03	2.39±5.43	1.57±3.36	2.49±3.65	7.31±13.33	4.28±4.80	6.59±8.01
	(0.00-13.24)	(0.01-4.41)	(0.02-28.4)	(0.00-25.12)	(0.02-14.93)	(0.03-15.65)	(0.00-54.58)	(0.07-21.30)	(0.14-35.38)

Note. Values for movement represent the coefficient of variation (CV) of two repeated measurements where the individual was moved between scans (scans 1 & 3). Still represents the CV of two repeated measurements where the subject was not moved between scans (scans 1 & 2). The combined data represents the CV of all four scans combined. Values are mean ± SD with ranges in parentheses. Abbreviations: BMC = bone mineral content, BMD = bone mineral density, FM = fat mass, LTM = lean tissue mass.

Table 3. Predictive models for chemically determined body composition variables in male *Trachemys scripta* (N = 25) determined by the regression of chemical estimates of body composition against DXA estimates for anesthetized individuals. Values for difference represent the average difference between actual tissue mass determined chemically and predicted tissue mass determined from the regression or cross-validation model. Values for percent difference represent the absolute difference represented as a percentage of total mass for the tissue component in question.

Model	r^2	Regression Model		Cross-validation	
		difference (g)	difference (%)	difference (g)	difference (%)
AM = 4.81*BMC_{DXA}-8.75	0.994	3.24±3.07	4.03±3.08	3.69±3.75	4.41±3.34
LTM = 0.98*LTM_{DXA}-39.66	0.979	31.62±24.17	6.44±3.99	34.14±25.41	6.89±4.14
LTM = 0.89*LTM_{DXA}+0.70FM_{DXA}-15.76	0.992	16.84±18.26	3.30±2.69	20.11±22.70	3.76±3.08
FM = FM_{DXA}+8.14	0.00	5.46±3.56	152.23±175.50	5.86±3.77	162.22±184.00
FM = 0.04*LTM_{DXA}-0.03*FM_{DXA}-0.62*BMC_{DXA}-1.51	0.528	3.60±2.68	85.40±87.70	4.43±3.11	100.81±94.98
FFM = 1.13*FFM_{DXA}-60.20	0.978	39.96±28.10	6.81±3.58	43.86±30.97	7.34±3.71
WM = 0.79*LTM_{DXA}-30.07	0.969	31.80±23.22	8.13±5.23	34.31±24.40	8.70±5.43
WM = 0.68*FM_{DXA}+0.71*LTM_{DXA}-7.11	0.987	19.34±16.85	4.86±3.7	22.72±20.87	5.48±4.06

Note. Predictor variables were selected using best-subsets regression. The simplest model was included and multivariate models were added if they had more explanatory power. Abbreviations: AM = ash mass, BMC = bone mineral content, LTM = lean tissue mass, FM = fat mass, FFM = fat-free tissue mass, WM = water mass.

Table 4. Literature review of the precision of DXA estimates of lean tissue mass, fat mass, and bone mineral content in several different animals.

Study	Animal	CV (%)		
		Lean Tissue Mass	Fat Mass	Bone Mineral Content
This study	Turtle	1.05	28.54	1.00
Nagy and Clair 2000	Mouse	0.86	2.20	1.60
Rose et al. 1998	Rat	2.88	12.16	6.34
Kastl et al. 2002	Rat humeri	-	-	0.90
Korine et al. 2004	Bird	1.28	4.92	-
Secor and Nagy 2003	Snake	0.6	-	1.0
Elowsson et al. 1998	Pig	0.94	13.51	1.91

Note. Intraindividual CV reported here for this study were based on anesthetized individuals where the subjected was not moved between two successive scans.

Figure Legends

Figure 1. The effects of immobilization method on DXA estimate of (A) bone mineral content, (B) bone mineral density, (C) fat mass, (D) lean tissue mass, and (E) body mass in male *T. scripta* (N=25). Significant differences ($\alpha < 0.05$) among treatment levels are indicated by different letters. Presented means indicate inverse $\log_{10}$-transformed data. Error bars represent 95% confidence intervals.

Figure 2. Regression of chemical estimates against DXA estimates of (A) lean tissue mass, (B) fat mass, and (C) bone mass for all three methods of immobilization in male *T. scripta* (N=25). The solid line represents a slope of 1.

Figure 3. Mean difference between DXA and chemical estimates of bone mineral content (BMC), fat mass (FM), lean tissue mass (LTM), and body mass (BM) for the three methods of immobilization in male *T. scripta* (N=25). Error bars represent 95% confidence intervals.

Figure 1

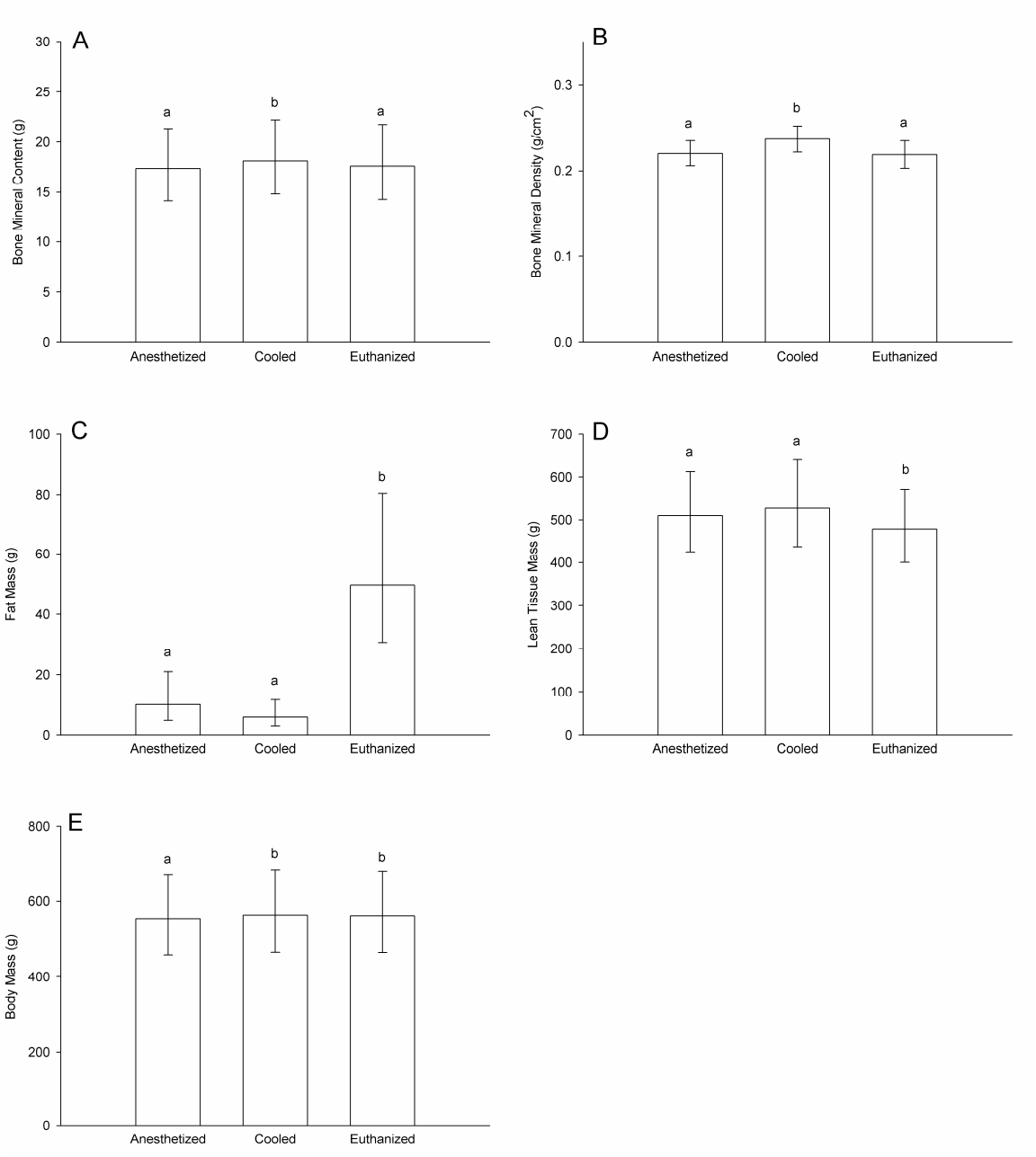

A
Bone Mineral Content (g)
30
25
20
15
10
5
0
a
b
a
Anesthetized
Cooled
Euthanized
B
Bone Mineral Density (g/cm2)
0.3
0.2
0.1
0.0
a
b
a
Anesthetized
Cooled
Euthanized
C
Fat Mass (g)
100
80
60
40
20
0
a
a
b
Anesthetized
Cooled
Euthanized
D
Lean Tissue Mass (g)
700
600
500
400
300
200
100
0
a
a
b
Anesthetized
Cooled
Euthanized
E
Body Mass (g)
800
600
400
200
0
a
b
b
Anesthetized
Cooled
Euthanized

Figure 2

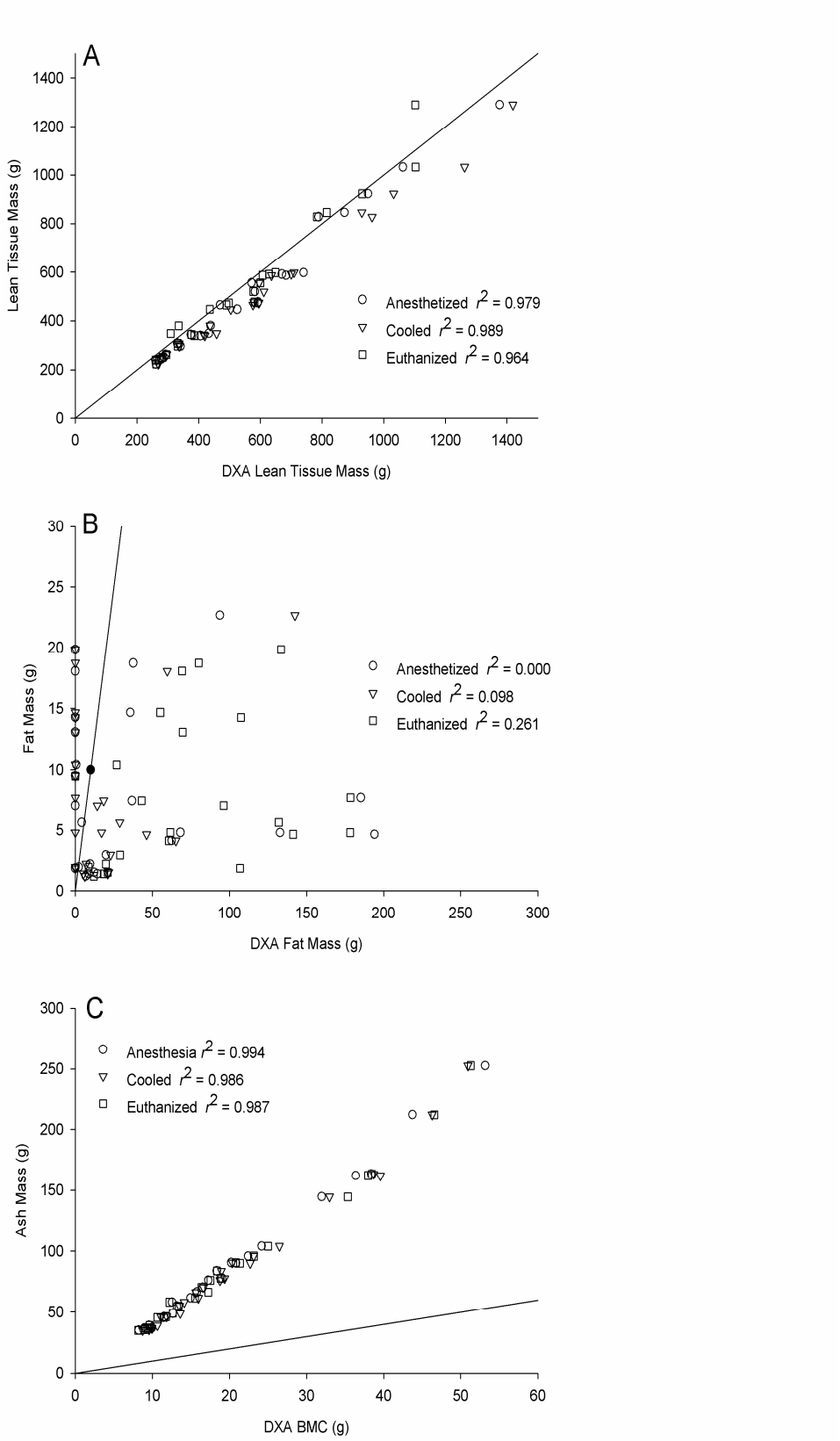
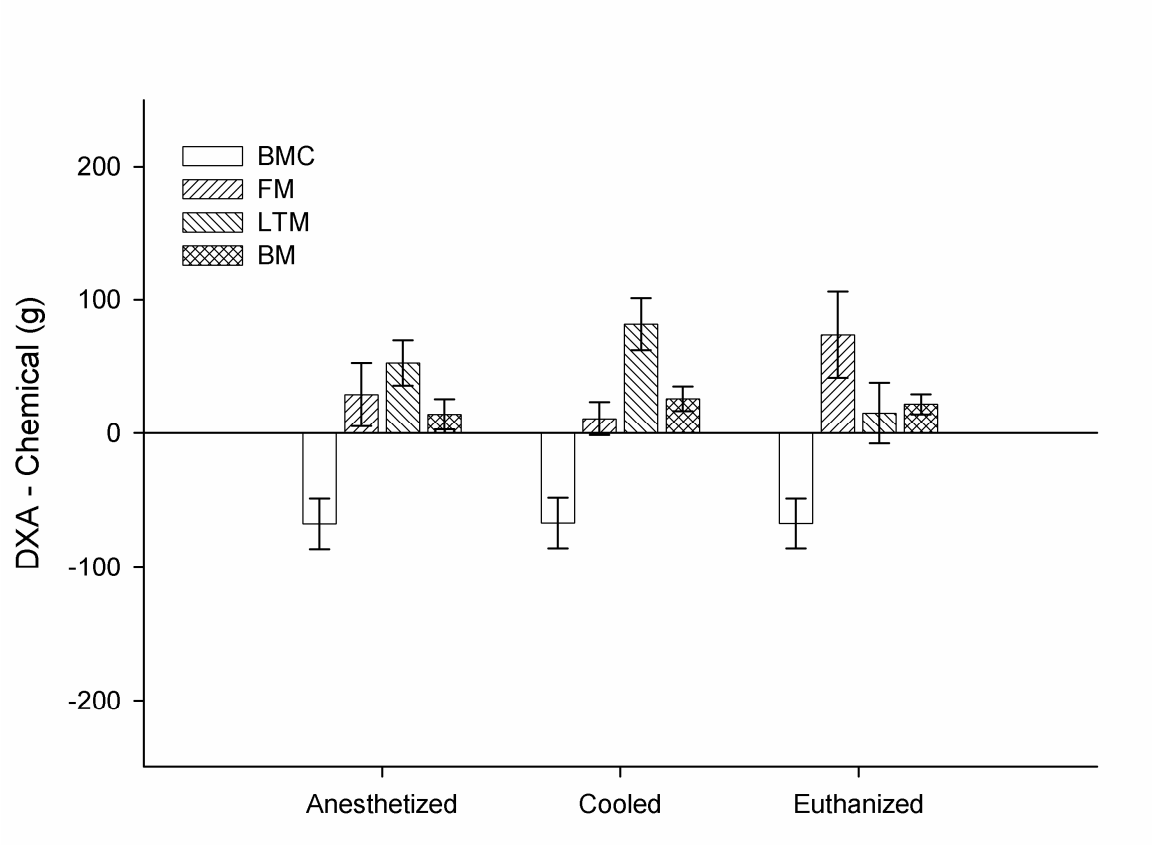

A
Lean Tissue Mass (g)
DXA Lean Tissue Mass (g)
Anesthetized r2 = 0.979
Cooled r2 = 0.989
Euthanized r2 = 0.964
B
Fat Mass (g)
DXA Fat Mass (g)
Anesthetized r2 = 0.000
Cooled r2 = 0.098
Euthanized r2 = 0.261
C
Ash Mass (g)
DXA BMC (g)
Anesthesia r2 = 0.994
Cooled r2 = 0.986
Euthanized r2 = 0.987

Figure 3


BMC
FM
LTM
BM
200
100
0
-100
-200
DXA - Chemical (g)
Anesthetized
Cooled
Euthanized

CHAPTER II

SEASONAL CHANGES IN CALCIUM PHYSIOLOGY OF THE TEXAS TORTOISE, *GOPHERUS BERLANDIERI*: INFLUENCE OF SEX, AGE, AND REPRODUCTIVE STATUS

Abstract

We measured the seasonal, sex, and age effects on plasma chemicals that are associated with calcium physiology in the Texas tortoise (*Gopherus berlandieri*). Female tortoises had higher concentrations of plasma calcium, phosphorus, magnesium, total protein, and alkaline phosphatase than did male tortoises. There were monthly changes in plasma calcium and phosphorus for female tortoises; plasma concentrations of these biochemicals were elevated post-oviposition, suggesting that females undergo vitellogenesis-induced hypercalcemia after eggs have been laid in mid-summer. No such seasonal change was observed for male tortoises. Although we did not specifically measure changes in bone structure, the investment of calcium into eggshell and yolk components may have important effects on bone density of female tortoises. In addition to measuring seasonal changes in blood biochemicals, we developed a hemogram of normal values for plasma calcium, phosphorus, magnesium, total protein, and alkaline phosphatase.

Introduction

Bone plays a number of important structural and physiological roles in vertebrates, including serving as a reservoir for, and maintaining homeostasis of, calcium. Calcium is well recognized for its importance in a variety of physiological processes, including muscle contraction, nerve transmission, and hemostasis. Calcium is also important during reproduction of egg-laying vertebrates by serving a structural role in the eggshell and providing a source of nutrients for the developing embryo. The influence of reproduction on maternal bone density has been heavily investigated in mammals and birds and to a lesser extent in reptiles (de Buffrenil and Francillon-Vieillot 2001). In some reptiles, egg production is related to a seasonal reduction of bone density in females, suggesting possible mobilization of calcium from bone to provide an adequate supply for vitellogenesis, the formation of the eggshell, or both (Edgren 1960; Suzuki 1963; Clark 1965; Wink et al. 1987; de Buffrenil and Francillon-Vieillot 2001).

Although the above studies document structural changes in bone associated with egg production, other studies have documented reproductive effects on plasma calcium without any changes in bone density. The administration of estrogen to male box turtles and female alligators induced rises in plasma calcium, but structural changes in bone density were not observed (Magliola 1984; Elsey and Wink 1986). These results suggest that estrogen produced during vitellogenesis facilitates calcium availability for egg production in reptiles. It is not certain whether this rise is supported by bone resorption, increased renal conservation, increased intestinal uptake, or any combination of these, but study results suggest bone resorption might not be necessary for vitellogenesis. Apparent

conflict between studies that suggest structural changes in bone density during egg production and those that do not might be explained by relative investment of calcium into the eggshell and yolk components of the egg. The rate of calcium transfer from mother to offspring is likely to differ between yolk and eggshell components due to the rate of formation of these components. Vitellogenesis is typically a long process, whereas eggshell formation is typically shorter in duration (Simkiss 1967).

The Texas tortoise, *Gopherus berlandieri*, is a threatened species in the state of Texas that might have particularly high calcium demands. Hellgren et al. (2000) documented a higher incidence of perforations through the costal bones of the posterior carapace in female *G. berlandieri*, and these authors suggested the perforations could be due to high demands for calcium during egg production coupled with diets poor in available calcium. Additionally, females exhibited higher mortality rates than female congeners leading to male-biased sex ratios in the older age classes. These observations may be due in part to increased disease susceptibility of females as a result of these carapacial perforations (Hellgren et al. 2000). The presence of carapacial perforations is not restricted to *G. berlandieri*. Occasionally, *Terrapene carolina triunguis* has perforations in the same region as *G. berlandieri* (personal observation). Perforations could be due to physical wearing of the superior end of the ilium against the most posterior costal bones of the carapace. Some emydid turtles with hinged shells have an ilial recess that accommodates the ilium when the posterior plastron is expanded away from the carapace (Bramble 1974). Thinning of this region to accommodate the pelvis might predispose species who possess carapacial kinesis to higher rates of carapacial perforations.

Gopherus berlandieri is a species that possesses a fair degree of carapacial kinesis thought to accommodate the oviposition of large eggs relative to body size (Ewert 1979; Rose and Judd 1991). Carapacial kinesis is the flexibility of bony portions of the carapace and plastron that is attained through thin bones and loose articulations (Rose and Judd 1991). However, there may be tradeoffs between the costs and benefits of thin bones. One cost can be reduced female survivorship when pelvic wearing breaks through the carapace, potentially rendering the tortoise vulnerable to disease, and one benefit can be increased shell kinesis allowing the laying of large eggs. Other suggested benefits include increased respiratory capacity and the ability to prevent extraction from the occasional burrow (Rose and Judd 1991).

We suggest that endogenous contributions of calcium to egg production by female *G. berlandieri* likely result in long-term reduction of bone mineral density and ultimately cause carapacial breakage due, in part, to pelvic wearing at the ilial recess. Resorption of bone as a calcium source may be an adaptive strategy to facilitate production of large eggs as proposed by Rose and Judd (1991) and Hellgren *et al*. (2000). Henen (2002) suggested that female desert tortoises might reduce reproductive output when food resources are scarce; similarly, one might expect that *G. berlandieri* would reduce egg production if calcium needs were not met. However, the investment of calcium in eggs at the expense of female bone density might be sustainable short-term. Although sustained calcium deficits could affect long-term survival, attempting reproduction when energy reserves are low would likely have immediate consequences on female survival.

Dietary deficiencies—potentially exacerbated by anthropogenic influences—also might contribute to thin bones in *G. berlandieri*. Anthropogenic disturbances generally

exhibit negative pressures on turtle populations and have resulted in population declines of many species; however, land management regimes in the range of *Gopherus berlandieri* have been suggested to have a positive or neutral effect on tortoises (Auffenberg and Weaver 1969; Ernst et al. 1994; Kazmaier et al. 2001). Prickly pear cactus (*Opuntia engelmannii*), a predominant dietary component of *G. berlandieri*, is positively affected by overgrazing and brush encroachment associated with management (Archer et al. 1988; Ernst et al. 1994; Kazmaier 2000). Although increasing cactus abundance would have positive effects on tortoise water regulation (Kazmaier 2000), high levels of oxalic acids in cacti (Ben Salem et al. 2002) may compromise the calcium status of *G. berlandieri* (Hellgren et al. 2000). Oxalates are known to form insoluble salts with calcium (James 1978; Ben Salem et al. 2002), therefore inhibiting the absorption of calcium (Weaver et al. 1987). Management regimes, such as root plowing, that cause increased cactus density may potentially shift tortoise food availability. Increased reliance on cactus as a food source might hamper the ability of *G. berlandieri* to obtain sufficient dietary calcium. Therefore, management regimes that cause increased reliance on cactus might affect survival and reproductive success of females and ultimately might have long-term implications for *G. berlandieri* populations.

Fully understanding the interaction between dietary intake of calcium and the potential tradeoffs between egg production, bone density, and carapacial kinesis were beyond the scope of this study. Instead, this study took the first step in understanding the effects of egg production on maternal calcium physiology. We report seasonal cycles in circulating metabolites involved in calcium metabolism. Specifically, we report the effects of season and age on male and female plasma calcium, magnesium, phosphorus,

total protein, and alkaline phosphatase. We predicted that plasma concentrations of calcium, magnesium, phosphorus, and total protein would increase in female tortoises during egg production to provide these nutrients to the offspring. Additionally, because alkaline phosphatase is an indicator of bone formation, we predicted that plasma alkaline phosphatase should decline during egg production because bone growth should decline to provide the calcium for investment in the eggshell and yolk components of the egg. Alkaline phosphatase should decline with age, because we expect growth rates to decline in older individuals. These biochemicals should not change in male tortoises if the seasonal changes in females are due to egg production.

Methods

Study Site and Field Methods—Texas tortoises were studied at the Chaparral Wildlife Management Area (CWMA) in Dimmit and LaSalle counties, Texas. For a detailed description of the study site, see Hellgren et al. (2000). Tortoise collection occurred during May–August 2004 and May–September 2005, and occurred during the majority of the peak activity period (Kazmaier 2000). All tortoises upon capture were brought to a nearby field laboratory and marked with a unique series of scute notches. Individuals were sought and primarily captured as they crossed roads, but were occasionally encountered when researchers were walking in the field. Age was determined for individuals based upon scute annuli. Although this technique can be problematic (Wilson et al. 2003), it has been validated in our population of Texas tortoises (Hellgren et al. 2000). All age estimation in this study was performed by either

RK or MS. Because of potential researcher bias in the estimation of age, RK and MS independently estimated the age of 29 tortoises. Age estimates did not vary between researchers (Paired t-test; t=1.09, P= 0.29). The mean difference between age estimates was 0.47 years for turtles that ranged in age from 2 to 17 years.

Morphometrics, including straight-line carapace length (SCL), greatest width (GW), and body mass (BM) were measured on all individuals, whereas the measurement of the carapacial plastron aperture (CPA) was only collected during 2005 captures (Table 1). The CPA is the straight-line distance between the plastron and carapace measured along the individual's midline at the caudal end and may give some measure of the maximum egg width capable of being oviposited. Length measurements were made to the nearest 0.1 mm using a vernier caliper and mass measurements were made to the nearest 10 g using a Pesola spring scale. In adult tortoises, sex was determined by the presence/absence of male secondary sexual characteristics (concave plastron, enlarged chin glands, and thickened anal scutes; Rose and Judd, 1982). Juvenile tortoises were distinguished from adults by body size as described by Hellgren et al. (2000). In all analyses involving gender we use the term "sex" loosely to describe gender differences in adult tortoises as well as juveniles from adult tortoises. The presence of eggs in females was determined with a 500V portable ultrasound scanner (Aloka Inc., Tokyo, Japan). The use of ultrasound to estimate the presence of eggs and clutch size was validated with the use of x-rays. Although the use of ultrasound tended to underestimate clutch size by an average of 1 egg, the use of ultrasound to detect presence/absence of eggs was precise.

Blood Collection, Handling and Analysis—Blood was collected from individuals via the subcarapacial vein with a 1-cc syringe. Although the time elapsed between

capture and phlebotomy was variable among individuals, blood was collected within 6 hours of initial capture for the majority of blood samples. All phlebotomy was performed by the same researcher (MS). After collection, blood samples were injected into vials containing lithium heparin and centrifuged for 4 minutes. A subsample of blood was collected in a hematocrit tube for the determination of packed cell volume. Hematocrit analyses were conducted on 2005 samples only. Upon centrifugation, plasma was separated from the cell fraction and then frozen at -20˚C. All samples were brought to the Oklahoma State University campus on dry ice to prevent thawing. An approximately 50-µL aliquot of plasma was used to determine concentration of calcium, inorganic phosphorus, magnesium, alkaline phosphatase, and total protein using an ACE® automated chemistry analyzer (Alfa Wassermann Inc., West Caldwell, New Jersey).

Statistical Analyses—We performed a multivariate cluster analysis of variables to have a better understanding of the "closeness" of variables and to inform subsequent analyses. Cluster analysis was performed using the "clusters of variables" procedure described by Minitab® statistical software (Minitab Inc., State College, PA). Variables loaded were age of subject, hematocrit, total protein, alkaline phosphatase, calcium, magnesium, phosphorus, time collected, month collected, julian date collected, blood osmolality, and date sample analyzed. Results of the multivariate analysis showed clustering of hematocrit with total protein and alkaline phosphatase. Additionally, calcium was linked with phosphorus and magnesium, but more closely with phosphorus (Figure 1). Due to this clustering, we performed a least-squares regression to test for effects of hematocrit on plasma biochemicals. This analysis allowed us to determine if plasma dilution might be a problematic issue in the accurate determination of blood

chemistry. Effects of hematocrit were significant for total protein and alkaline phosphatase (see Results); therefore, we used ANCOVA with hematocrit as a covariate to analyze effects of sex and month on these variables. Because hematocrit data were collected only during the 2005 field season, we could not test for the effect of year in these analyses. Moreover, we had insufficient sample sizes of juveniles and their removal from hematocrit analysis was required. We tested the assumption of homogeneity of regression coefficients and determined there was no difference among slopes for total protein ($F_{1,68}$=0.03, P=0.85) and alkaline phosphatase ($F_{1,63}$=0.02, P=0.89), so a linear-slopes model was appropriate for these analyses. One assumption of ANCOVA is that the covariate is independent of the treatment. In this case, we cannot be certain that this assumption was not violated. Therefore we analyzed both of these variables with and without hematocrit included as a covariate and we present the results of both of these analyses.

We analyzed dependent variables (calcium, magnesium, and phosphorus) with 3-way ANOVA to examine for sex and temporal effects. Categorical variables sex, month, and year were analyzed as independent variables. Analysis of effects on hematocrit was similar, but did not include year as an independent variable because these data were collected only during the 2005 field season. Individuals of unknown sex were excluded from analyses involving sex effects. Additionally, we removed samples collected during September from analysis because of insufficient sample sizes needed to run higher-order interactions. All variables were assessed for violation of test assumptions before analysis. Normality and homogeneity of variance assumptions were assessed using the Anderson-Darling and Levene's Test, respectively. All blood variables satisfied the test statistic

assumptions of normality (Anderson-Darling Test; P>0.051) and homogeneity of variance (Levene's Test; P>0.106). Due to unequal sample sizes and insufficient subjects for some treatments, tests for higher-order interactions could not be performed. Instead, we elected to combine years for *post hoc* comparison because year effects were not detected (year, $F_{1,172}$=1.59, P=0.209; year-sex interaction, $F_{2,172}$ =1.04, P=0.357) and were of less interest. Our focus was on sex and seasonal variation in concentrations of plasma calcium, magnesium, and phosphorus levels.

We assessed the effects of age on circulating levels of plasma biochemicals using least-squares regression. Dependent variables calcium, magnesium, phosphorus, total protein, alkaline phosphatase, and hematocrit were regressed against age. We assessed the influence of egg production on all plasma biochemicals with one-way ANOVA using number of eggs produced (0-4) by females as treatment levels.

Statistical analyses were performed using Minitab version 13.20 (Minitab Inc., State College, Pennsylvania). *Post-hoc* comparisons among treatment levels, when applicable, were compared using Tukey tests. Statistical analyses were considered significant if P<0.05 and marginally significant if P<0.10. Mean values are given ± 1SD unless stated otherwise.

Results

A total of 263 individual tortoises (137 female, 60 male, 59 juvenile, and 7 unknown) were captured over the two-year period resulting in a total of 286 blood samples collected. Some tortoises were collected and sampled multiple times throughout

the field season. In such instances, only the first blood sample was included in analyses. Of the females examined for the presence of eggs, 34 females were observed gravid (11 in 2004 and 23 in 2005). Gravid females had on average 2.0±0.9 (range: 1-4) eggs present.

Hematocrit—Result of least-squares regression suggest that hematocrit had a significant effect on total protein (Figure 2D; n=94, F=48.46, P<0.001) and alkaline phosphatase (Figure 2E; n=94, F=8.93, P=0.004), a marginal effect on calcium (Figure 2A; n=94, F=3.56, P=0.062) and phosphorus (Figure 2C; n=94, F=3.62, P=0.06), and no effect on magnesium (Figure 2B; n=94, F=0.00, P=0.974). Hematocrit did not vary by a month-sex interaction (two-way ANOVA; $F_{3,65}$=1.67, P=0.18), nor did hematocrit vary across months (Figure 3F; $F_{3,65}$=0.99, P=0.40). Hematocrit varied between sexes ($F_{1,65}$=6.84, P=0.011), with mean hematocrit being higher in male tortoises (24.3±7.1%) than in females (20.3±5.0%).

Calcium—Plasma calcium varied by a sex-month interaction (3-way ANOVA; $F_{6,172}$=3.39, P=0.003). For male tortoises, plasma calcium did not vary across months (Figure 3A; P=1.000). However, female plasma calcium rose from its lowest concentration in May to June (t=3.51, P=0.028) and also from June until July (t=4.15, P=0.003), where it began to level off until reaching its apex in August (Figure 3A). Overall, plasma calcium varied among sexes ($F_{2,172}$=8.28, P<0.001), with females having higher plasma calcium than males (Table 2; t=3.2, P=0.004) and juveniles (t=3.0, P=0.009). Plasma calcium in males did not differ from juveniles (t=0.9, P=0.613). No significant main effect of month ($F_{3,172}$=1.59, P=0.194) or year ($F_{1,172}$=1.59, P=0.209) was detected.

Phosphorus—Plasma phosphorus followed similar trends to that of calcium, with a marginally significant interaction between sex and month ($F_{2,172}$=2.10, P=0.056). Phosphorus tended to vary more across months in females than in males, which remained relatively stable (Figure 3C). We found no interaction between sex and year ($F_{2,172}$=1.62, P=0.2). Overall, we found a significant effect of sex ($F_{2,172}$=10.36, P<0.001), where females had higher plasma phosphorus than males (t=3.9, P<0.001) and juveniles (t=3.0, P=0.008), but juveniles were not different from males (t=0.6, P=0.8). We found no main effect of month ($F_{3,172}$=0.45, P=0.72) or year ($F_{1,172}$=0.60, P=0.43).

Magnesium—Plasma magnesium varied by a marginal sex-year interaction (Figure 3B; $F_{2,172}$=2.86, P=0.06) but not a sex-month interaction ($F_{6,172}$=0.61, P=0.72). Comparisons between years within sexes indicated that mean plasma magnesium was higher in 2005 than 2004 for females (t=3.03, P=0.03). No differences between years were observed for males (t=0.73, P=0.97) and juveniles (t=1.20, P=0.83). Plasma magnesium varied among the sexes ($F_{2,172}$=9.43, P<0.001), with females having higher magnesium than males (t=4.20, P<0.001). Magnesium concentrations did not differ between females and juveniles (t=1.86, P=0.15) or between males and juveniles (t=0.62, P=0.81). There were no significant differences among months ($F_{3,172}$=0.06, P=0.98) or between years ($F_{1,172}$=0.21, P=0.65).

Total Protein—Total protein did not vary by a month-sex interaction (ANCOVA; $F_{4,69}$=1.75, P=0.15). Total protein was different between male and female tortoises ($F_{1,69}$=5.66, P=0.02), with females (least squares mean±SE; 3.39±0.10) having higher total protein than males (2.97±0.14). There was no significant effect of month (Figure 3D; $F_{4,69}$=0.86, P=0.49). The effect of hematocrit was significant, suggesting the

importance of including hematocrit as a covariate ($F_{1,69}$=49.77, P<0.001). The removal of hematocrit from analysis altered the results of the study. A sex-month interaction was detected ($F_{6,172}$=2.66, P=0.017); however, simple effects among levels within sex and within month were not different (P>0.15).Year effects were significant ($F_{1,172}$=8.40, P=0.004), with tortoises captured in 2004 (3.49±0.88 g/dL) having higher total protein than those captured in 2005 (3.17±0.85 g/dL). Total protein did not vary between sexes ($F_{2,172}$=1.97, P=0.14).

Alkaline Phosphatase—Analysis of alkaline phosphatase activity showed no sex-month interaction (ANOVA; $F_{6,172}$=0.84, P=0.543; Figure 3E) or sex-year interaction ($F_{2,172}$=0.70, P=0.499). Additionally, there was no main effect of sex (ANOVA; $F_{2,172}$=0.90, P=0.408) or month ($F_{3,172}$=0.49, P=0.69). There was an effect of year ($F_{1,172}$=7.18, P=0.008), with tortoises collected in 2004 (38.8±15.6 U/L) having higher levels of alkaline phosphatase than 2005 (33.5±13.3 U/L). Using ANCOVA to control for variation in hematocrit, we found no significant sex-month interaction ($F_{3,64}$=0.92, P=0.44). There was a marginal main effect of sex ($F_{1,64}$=3.53, P=0.065), with levels of circulating alkaline phosphatase marginally higher in female tortoises (least squares mean±SE; 35.01±1.84 U/L) than in males (28.56±2.82 U/L). There was no main effect of month on alkaline phosphatase ($F_{3,64}$=1.85, P=0.15).

Age—Age had no significant influence on calcium (least-squares regression, n=236, F=0.36, P=0.55), phosphorus (n=236, F=0.00, P=0.963), total protein (n=236, F=0.49, P=0.483), and hematocrit (n=120, F=0.92, P=0.34). Age had a weak positive (slope = 0.11±0.03 SE) influence on magnesium (n=236, F=17.31, P<0.001, r^2=0.064)

and negative (slope = -0.72±0.23 SE) influence on alkaline phosphatase (n=236, F=9.33, P=0.002, r^2=0.035).

Egg Production—The influence of egg production on plasma biochemicals was varied. Magnesium was marginally influenced by the number of eggs that females had present in the oviduct (one-way ANOVA, $F_{4,30}$=2.30, P=0.082, Figure 4). Plasma magnesium was higher in females with 4 eggs present (7.0±3.1 mEq/L) than in females who did not have eggs present (3.8±1.6; t=2.92, P=0.048). The number of eggs produced had no influence on calcium ($F_{4,30}$=0.09, P=0.99), phosphorus ($F_{4,30}$=0.99, P=0.43), total protein ($F_{4,30}$=0.82, P=0.52), or alkaline phosphatase ($F_{4,30}$=2.02, P=0.117).

Discussion

We predicted that plasma calcium, magnesium, phosphorus, and total protein would rise and that alkaline phosphatase would decline during egg production in female tortoises. We also predicted that no such changes should be evident in males if such changes in females were a result of egg production. The findings of this study support our predictions for calcium and phosphorus, where we observed seasonal increases of these nutrients in females but not in males. We suggest these elevated concentrations are a result of vitellogenesis. We did not detect any seasonal changes in total protein and alkaline phosphatase for either sex, which is contrary to our initial predictions. This discrepancy might be explained by the possible dilution of samples with lymph.

Blood Chemistry Comparisons with Other Turtle Species—To our knowledge, ours is the first study to measure blood chemistry of the Texas tortoise. The values we

obtained in this study are comparable to, and fall within, the range of values determined for other species of turtles (Table 2). Many of our values were on the lower end of the range in turtles; however, several studies reported values equal to or lower than ours. In our literature search, the range of hematocrit, calcium, magnesium, phosphorus, total protein, and alkaline phosphatase were 16-37%, 3.5-18.3 mg/dL, 1.1-4.9 mg/dL, 1.6-13.8 mg/dL, 2.0-6.1 g/dL, and 13-465 U/L, respectively (Table 2). Our averages were well within these ranges and therefore we feel our values aid in the establishment of a normal hemogram for this species. We recommend caution, however, with the interpretation of absolute values for total protein and alkaline phosphatase as these values can be influenced by lymphatic dilution (see discussion below).

Validity of Normal Values—An important consideration for the accuracy of our blood plasma variables is the potential for lymphatic dilution. Collection of blood samples in tortoises is challenging at best. A variety of collection sites exist, each with certain drawbacks. Major drawbacks are the general difficulty of easily obtaining a blood sample without the use of repeated attempts on individuals, or the challenge of obtaining whole blood without contamination of lymph. Generally, sites that are easily accessed are those with a higher probability of lymphatic dilution, whereas sites that have a lower probability of dilution are generally more challenging to venipuncture. We elected to draw blood from the subcarapacial vein. Although there is risk of lymphatic dilution from this site, it is easily accessed compared to other sites where a struggling tortoise can make venipuncture challenging or impossible (e.g., the jugular vein). The possibility of not being able to collect whole blood is a serious issue. Diluted samples might lead to erroneous estimates of certain blood parameters, especially those that are protein based

and would not likely enter lymphatic circulation (Crawshaw and Holz 1996). Our data suggest that lymphatic dilution might have occurred because we found relationships between hematocrit and plasma protein and hematocrit and alkaline phosphatase, as well as the presence of some samples with unusually low hematocrit. The results of cluster analysis corroborate this view, with hematocrit clustering more with total protein and alkaline phosphatase (similarity level = 76.06) than other plasma variables. Despite the probability of lymphatic dilution in some samples, we believe that comparisons among treatments are valid.

We can postulate how the probability of collecting lymph-diluted samples could change among years or month. For example, if we were refining our skills in phlebotomy as time progresses (i.e., getting better at obtaining whole blood), then a month effect should be evident with hematocrit, as well as total protein and alkaline phosphatase, increasing across months. Our data suggest that this is not occurring as hematocrit does not vary across months. We suggest that the probability of lymphatic dilution is random, and is mainly responsible for increasing error variance rather than between treatments. Moreover, hematocrit determined in this study was within normal ranges of other turtles (Peterson 2002).

Sex, Season, and Year Effects on Plasma Chemistry—Hematocrit or packed cell volume is known to be variable among turtle species and populations, and seasonally varies within individuals. We observed effects of sex on hematocrit but did not observe any temporal effects. A significant sex effect on hematocrit is corroborated by previous studies. Male desert tortoises (*Gopherus agassizii*) had equal or higher packed cell volume than females and such dimorphism is suggested to be a result of complex

interactive influences including hormone and nutritional influences (Peterson 2002). Although multiple influences may dictate variance in hematocrit, hydration status is likely an important factor (Peterson 2002). In the present study population, male tortoises had higher plasma osmolarity than female tortoises (Stone unpublished data). Dehydration of male tortoises could be accounting for elevated plasma osmolarity and hematocrit. We did not detect seasonal variation in hematocrit as has been documented in some other species (Peterson 2002); however, our sampling was restricted to months of high turtle activity.

The results from the analysis of circulating alkaline phosphatase did not correspond with our initial predictions. We hypothesized that female tortoises would have reduced levels of alkaline phosphatase when compared to male tortoises because bone growth would be reduced during the egg-laying season as a result of calcium investment into egg production rather than in somatic growth. Depending on the analysis, we found either no effect of sex or higher alkaline phosphatase values in female tortoises. Total alkaline phosphatase is an indicator of bone formation; however, assays of the bone isoform are a more reliable indicator because liver, intestine, spleen, and kidney all contribute to circulating levels of total alkaline phosphatase (Khosla and Kleerekoper 2003). Despite this potential caveat, liver and bone isoforms are the primary contributors to elevated levels of total alkaline phosphatase (Khosla and Kleerekoper 2003).

We suggest that our observation of higher 2004 concentrations of alkaline phosphatase were a result of higher bone growth during the 2004 field season compared to 2005. Rainfall events were more frequent and tortoise activity was higher during the 2004 field season as indicated by higher catch rates. Differences in catch rates between

2004 (152 captures) and 2005 (111 captures) are not a result of trapping effort because the 2005 season was sampled earlier and later in the year and more intensively than the 2004 season. We suggest that increased available forage and less extreme environmental conditions led to increased growth of tortoises of both sexes and were detectable by elevated concentrations of alkaline phosphatase. Age effects on alkaline phosphatase followed our expectations. Younger individuals tended to have higher levels of alkaline phosphatase; a result that is likely due to higher growth rates in juvenile tortoises.

Correspondence Between Plasma Chemistry and Reproductive Cycle—Little is known about the reproductive cycle of the Texas tortoise (Rose and Judd 1982). The most comprehensive studies of reproduction are limited to data on nesting chronology and clutch sizes. These studies suggest that female tortoises are gravid from April until July, laying up to 2 clutches per season of 1 to 5 eggs per clutch (Judd and Rose 1989; Hellgren et al. 2000). Although some data suggested tortoises might remain gravid until November (Auffenberg and Weaver 1969), these observations are from southern Texas coastal populations and there are no data on the frequency of this occurrence. Furthermore, a study on *G. berlandieri* in nearby locations suggested that nesting ceases after July, and investigators found no gravid females in the months of Aug-Oct (Judd and Rose 1989). Data for northern inland populations corroborate this and suggest the majority of females are no longer gravid by late July-early August (Hellgren et al. 2000). In our study, the latest we observed a gravid female was 8 August. In light of this, our data suggest that elevated levels of calcium and phosphorus are likely associated with vitellogenesis rather than eggshell production because we observed our lowest values of measured calcium during the period (May) where the majority of females contained

shelled or shelling eggs within the oviduct and our highest levels occurred when females were no longer gravid.

Little is known about the endocrinology and timing of gonad cycles for the Texas tortoise, but much can be assumed from what is known about the timing of their egg production as well as reproductive cycles of other *Gopherus*. In the desert tortoise, vitellogenesis occurs in the fall prior to hibernation (Rostal et al. 1994). We assume a similar pattern is occurring with *G. berlandieri* and that the increase in calcium and phosphorus we observed is associated with follicular development. It is well known that during vitellogenesis total calcium and phosphorus levels are elevated because of increases in the amount of plasma proteins, primarily vitellogenin, that bind these ions (Alcobendas et al. 1992; Rostal et al. 1994; Rostal et al. 1998; Wysolmerski 2002; Lv et al. 2006). Interestingly, we did not observe significant increases in total protein or magnesium concurrent with elevated calcium and phosphorus as would be expected to occur during vitellogenesis. Estrogenic induction of vitellogenesis has been implicated to increases in total protein and magnesium in addition to that of calcium (Lv et al. 2006). However, females had greater concentrations of total protein and magnesium levels than males; vitellogenesis might account for this difference. Additionally, it is possible that such seasonal effects are present but the dilution of samples with lymph might have resulted in increased error variance, making treatment effects undetectable.

Long-term Effects of Reproduction on Maternal Bone Density—The long-term effects of reproduction on maternal bone density is variable among and within species. In crocodilians, it appears that female bone density is affected by egg production, specifically eggshell formation (Wink et al. 1987). Similar trends were also observed in

lizards (de Buffrenil and Francillon-Vieillot 2001) and turtles (Edgren 1960). It appears that the deposition of an eggshell composed primarily of calcium carbonate imposes calcium demands on some reptiles, such that increased intestinal absorption and kidney conservation is insufficient to meet daily demands, thus necessitating bone resorption. Whether reptiles are able to recover to prereproductive levels is not so clear. In the Nile monitor (*Varanus niloticus*), it appears that reproductive demand reduces bone compactness in females as they grow larger (age); males, on the other hand, have increased bone compactness with age (de Buffrenil and Francillon-Vieillot 2001). Those findings were complicated by the fact that the effect of sex was population-dependent. Populations in Nigeria and Chad showed sex differences in the rate that bone compactness changed with age; however, in Mali populations, the rate at which bone compactness changed with age was similar between sexes. Thus, even within a species, the effect of long-term egg production in females is not constant. Age likely interacts with environmental conditions and evolved reproductive strategies of the organism. There is also evidence for an inability of females to compensate for the calcium demands of reproduction in the American alligator (Wink et al. 1987). The evidence in alligators is not as strong as in monitors, but juvenile alligators have more robust bones than reproductively quiescent adult females, suggesting females that previously laid eggs might not be able to return to levels previously held as juveniles (Wink et al. 1987). Unfortunately, no control study examined bone porosity in adult males to rule out a similar trend in adult males. Although we did not investigate bone density changes in *G. berlandieri*, this species may have a reduced calcium reservoir compared to other turtles due to their thin carapacial bones and therefore might be more prone to a bone deficiency

if there are any perturbations to calcium availability either through food availability or physiological constraints (i.e., high dietary oxalate intake restricting intestinal absorption rates of calcium) as per Hellgren et al. (2000).

Summary and Future Research on Gopherus berlandieri—We have documented seasonal changes in calcium physiology in *G. berlandieri* that are likely a result of egg production. These physiological changes might have important implications for the maintenance of maternal bone density. However, to fully address calcium physiology in this species and the larger question of tradeoffs between reproduction and survival in female tortoises tied to egg production, the documentation of a calcium budget will be necessary. Specifically, we need to know the total amount of calcium allocated each year for the production of eggs, and the proportional allocation to eggshell and yolk components. These data would be a logical first step to help estimate total calcium requirements of females.

No studies exist that estimate calcium in any portion of the egg of the Texas tortoise, so we calculated estimates using data derived from other closely related species. Eggshell ash mass expressed as a percent of total egg dry mass is 18.98% for *Gopherus polyphemus* (Lamb and Congdon 1985). We assume that the entire inorganic content of eggshell is composed of calcium because the inorganic portion of turtle eggshells is composed primarily of calcium carbonate (Congdon and Gibbons 1990). Wet-to-dry mass ratios of the eggs of *G. berlandieri* are not known; therefore, we assumed that eggs were composed of 68.8% water, the average for several species of turtles (Congdon and Gibbons 1990). Mean wet mass of *G. berlandieri* eggs is 26.9±1.06 g (range 18.7-30.37; Judd and Rose 1989). Based on these values, we estimate that eggshell calcium of *G.*

berlandieri averages 1.593 g/egg. Using a mean clutch size of 2.07 eggs and an annual clutch rate of 1.34 (Hellgren et al. 2000), we estimate a total annual calcium demand of 4.419 g/year for the production of eggshell components. We recognize this as a rough estimate, but it is currently our best one based upon the available data, and is only the first step in understanding calcium needs of *G. berlandieri*. In order to better understand these needs, much research is needed on the dynamics of intestinal assimilation and renal excretion of calcium for this species.

Additionally, we need more evidence that bone resorption is occurring during egg production (either during egg-laying or vitellogenesis) to provide the embryo with calcium. Although the documentation of reduced bone density during eggshelling would provide some evidence, other techniques could provide better evidence that this is occurring. Currently, a number of biochemical markers are available for mammalian research that can quantify bone turnover. The most reliable markers of bone resorption are the metabolic products of collagen degradation, which can be assayed from both blood and urine depending on the indicator of interest (Khosla and Kleerekoper 2003).

Finally, identification of the potential long-term effects of egg production in *G. berlandieri* needs to be addressed. A variety of tools exist that allow for the quantification of bone density. Determining seasonal and age effects on bone density of this species would offer insight to whether annual production of eggs results in a balance between bone resorption and formation or whether females exhibit a lifetime deficit in bone density as a result of egg production. Compared to other species of turtles, the relatively thin nature of *G. berlandieri* bones might offer little long-term buffer to negative calcium balance if reproductive demands for calcium are higher than can be

obtained from dietary sources. For example, evidence indicates that for some squamates, embryos might rely more heavily on yolk calcium than that derived from the eggshell (Packard et al. 1984); however, in many species of turtles, reliance is greater on eggshell calcium (Simkiss 1962; Packard and Packard 1991).

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Table 1. Descriptive statistics of tortoise morphometrics from *G. berlandieri* captured at the Chaparral Wildlife Management Area during the 2004 and 2005 active season.

Metric	Sex	N	Mean	SD	Range
Mass (g)	Juvenile	58	274	116	28.5-470
	Male	59	824	206	400-1320
	Female	135	677	174	340-1180
	Combined	257	616	260	28.5-1320
SCL (mm)	Juvenile	57	102.0	17.2	47.8-122.3
	Male	58	154.9	13.6	122.0-178.6
	Female	133	142.1	12.0	115.0-174.6
	Combined	253	135.7	23.4	47.8-178.6
GW (mm)	Juvenile	57	88.5	13.6	46.7-107.4
	Male	57	130.2	11.9	104.3-155.2
	Female	133	123.6	11.1	96.3-149.0
	Combined	252	116.9	19.7	46.7-155.2
CPA (mm)	Juvenile	19	14.1	3.1	9.9-21.7
	Male	23	22.2	2.7	17.6-28.4
	Female	47	19.6	3.2	13.6-25.9
	Combined	92	19.0	4.2	9.9-28.4
Age (years)	Juvenile	58	3.6	0.9	1-5
	Male	56	9.7	3.8	4-20
	Female	122	7.8	3.3	4-18
	Combined	241	7.2	3.7	1-20

Combined data include individuals whose sex is unknown, thus these values are not the sum of juvenile, male, and female data. Abbreviations: SCL=straight carapace length, GW=greatest width, CPA=carapace plastron aperture

Table 2. Literature review of normal plasma chemistry concentrations for hematocrit (Ht), calcium (Ca), magnesium (Mg), inorganic phosphorus (Phos), alkaline phosphatase (ALP), and total protein (TP) in turtles. Values are stated as mean±SD unless otherwise noted. DCV=dorsal coccygeal vein, JV=jugular vein, POVP=post occipital venous plexus, DCS=dorsal cervical sinus, OP=occipital plexus, CP=cardiac puncture, SCA=severed carotid artery, NC=nail clip, AA=axilary area, CA=caudal artery, SCS=subcarapacial sinus.

Study	Species	Sample Site	Season	Sex	Ht (%)	Ca (mg/dL)	Mg (mg/dL)	Phos (mg/dL)	TP (g/dL)	ALP (U/L)
This study	*Gopherus berlandieri*	SCS	SU	F	20.1	10.2±5.0	4.1±1.6	3.2±1.7	3.4±0.9	37.6±15.0
				M	23.6	7.8±2.6	3.1±1.2	2.0±0.8	3.3±0.9	33.1±14.2
				Juv	21.3	7.3±2.7	3.1±1.0	2.0±0.7	3.0±0.6	37.5±16.0
(Pages et al. 1992)	*Mauremys caspica*	CP	SU	M	16	3.5±0.3	1.1±0.5	13.8±3.5	3.29±1.27	NP
			FA	Both	24	7.9±0.4	3.0±0.2	7.0±1.2	4.15±0.41	NP
(Christopher et al. 1999)[1]	*Gopherus agassizii*	JV	SP	M	27	11.1	4.9*	2.8	3.4	39*
				F	22.5	17.5		4.6	3.8	
			SU	M	27	10.5	4.2*	2.1	3.3	50*
				F	25	18.3		3.1	4.0	
(Taylor, Jr. and Jacobson 1982)[2]	*Gopherus polyphemus*	CP	FA,SP	Both	22.7	11.8±0.6	4.1±0.2	2.1±0.2	3.11±0.23	276±28**
(Gottdenker and Jacobson 1995)[2]	*Gopherus agassizii*	POVP	?	?	17.8	8.7±0.4	NP	1.6±0.3	1.96±0.24	18.2±2.5
		JV	?	?	26.6	10.0±0.4	NP	2.1±0.3	3.61±0.21	31.7±3.0
(Aguirre et al. 1995)	*Chelonia mydas*	DPOS	FA	Juv.	NP	8.42±1.0	NP	7.9±0.9	4.32±0.6	42.4±10.7
(Bolten and Bjorndal 1992)	*Chelonia mydas*	DCS	SP	Both	35.2	9.1±2.1	NP	6.7±1.2	5.1±0.8	43±16
(Bolten et al. 1992)	*Caretta caretta*	DCS	?	Juv, Both	NP	6.8±1.2	NP	8.0±2.0	4.1±1.3	13±3
(Raphael et al. 1994)	*Malacochersus tornieri*	OP	Feb	Juv	34	12.7±2.2	NP	3.1±0.6	3.1±0.5	NP
(Ghebremeskel et al. 1991)	*Geochelone gigantea*	DCV	?	?	NP	9.3±2.5	NP	2.2±0.7	2.1±1.1	26.3±17.0**

(Knotkova et al. 2002)	*Agrionemys horsfieldi*	DCV	?	Both	NP	10.0±3.6	NP	4.3±1.2	4.5±0.7	264.7±117.6
(Oyewale et al. 1998)[2]	*Kinixys erosa*	SCA	?	M	34	8.4±0.0	NP	4.6±0.1	6.1±0.1	152.2±3.3
				F	26	8.4±0.1	NP	4.5±0.1	6.1±0.1	162.0±2.6
	Gopherus agassizii			M	28	8.4±0.1	NP	4.6±0.1	6.1±0.1	159.0±8.3
				F	30	8.4±0.1	NP	4.6±0.0	6.1±0.0	163.7±5.4
(Rosskopf 1982)	*Gopherus agassizii*	NC, AA	?	Both***	23-37	9.0-17.0	NP	NP	2.2-5.0	NP
(Aguirre and Balazs 2000)	*Chelonia mydas*	DPOS	FA	Juv	NP	9.1±1.7	NP	8.2±1.3	4.2±0.6	33.5±12.2
						11.2±2.2		5.0±1.3	5.0±0.7	18.2±8.5
(Marks and Citino 1990)	*Testudo radiata*	JV,AA	SU	Both	31	12.2±0.9	NP	3.2±0.5	4.0±0.5	92.7±14.4
(Mundim et al. 1999)	*Podocnemis expansa*	CA	?	Both	NP	9.3±1.4	NP	2.8±0.5	3.2±0.5	107.0±45.7
(Samour et al. 1986)	*Geochelone sp.*	DCV	Captive	?	?	11.6±4.0	NP	4.3±3.1	4.1±1.8	111±46
(Whitaker and Krum 1999)	*Lepidochelys kempii*	?	Captive	?	?	6.3±1.3	NP	8.7±1.7	3.2±0.7	465±306
	Caretta caretta	?	Captive	?	?	7.2±0.8	NP	5.1±3.3	2.0±0.8	74±37
(Raiti and Haramati 1997)	Geochelone pardalis	?	Captive	M	NP	10.0±0.5	NP	2.8±0.4	4.5±0.6	94.3±21.8
				F	NP	11.3±1.5	NP	2.8±0.7	3.4±0.2	78.0±25.6

NP: Not performed.
[1]Results from this study are given as median values.
[2]Results from this study are mean ± SE
*These values are derived from both sexes.
**Values converted from King-Armstrong units
***Not implicitly stated in study but assumed

Figure Legends

Figure 1. Results of the cluster analysis of variables for *G. berlandieri* collected in southern Texas during 2004 and 2005 activity seasons. Dendrogram represents similarity of blood and collection variables as a result of the cluster analysis. Abbreviations: HT=hematocrit, TP=total protein, ALP=alkaline phosphatase, Ca=calcium, Phos=phosphorus, Mg=magnesium, Time Col=time of tortoise collection, Month=month of collection, Date Col=date of collection, Osm=blood osmolarity, Date Run=date the sample was analyzed.

Figure 2. Results of the regression of plasma A) calcium, B) magnesium, C) inorganic phosphorus, D) total protein, and E) alkaline phosphatase against hematocrit for *G. berlandieri* collected in southern Texas 2004-2005. Note: data in all graphs contain replicates of individuals and could violate the assumption of independence of errors.

Figure 3. Monthly changes in tortoise plasma A) calcium, B) magnesium, C) inorganic phosphorus, D) total protein, E) alkaline phosphatase, and F) hematocrit for male (circular symbols), female (triangular symbols), and juvenile (square symbols) *G. berlandieri* collected at the Chaparral Wildlife Management Area 2004-2005. Symbols represent mean ± 95% confidence intervals.

Figure 4. Effects of egg production on plasma A) calcium, B) magnesium, C) inorganic phosphorus, D) total protein, and E) alkaline phosphatase in female *G. berlandieri* collected in southern Texas during 2004 and 2005 active seasons.

Figure 1

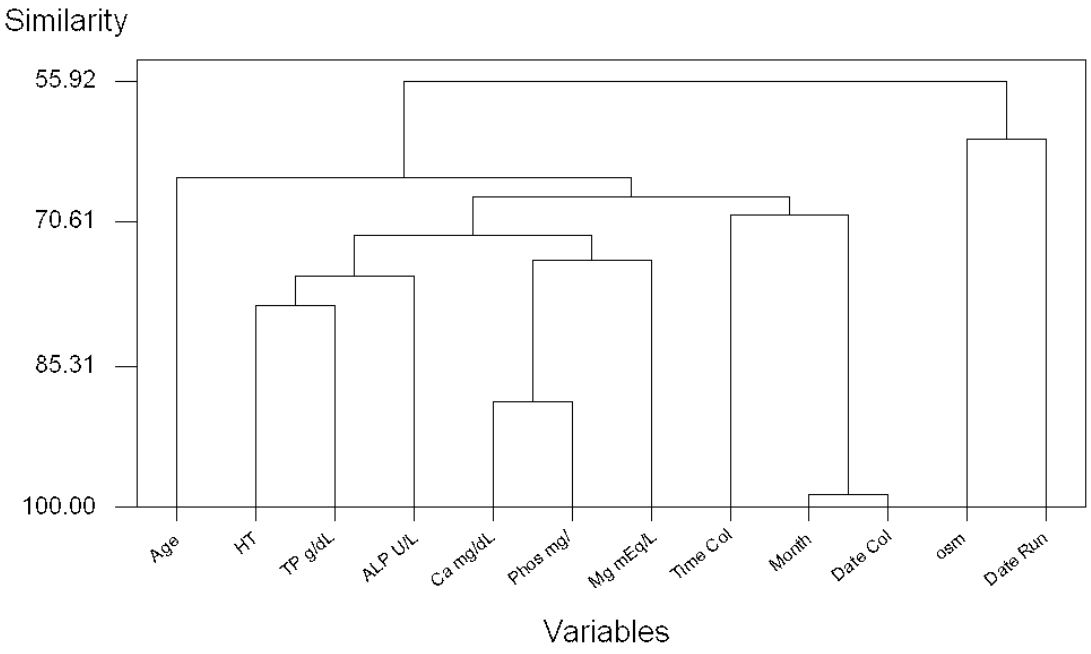

Similarity
55.92
70.61
85.31
100.00
Age
HT
TP g/dL
ALP U/L
Ca mg/dL
Phos mg/
Mg mEq/L
Time Col
Month
Date Col
osm
Date Run
Variables

Figure 2

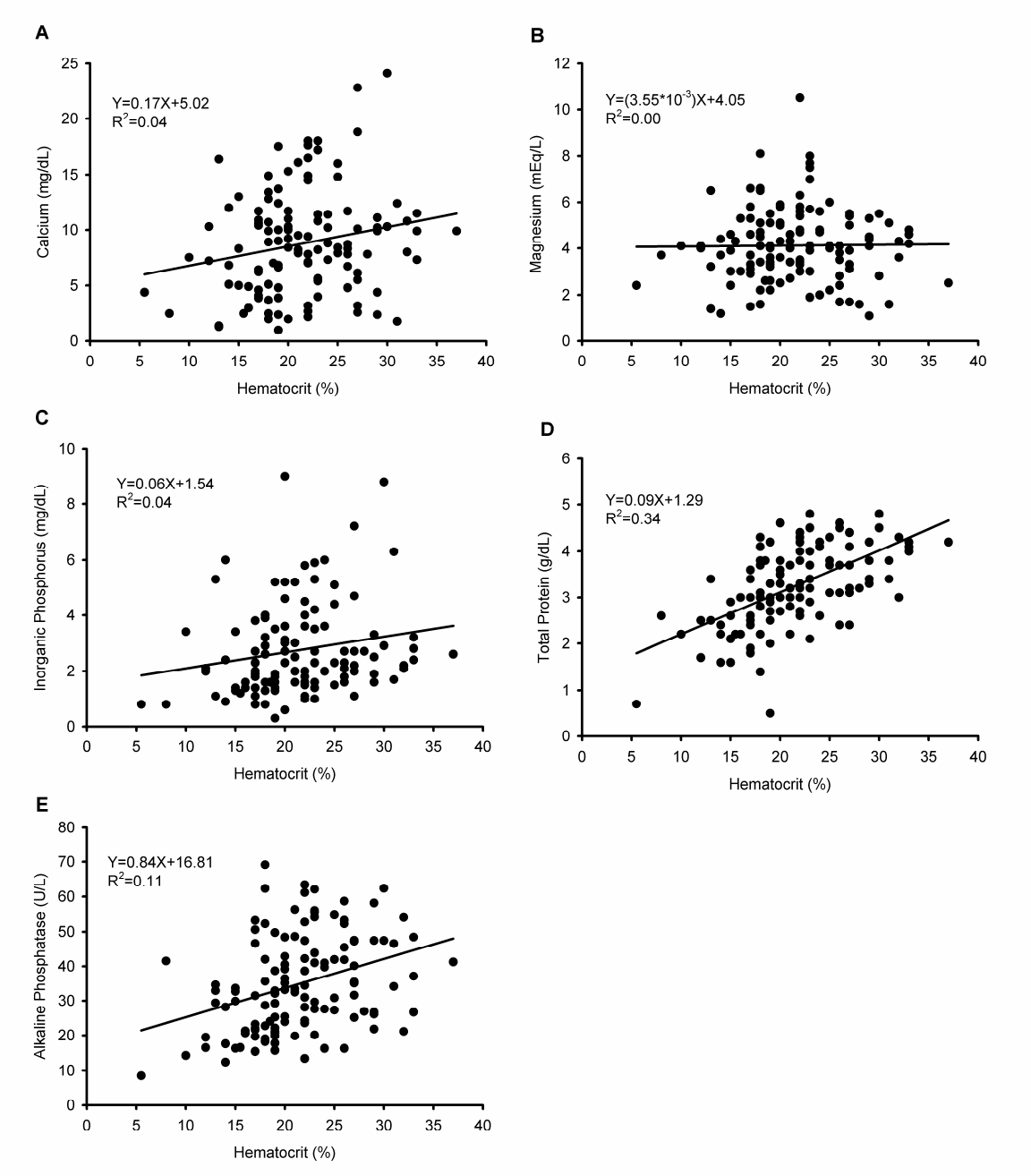

A
Y=0.17X+5.02
R2=0.04
Calcium (mg/dL)
Hematocrit (%)
B
Y=(3.55*10-3)X+4.05
R2=0.00
Magnesium (mEq/L)
Hematocrit (%)
C
Y=0.06X+1.54
R2=0.04
Incrganic Phosphorus (mg/dL)
Hematocrit (%)
D
Y=0.09X+1.29
R2=0.34
Total Protein (g/dL)
Hematocrit (%)
E
Y=0.84X+16.81
R2=0.11
Alkaline Phosphatase (U/L)
Hematocrit (%)

Figure 3

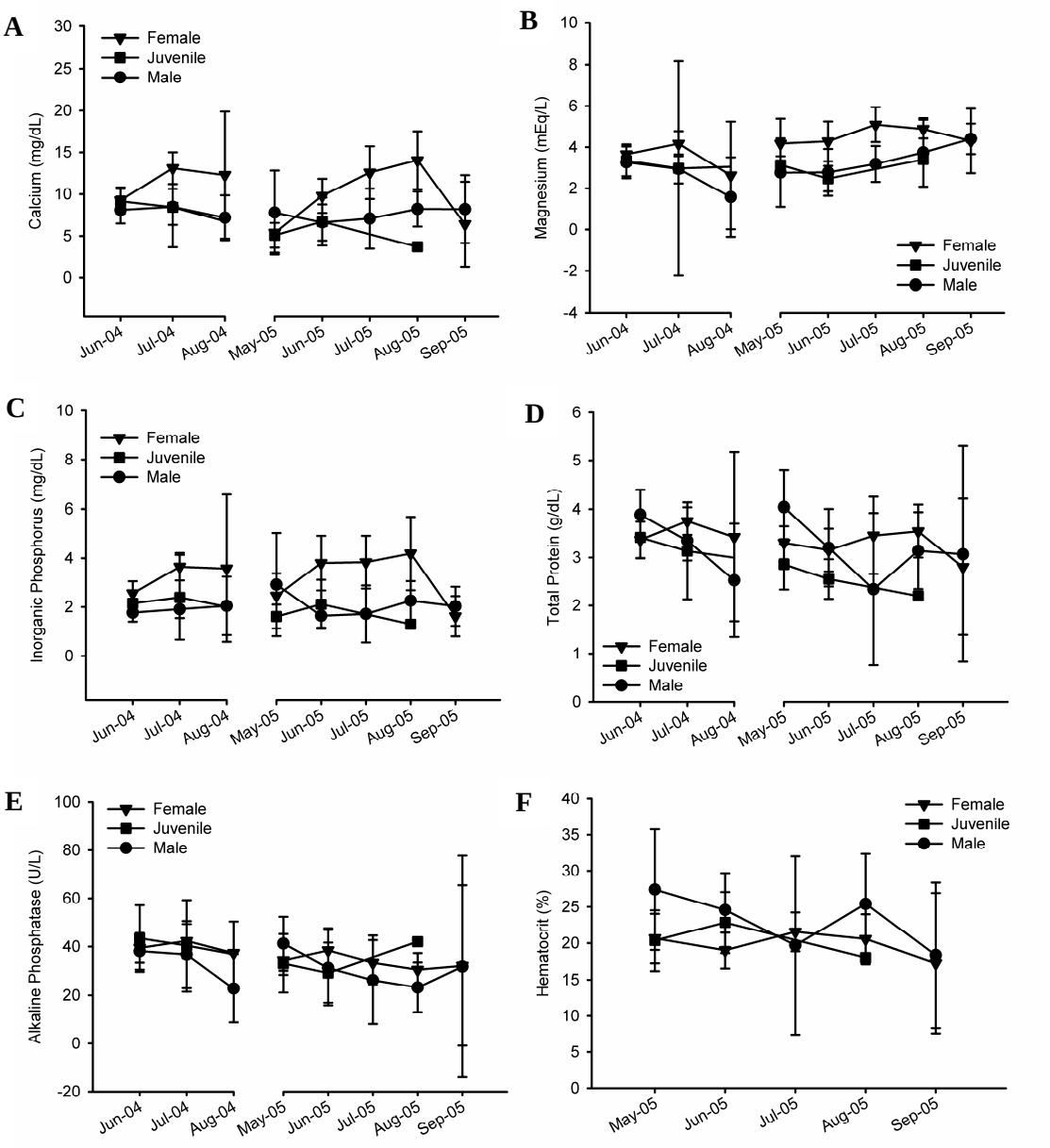
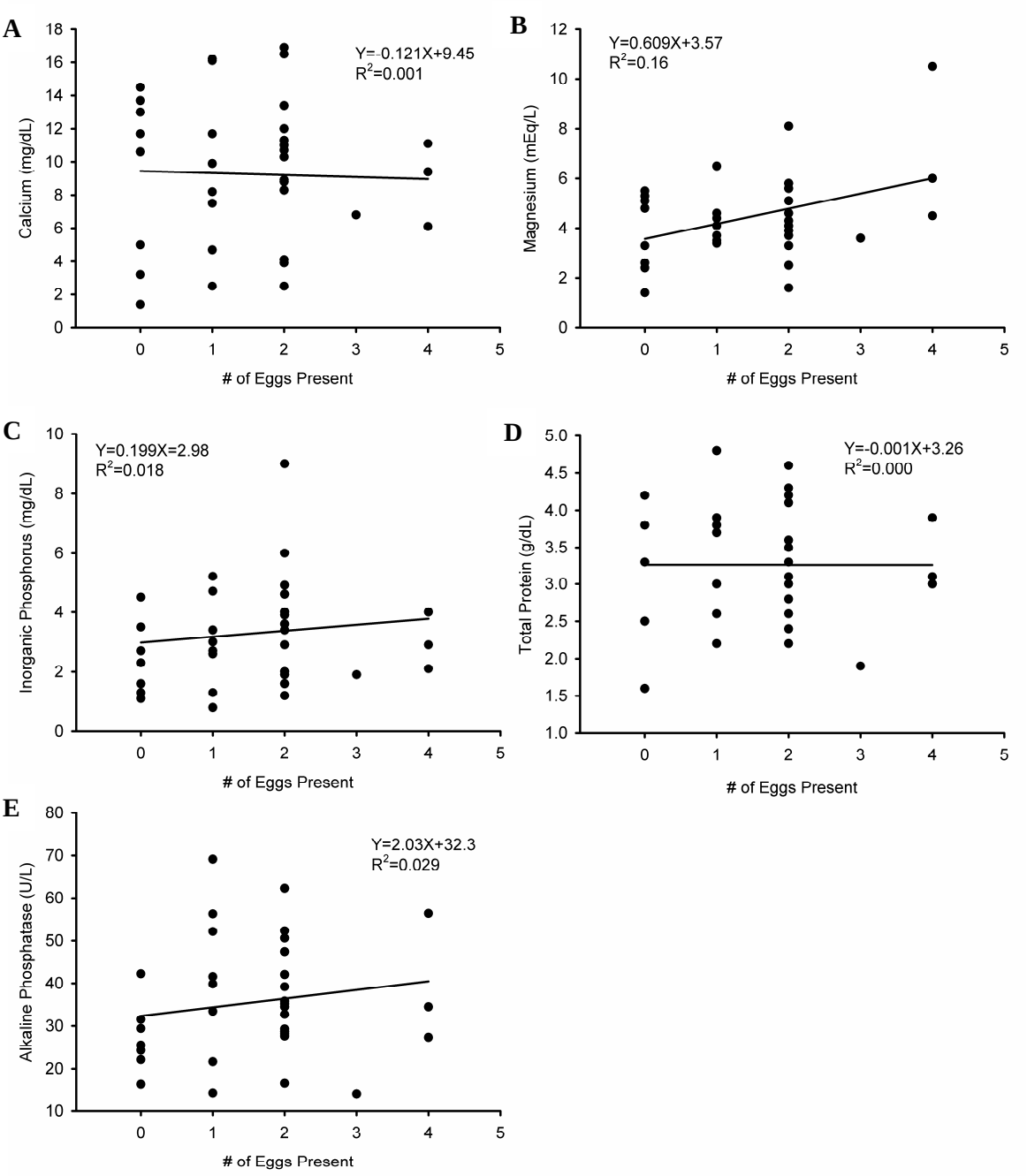

A
Calcium (mg/dL)
Female
Juvenile
Male
B
Magnesium (mEq/L)
C
Inorganic Phosphorus (mg/dL)
D
Total Protein (g/dL)
E
Alkaline Phosphatase (U/L)
F
Hematocrit (%)
Jun-04
Jul-04
Aug-04
May-05
Jun-05
Jul-05
Aug-05
Sep-05

Figure 4


A
Y=-0.121X+9.45
R2=0.001
Calcium (mg/dL)
# of Eggs Present
B
Y=0.609X+3.57
R2=0.16
Magnesium (mEq/L)
# of Eggs Present
C
Y=0.199X=2.98
R2=0.018
Inorganic Phosphorus (mg/dL)
# of Eggs Present
D
Y=-0.001X+3.26
R2=0.000
Total Protein (g/dL)
# of Eggs Present
E
Y=2.03X+32.3
R2=0.029
Alkaline Phosphatase (U/L)
# of Eggs Present

CHAPTER III

DO SEX, AGE, OR REPRODUCTIVE STATUS INFLUENCE CALCIUM PHYSIOLOGY AND BONE DENSITY IN RED-EARED SLIDERS (*TRACHEMYS SCRIPTA*)?

Abstract

Calcium is an important nutrient for a variety of physiological processes and plays an important structural role in bone. During reproduction, calcium is transferred from mother to eggs and offspring, and this process might influence maternal bone reserves. We measured changes in bone density and plasma calcium, phosphorus, magnesium, total protein, and alkaline phosphatase in a population of red-eared sliders (*Trachemys scripta*) to determine how reproductive investment of calcium into offspring influenced maternal calcium physiology. We observed a seasonal rise in plasma calcium, phosphorus, and alkaline phosphatase during the active season, but this pattern was present for both male and female turtles, suggesting a pattern driven by diet rather than egg production. We noted that gravid females had higher plasma calcium and phosphorus than non-gravid females. Mass-specific bone mass followed a sigmoidal growth pattern with straight carapace length. We found that this growth rate declined in both sexes at the approximate size at which females obtain sexual maturity, a finding contrary to previous studies of other species. We also did not detect any effect of season on bone density for either sex, suggesting that egg production in females does not adversely influence bone density in this population. This finding is also contrary to some previous studies of this and other

species. This finding might be a result of the production of an eggshell containing relatively less calcium than some other species of turtles that have been previously studied.

Introduction

Parental investment is the provisioning of resources to offspring that increases offspring survival and reproductive success at the expense of parental ability to invest in other offspring (Trivers 1972). Maternal investment is often expressed in terms of energy allocation to offspring; however, investment of other nutritional components such as calcium also might be important in determining a mother's ability to invest in future offspring. Calcium plays a variety of important roles in the body, including serving as a structural component of bone and an important cellular signal in muscle and nervous function. The importance of parental investment of calcium has been recognized and well studied in mammals (Garel 1987). Birds have received considerable attention in part due to the unique constraint that flight has imposed in restricting the production of a large calcium reserve in bone to minimize mass. The energetic cost of flight has limited the degree of bone mineralization in birds, yet they still continually invest large quantities of calcium into offspring (Simkiss 1967). Non-avian reptiles have received relatively less attention. In this group, research on the effects of egg production on maternal bone reserves has been investigated in turtles (Edgren 1960; Magliola 1984), alligators (Elsey and Wink 1985; Wink and Elsey 1986; Wink et al. 1987; Schweitzer et al. 2007), and lizards (Allen et al. 1993).

Maternal investment can be separated into preovulatory and postovulatory categories (Fischer et al. 1991). In the case of calcium investment in egg-laying species, these categories represent the investment of calcium into the yolk and eggshell, respectively. Understanding the relative investment into these two components is important. We know that growing embryos utilize calcium from both yolk and eggshell components; the relative contribution of eggshell and yolk calcium to the developing embryo is variable among species and among groups at higher taxonomic levels (Packard et al. 1977; Packard et al. 1984a; Packard et al. 1984b; Packard et al. 1985; Packard and Packard 1991a; Packard and Packard 1991b). The rate of calcium transfer from mother to offspring is likely to differ between yolk and eggshell components due to the rate of formation of these components. Vitellogenesis is typically a long process, while eggshell formation is typically shorter in duration (Simkiss 1967). Vitellogenesis generally occurs over a span of several months, while eggshell formation is completed over several days to weeks. The ability of females to derive sufficient calcium solely from dietary sources rather than relying on bone reserves is also likely to differ between these two phases of maternal-offspring transfer. This difference is best evidenced in the extreme case of birds where a specialized form of bone, medullary bone, increases during vitellogenesis, but is resorbed during eggshell formation (Dacke et al. 1993).

Investigation of maternal-offspring transfer of calcium in turtles is important because this taxonomic group owes much of its success to the presence of a protective carapace. Turtles are a morphologically unique taxonomic group characterized, in most species, by the presence of a robust carapace and plastron formed by the fusion of dermal and endochondral bone that encases the internal organs and both limb girdles. Because of

the protective nature of the turtle shell, understanding how investment of calcium in offspring affects maternal bone density could have important implications to survival in turtles. Available evidence suggests that non-avian vertebrates, including turtles, do not have the ability to produce medullary bone (Edgren 1960; Simkiss 1961b; Suzuki 1963; Magliola 1984; Elsey and Wink 1986) and are reliant on dietary sources and normal skeletal reserves to supply calcium for reproduction. For turtles, the carapace might provide a considerable reservoir of calcium and may buffer against excessive loss due to reproduction. The proportion of bone relative to body mass is higher in turtles than most other vertebrates (Iverson 1984). Evidence suggests that a seasonal decline in bone density of female turtles is associated with a rising need for calcium during egg production, specifically eggshell formation (Edgren 1960). Despite that study and many others that have investigated plasma calcium dynamics, few have shown direct evidence linking the dynamics of bone density to reproduction in turtles (e.g., Edgren 1960; Suzuki 1963).

The goal of the present study was to examine the link between bone density, the nutrients involved in bone metabolism, and the reproductive cycle of turtles. Additionally, we investigated the relationship between bone density and age in turtles to determine if continuous reproductive investment of calcium into offspring has long-term consequences for bone density. To achieve this, we monitored an Oklahoma population of red-eared sliders, *Trachemys scripta*, over four years. We measured morphometrics to determine age and growth of individuals. We also measured bone density, egg production, and plasma concentrations of calcium, magnesium, phosphorus, total protein, and alkaline phosphatase. We monitored seasonal changes in these variables in both

sexes. We predicted that plasma concentrations of calcium, magnesium, phosphorus, and total protein would increase in female turtles during egg production to provide these nutrients to the offspring. Additionally, because alkaline phosphatase is an enzyme associated with osteoblast activity and an indicator of bone formation, we predicted that plasma alkaline phosphatase would decline during egg production because bone resorption should increase to supply the calcium for investment in the egg. If our prediction of increased bone resorption is correct, bone density should decline with alkaline phosphates in female tortoises. We predicted that all of these patterns would be absent in male turtles.

Methods

To determine the relative importance of season, age, and sex influences on bone density and plasma metabolites, we studied a population of *T. scripta* between April 2004 and October 2007. Turtles (N=147) were collected from a pond (36°07’51.52”N, 97°05’56.91”W) located near the Oklahoma State University campus in Payne County. Subjects were also captured through fortuitous encounters as turtles crossed roads within Payne County. All turtles were marked with a unique series of scute notches. Upon capture, turtles were brought to a field laboratory where we collected morphometric data and blood. We measured straight carapace length (SCL), straight plastron length (SPL), greatest width (GW), length of left middle claw (LMC), plastron to cloaca length (PTC), plastron to tail tip length (PTT), the midline distance between the carapace and plastron at the caudal end of the turtle (CPA) using a vernier caliper; all measurements were recorded to the nearest 0.1 mm. Body mass was measured to the nearest 10 g with a

Pesola spring scale. We used body size (SCL) as an estimate of age of individuals. The number of shelled eggs present in female oviducts was assessed with a 500V portable ultrasound scanner (Aloka Inc., Tokyo, Japan) and by dual-energy X-ray absorptiometry (DXA). Additionally, large (>1.5 cm in diameter) and small (<1.5 cm) ovarian follicles were counted when possible.

Blood Collection and Analyses--Up to 1 mL of blood was collected from each individual from the subcarapacial sinus. Blood was placed in lithium heparin vials and centrifuged for 5 minutes. Prior to centrifuging the sample, a subsample of blood was transferred to a microhematocrit tube and separately centrifuged for determination of hematocrit. For the primary sample, plasma was separated from cellular components and then frozen and stored at -20°C prior to chemical analysis. Plasma was analyzed for calcium (Ca), magnesium (Mg), inorganic phosphorus (Phos), total protein (TP), and alkaline phosphatase (ALP) with an Alfa Wasserman ACE® clinical chemistry analyzer. Samples were analyzed using standard procedures provided by the manufacturer.

Bone Analyses –We measured bone density of each turtle 4-14 days after its capture using dual-energy x-ray absorptiometry (DXA) to monitor changes in bone mass and density throughout the activity season. The use of DXA to measure bone mass has been validated in turtles (Stone unpublished data). After capture, turtles were individually housed in 19-L plastic buckets containing approximately 5 cm of water. Turtles were fasted for at least 4 days prior to determination of body composition because food contents in the intestine can influence DXA estimates. Bone mineral content (BMC; g) and bone mineral density (BMD; g/cm^2) were measured with a Hologic® QDR 4500A fan-beam scanner equipped with a small-animal software program. Prior to scanning, the

densitometer was quality-checked each day using Hologic® calibration models (anthropomorphic-spine phantom and small-step phantom). Calibration procedures followed those provided by the manufacturer. Subjects were immobilized on the scanning bed by cooling them at 4°C for at least 8 hours prior to scanning. Cooling was used as the preferred method of immobilization because of its ease of use and cost effectiveness (Stone unpublished data). Individuals were scanned a minimum of 2 times without repositioning, and the average of each scan was used for analysis. The scanning area for small-animal software was limited to a maximum width of 183 mm. Only two captured females exceeded this size limit and could not be included in bone analysis; therefore, this method will exclude the largest individuals, but this bias is small. After DXA analysis, turtles were released at their site of capture.

Statistical Analyses— We regressed all plasma biochemistry variables against hematocrit using least-squares regression to determine if plasma samples were potentially diluted with lymph and to determine if there were any effects on plasma biochemicals. Plasma biochemicals were also compared among months (April-Oct) and between sexes with two-way ANOVA. Total protein was analyzed with ANCOVA with hematocrit as the covariate to control for possible dilution of samples with lymph. Prior to analysis, calcium, phosphorus, and alkaline phosphatase data were log-transformed to satisfy assumptions of normality and homogeneity of variance.

Statistical analyses of bone data were performed on corrected bone mineral content and bone mineral density. Corrected bone data were used because DXA estimates of bone mineral density and bone mineral content, although precise, are inaccurate (Stone unpublished data). The following relationship was used to correct for the inaccuracy of

bone mineral content: BMC = 4.8103(BMC_{DXA})-8.7535. Bone mineral density was corrected by dividing corrected bone mineral content by the 2-D surface area of the subject determined by DXA. To examine the relationship between body size and bone mass and to examine the potential differences between the sexes for this relationship, we plotted corrected bone mineral content, hereafter referred to simply as bone mineral content, against straight carapace length. The data were fitted to the power function: $y=ax^b$. I also plotted the relationship between relative bone mass, defined as the ratio of bone mineral content to body mass (Iverson 1982), and straight carapace length. These data were fitted to the sigmoid function:

$$y = \frac{a}{1 + e^{-\frac{x - x_o}{b}}}$$

This analysis was performed twice for each sex, both with and without the inclusion of juvenile turtle data. These non-linear regressions were performed on Sigma Plot version 8.0 (Systat Software Inc., San Jose, CA). We used two-way ANCOVA to examine the effects of month (April-October) and sex (male or female) on bone mineral content, bone mineral density, and relative bone mass. Straight carapace length was used as the covariate for analysis of bone mineral density and relative bone mass, but for the analysis of bone mineral content, straight carapace length was cubed to linearize the relationship between covariate and dependent variable. Relative bone mass was log-transformed prior to analysis to linearize the relationship between covariate and dependent variable.

We assessed each female as “yes” or “no” for 3 possible conditions (shelled eggs, large ovarian follicles, and small ovarian follicles) to examine the effects of reproduction on blood biochemistry and bone. For each condition, calcium, magnesium, phosphorus,

total protein, alkaline phosphatase, relative bone mass, bone mineral content, and bone mineral density were compared between “yes” and “no” groups with two-sample t-tests. Normality was assessed with a normality probability plot and homogeneity of variance was assessed with Levene’s tests. For all tests of egg presence, homogeneity of variance was satisfied ($P>0.208$) except for phosphorus data ($P=0.015$), which were log-transformed prior to analysis. For analysis of large-follicle presence, all tests satisfied these assumptions ($P>0.062$), except for alkaline phosphatase data ($P=0.007$), which were log-transformed prior to analysis. For all tests of small-follicle presence, test assumptions were satisfied ($P>0.110$) except for phosphorus ($P=0.057$) and alkaline phosphatase data ($P=0.028$), which were log-transformed prior to analysis. Log-transformations were successful in satisfying test assumptions in all cases.

Statistical analyses were performed using Minitab version 13.20 (Minitab Inc., State College, PA). Post-hoc comparisons among treatment levels, when applicable, were performed using Tukey tests. Means are presented ± 1SD, unless stated otherwise. All analyses were considered significant if $P<0.05$ and marginally significant if $P<0.10$.

Results

We captured 147 (3 juvenile, 54 female, 90 male) individual turtles 285 times between 5 April 2004 and 30 Oct 2007 (Table 1). Captures were male-biased, with males and females accounting for 67% and 33% of total captures, respectively. Juveniles were captured only 4 times and were excluded from most analyses. A total of 268 blood samples was collected and analyzed for blood chemistry. Of the 285 turtle captures, 182 were scanned for body composition using DXA (Table 2).

Blood Analyses—Results of least-squares regressions suggest that hematocrit had an effect on total protein (Figure 1D; n=135, R^2=0.39, F=83.92, P<0.001), weak effects on calcium (Figure 1A; n=135, R^2=0.06, F=9.47, P=0.003) and phosphorus (Figure 1C; n=135, R^2=0.04, F=5.42, P=0.021), and no effect on alkaline phosphatase (Figure 1E; R^2=0.01, n=135, F=1.79, P=0.183) and magnesium (Figure 1B; R^2=0.01, n=135, F=0.72, P=0.397). Hematocrit did not vary by sex (two-way ANOVA, $F_{1,119}$=0.15, P=0.695) or by month (Figure 2F, $F_{6,119}$=1.06, P=0.393). There was no interactive effect of month and sex on hematocrit ($F_{6,119}$=0.57, P=0.754).

Plasma calcium did not vary significantly by a sex-month interaction (two-way ANOVA; $F_{6,93}$=1.30, P=0.265). Plasma calcium did not vary between male and female turtles ($F_{1,125}$=2.20, P=0.141), but did vary by month (Figure 2A; $F_{6,93}$=4.74, P<0.001). Similarly, plasma magnesium did not vary by an interaction between month and sex (two-way ANOVA; $F_{6,125}$=0.52, P=0.794), nor did magnesium vary between sexes ($F_{1,125}$=0.44, P=0.506). Month influenced plasma magnesium ($F_{6,125}$=2.17, P=0.050). Plasma magnesium tended to decline from April to August, where it reached its lowest point, after which magnesium began to rise to its apex in October (Figure 2C). However, main-effect contrasts among months were not significant at α=0.05 perhaps due to the conservative nature of Tukey's test. Plasma magnesium was marginally different between October and August (t=2.779, P=0.088). Plasma inorganic phosphorus did not vary by a sex-month interaction (two-way ANOVA; $F_{6,125}$=0.29, P=0.941). Plasma phosphorus was different between the sexes, with female turtles having on average 1.16 mg/dL more than males ($F_{1,125}$=4.87, P=0.029). Phosphorus varied among months ($F_{6,125}$=4.57, P<0.001).

Phosphorus was lowest in April and rose significantly in May (t=3.004, P=0.049), where it remained relatively stable for the following months (Figure 2B).

The effect of hematocrit on plasma protein was significant, indicating it was an appropriate covariate ($F_{1,111}$=83.06, P<0.001). Additionally, non-significant covariate-sex ($F_{1,111}$=0.07, P=0.798) and covariate-month ($F_{6,111}$=1.09, P=0.371) interactions indicate the parallel-slopes model was appropriate. Total protein varied by a month-sex interaction (Figure 2D; $F_{6,111}$=2.22, P=0.046); however, post-hoc analysis suggests all simple-effect contrasts were not significantly different. Plasma protein did not differ between sexes ($F_{1,111}$=0.22, P=0.644) or among months ($F_{6,111}$=0.81, P=0.566). Alkaline phosphatase did not vary by a month-sex interaction (two-way ANOVA; $F_{6,124}$=1.67, P=0.134) or between the sexes ($F_{1,124}$=0.90, P=0.345). Alkaline phosphatase was significantly different across months (Figure 2E; $F_{6,124}$=8.98, P<0.001). For both sexes, alkaline phosphatase tended to gradually rise from its lowest point in April, and reached its apex in September, and then declined in October and November (Figure 2E).

Body Composition—Bone mineral content was positively related to straight carapace length for male (Figure 3A; $BMC=4.423*10^{-5}(SCL)^{2.878}$; R^2=0.92, P<0.001) and female turtles ($BMC=1.571*10^{-6}(SCL)^{3.504}$; R^2=0.88, P<0.001). Relative bone mass was also positively related to straight carapace length (Table 3). Inclusion of juveniles in the analysis strengthened this relationship (Table 3). The relationship between relative bone mass and straight carapace length followed a sigmoidal pattern. At approximately 150-mm straight carapace length, the increase in relative bone mass began to decline in rate for both of the sexes (Figure 3C). For the analysis of relative bone mass with ANCOVA, we found no significant sex-month interaction ($F_{6,88}$=1.31, P=0.261) or main effect of

month ($F_{6,88}$=1.22, P=0.306). Relative bone mass varied between the sexes ($F_{1,88}$=7.85, P=0.006) with males (0.1627±0.0234 g) having higher relative bone mass than females (0.1622±0.0193g). The effect of the covariate was significant ($F_{1,88}$=80.46, P<0.001). A non-significant interactive effect between covariate and independent variables month ($F_{6,81}$=0.59, P=0.738) and sex ($F_{1,81}$=1.14, P=0.288) suggest a parallel-slopes model was appropriate.

Bone mineral content was affected by an interaction between sex and month ($F_{6,88}$=2.64, P=0.021), although there was no main effect of sex ($F_{1,88}$=0.05, P=0.822) or month ($F_{6,88}$=1.49, P=1.91). Bone mineral content tended to remain relatively constant across months for males, whereas it varied more among females (Figure 4A); however, all simple-effect contrasts among levels within sex or within month were not significantly different (P>0.279). The effect of the covariate was significant ($F_{1,88}$=844.55, P<0.001). Non-significant covariate- month ($F_{6,81}$=0.14, P=0.991) and covariate-sex ($F_{1,81}$=0.04, P=0.841) interactions suggest a parallel-slopes model was appropriate.

Bone mineral density followed a pattern similar to that of bone mineral content (Figure 4B), although without an interactive effect of sex and month ($F_{6,88}$=1.68, P=0.135). There was no significant main effect of sex ($F_{1,88}$=0.07, P=0.791) or month ($F_{6,88}$=0.96, P=0.456). The effect of the covariate was significant ($F_{1,88}$=415.97, P<0.001). Non-significant covariate-month ($F_{6,81}$=0.14, P=0.991) and covariate-sex ($F_{1,81}$=0.04, P=0.841) interactions suggest a parallel-slopes model was appropriate.

Influence of Reproduction—Hematocrit did not differ between gravid and non-gravid females (two-sample t-test; t_{67}=-1.17, P=0.124). Plasma calcium was higher (t_{67}=2.19, P=0.032) in gravid females (7.53±4.67 mg/dL, n=19) than females lacking

eggs (5.25±3.52 mg/dL, n=50). Phosphorus was also higher (t_{68}=-3.40, P=0.001) for gravid females (3.29±1.49 mg/dL, n=20) than for non-gravid females (2.19±0.97 mg/dL, n=50). Gravid females were marginally higher in plasma protein than non-gravid females (t_{68}=-1.79, P=0.078), but were not significantly different than non-gravid females for plasma magnesium (t_{68}=-0.65, P=0.519), plasma alkaline phosphatase (t_{68}=0.84, P=0.405), relative bone mass (t_{41}=0.13, P=0.901), bone mineral content (t_{42}=-1.07, P=0.291), and bone mineral density (t_{42}=-1.32, P=0.193).

For comparison between females with and without large follicles, we found no significant difference among females for hematocrit (two-sample t-test; t_{57}=1.40, P=0.917), calcium (t_{56}=0.88, P=0.809), phosphorus (t_{57}=0.52, P=0.698), total protein (t_{57}=0.09, P=0.538), alkaline phosphatase (t_{38}=2.89, P=0.997), and relative bone mass (t_{34}=0.21, P=0.584). Plasma magnesium was marginally different between females (t_{57}=-1.57, P=0.061), with females possessing large follicles having marginally higher magnesium concentrations (1.55±1.11 mEq/L) than those without (1.06±1.30 mEq/L). Bone mineral density was also marginally different between females (t_{35}=-1.55, P=0.065), with females possessing large follicles (0.3215±0.031 g/cm^2) having marginally higher bone mineral density than those without (0.2998±0.054 g/cm^2). Bone mineral content varied between female groups (t_{35}=-1.88, P=0.034), with females possessing large follicles (44.8±10.6 g) having higher bone mineral content than those without (36.9±14.6 g).

For comparison between females with and without small follicles, we found no significant difference among females for hematocrit (2-sample t-test; t_{47}=-0.02, P=0.492), calcium (t_{47}=2.09, P=0.979), phosphorus (t_{48}=2.58, P=0.994), total protein (t_{48}=-0.16,

P=0.438), alkaline phosphatase (t_{47}=1.76, P=0.957), relative bone mass (t_{30}=1.50, P=0.928), bone mineral content (t_{31}=-0.88, P=0.192), and bone mineral density (t_{31}=-0.81, P=0.211). Plasma magnesium was marginally different between females (t_{48}=-1.39, P=0.086), with females possessing small follicles (n=20, 1.61±1.16 mEq/L) having marginally higher magnesium than females without (n=30, 1.16±1.11 mEq/L).

Discussion

We predicted that plasma calcium, magnesium, phosphorus, and total protein would rise and that alkaline phosphatase would decline during egg production in female tortoises. We also predicted that no such changes should be evident in males if such changes were a result of egg production. The findings of this study do not support our predictions. Although we observed seasonal increases in calcium and phosphorus, these seasonal changes cannot be explained solely by egg production because such changes were found in both sexes. We suggest the temporal changes in these nutrients are likely related to increased activity levels, including dietary intake, during warmer months. Also contrary to our predictions, egg production did not appear to adversely influence maternal bone density in this population. We did not observe any significant changes in bone density during egg production, perhaps due to a reduced demand for calcium by producing eggs containing relatively less calcium than some other species of turtle.

Blood Biochemistry—Blood biochemicals were generally either within normal ranges or tended to be slightly low compared to other studies of turtles (for review, see Chapter 2, Table 2). Hematocrit tended to be lower that that of other studies, potentially indicating lymphatic dilution of blood sample; however, the majority of these studies

were examining turtles in the families Cheloniidae and Testudinidae. A study of the emydid turtle *Pseudemys rubriventris* (Innis et al. 2007) measured hematocrit values that were similar to our study (19% vs. 16% in our study). Plasma calcium was generally lower than of most other turtles studied. We believe this is due, in part, to the contamination of blood with lymph, because we found that hematocrit significantly influenced plasma calcium. Because only a portion of circulating calcium is protein-bound, this influence is reduced relative to other parameters such as total protein, which is largely restricted from entering tissue fluid through capillary membranes. A previous hematological study of red-eared sliders examined the effects of lymph dilution on blood biochemistry. They found no significant difference between whole blood and blood mixed with lymph for concentrations of calcium, phosphorus, and alkaline phosphatase; however, total protein was significantly lower in blood-lymph mixtures (Crawshaw and Holz 1996). Although we believe that hemodilution might result in underestimates of some blood parameters, we believe this effect to be small, with the exception of total protein, and do not believe this to influence treatment effects (see discussion in Chapter 2). The availability of magnesium data for turtles is relatively limited; however, our values were on the lower end of the published range. Inorganic phosphorus values that we obtained in this study were within ranges obtained in previous studies. Blood values for alkaline phosphatase were well within normal ranges for other species of turtles. Total protein, despite the possibility of being influenced by hemodilution, was within normal ranges.

The seasonal patterns of blood biochemicals exhibited by *T. scripta* differed from that of other turtles. For plasma calcium and phosphorus, we observed a seasonal rise that

is loosely associated with the reproductive period. Female *T. scripta* lay eggs primarily from mid-April to mid-July, with a peak in late May to early June (Gibbons and Greene 1990). Our study observed similar trends in reproductive timing, as we captured gravid females between 9 April and 13 July. The peak number of gravid females occurred during June. Although the rise in calcium and phosphorus during egg production itself is similar to previous studies of other turtles, the fact that we found this pattern in both sexes is not. Female *Gopherus berlandieri* exhibited a seasonal rise in plasma calcium and phosphorus that is likely associated with vitellogenesis, but no seasonal rise was observed in male tortoises (Stone unpublished data). Hypercalcemia during reproduction in females is a common phenomenon and has been documented in birds (Riddle and Reinhart 1926), fish (Hess et al. 1928), amphibians (Zwarenstein and Shapiro 1933), and non-avian reptiles (Dessauer and Fox 1959; Clark 1967). This seasonal hypercalcemia has been attributed to an estrogen-dependent rise in plasma proteins, primarily vitellogenins, and subsequently an increase in total calcium due to increases in protein-bound fraction (Schjeide 1985). Ionized calcium concentrations typically remain stable; changes associated with reproduction have not been documented nor has estrogen manipulation influenced ionized calcium levels (Simkiss 1961a; Feinblatt 1982; Schjeide 1985; Wysolmerski 2002). The rise in total calcium as a result of increased vitellogenins is a process that allows calcium to be transferred to and stored in the yolk for use by the developing embryo (Wysolmerski 2002).

The monthly changes that we observed for most blood chemicals followed a similar pattern for both sexes, and suggest a pattern driven by non-reproductive processes. We suggest that increased dietary intake of nutrients during the active season

is responsible for elevated levels of calcium and phosphorus in *T. scripta*. A seasonal rise in plasma phosphorus has been documented during the activity season for both sexes of *Vipera aspis* (Alcobendas et al. 1992) and suggests a pattern driven, at least partially, by processes other than bone resorption for egg production. Increased calcium might be a function of seasonally elevated vitamin D_3 production. Cutaneous vitamin D_3 production has both seasonal and daily cycles and is influenced by photoperiod (Holick 2003). Conversely, *Gopherus berlandieri* exhibited the predicted and the typical sexually dimorphic pattern of significant monthly changes in calcium and phosphorus (Stone unpublished data). Despite that we detected a similar seasonal pattern in blood biochemistry for both male and female turtles, higher concentrations of calcium, phosphorus, and total protein in gravid females than nongravid females suggests an influence of egg production on plasma chemistry for *T. scripta*.

Circulating levels of alkaline phosphatase suggested a pattern that differs from our original prediction of a seasonal decline in bone formation during eggshell deposition. Alkaline phosphatase increased during the reproductive season where calcium demands of reproduction are most likely to be highest. A possible interpretation of these results is that the demands of calcium required for egg production did not require resorption of bone, and calcium availability from dietary sources was sufficient to allow for increased bone growth in summer months. Growth in body size of both male and female *T. scripta* occurs throughout the activity season (Stone unpublished data). Thus normal body growth likely accounts for increased levels of alkaline phosphatase activity during our collection period. Interestingly, although not statistically different, male

alkaline phosphatase activity increased at a greater rate during the summer months while females increased less so.

Bone Density—Our data suggest that for this population of *T. scripta* there is no seasonal effect of bone density in either sex. This finding is contrary to that seen in *Sternotherus odoratus*, where female bone density was reduced in correspondence with the approximate timing of eggshell production (Edgren 1960). The reason for this disparity is unknown, but could be accounted for by a variety of factors. We measured bone content and density with a non-destructive technique. Although DXA is generally considered a relatively precise estimator of bone mineral content, it is possible that intra-individual variation is too high to detect small changes in bone density. We cannot rule out that our observed lack of statistical significance is a result of low statistical power as opposed to no effect being present. However, *T. scripta* is among the number of species that produce a flexible-shelled egg, whereas *S. odoratus* produces eggs that possess rigid eggshells (Congdon and Gibbons 1990). The calcium content of rigid-shelled eggs might impose considerably higher calcium demands than that of flexible-shelled eggs. The inorganic content of the eggshell, expressed as a proportion of total dried egg mass, is greater in turtles producing rigid eggshells compared to those producing flexible eggshells (Lamb and Congdon 1985). Thus, the discrepancies between *T. scripta* and *S. odoratus*, for the seasonal pattern of bone density, might be a result of differing calcium demands and the manifestation of those demands on maternal bone density, rather than a lack of statistical power or instrumental precision in this study. It has been determined previously that the degree of eggshell calcification can influence maternal ionized

calcium concentration of birds, lending further support for this interpretation (Ozpinar 1997).

Other studies have contradicted our findings. Suzuki (1963) reported thinner cortical bone and increased porosity of cancellous bone of femurs from female *T. scripta* with oviducal eggs compared to immature females and males. That study suggested a possible effect of eggshell formation on bone mineral content; however, those findings were primarily qualitative. A previous study of *Chrysemys picta* lends some support to our observations. Clark (1965) found no significant differences in the calcium content of fibulas from females containing well-developed follicles (late vitellogenesis during May) and females just after the breeding season (July). These results suggest that eggshell formation alone had either no effect on skeletal calcium of the fibula, or that by the point of the completion of the egg-laying cycle bone reserves have returned to those prior to eggshell production. We suggest the former interpretation is the most plausible explanation. We postulate this because, like *T. scripta*, *C. picta* lays flexible-shelled eggs.

Another potential interpretation of why we were not able to detect changes in bone density is methodological. The DXA method we used to calculate bone density determines average concentration of bone mineral across all bone. Therefore, we might not be able to detect subtle bone contributions if such contributions are not distributed evenly among all bones of the body. We know that change in bone density varies among bones within individuals (de Buffrenil and Francillon-Vieillot 2001). Although DXA possesses the capability to measure regional bone density (i.e., specific bones), the use of this tool is challenged by the unique morphology of turtles. The use of regional analysis was not possible in our study because individuals almost always had their limbs retracted

within the shell and therefore, bone mineral content of the appendages could not be separated from that of the carapace and plastron.

Influence of Age on Bone Density— Age is positively related to straight carapace length and straight plastron length in turtles (Gibbons et al. 1981), although we were unable to make more direct estimates of age through the counting of annuli. We detected a sigmoidal pattern between relative bone mass and straight carapace length, which is similar to the pattern detected in the painted turtle, *Chrysemys picta* (Iverson 1982). This pattern indicates that the rate of bone production, although slow in early development, begins to increase in rate at an approximate straight carapace length of 50-80 mm. At about 150-180 mm straight carapace length, the rate of relative bone mass growth has declined. This decrease in bone production, which is observed in male and female turtles, corresponds with the size at which females attain sexual maturity. Male *T. scripta* mature at a straight plastron length of 90-100 mm (≈99-110 mm SCL), whereas females mature at 150-195 mm (≈164-213 mm SCL; Cagle 1944). This pattern is contrary to findings of Iverson (1982), where relative bone mass in *C. picta* leveled off at the size where males attained sexual maturity. Additionally, he found that male *C. picta* exhibited less variation in relative bone mass than females, and suggested this might be a function of the physiological demands of calcium for egg production. We found that males and females had similar variation in relative bone mass (Levene's Test P=0.176), bone mineral content (P=0.302), and bone mineral density (P=0.617). Therefore, it is likely that the calcium requirement for reproduction does not result in increased bone density variation in this population of *T. scripta*.

Overall, the findings of our study suggest that bone density and calcium reserves of female *T. scripta* are not adversely affected by egg production on both short-term (seasonal) or long-term scales (lifetime). We suggest that the production of a pliable eggshell could account for our results, which contrast with findings from studies of other species of turtles that suggested significant loss of bone as a result of egg production. Although we documented seasonal rises in plasma calcium, phosphorus, and alkaline phosphatase, these changes are unlikely related to egg production because such observations were observed in both sexes. We suggest this rise in plasma biochemicals is a result of increased activity, primarily increased dietary intake, during warmer months.

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Table 1. Descriptive statistics of *T. scripta* morphometrics from Payne Co., OK. These values include repeated measures of individuals over a 3.5 year period (April 2004-October 2007).

Metric	Sex	N	Mean	SD	Range
Mass (g)	Female	89	1175.4	418.8	286-2400
	Male	167	873.8	330.2	240-1620
	Combined	256	978.7	390.1	
SCL (mm)	Female	85	199.1	28.5	124.9-249.7
	Male	158	186.8	27.0	119.3-252.5
	Combined	243	191.1	28.1	
SPL (mm)	Female	85	185.1	25.9	116.9-231.0
	Male	159	170.3	24.2	94.5-206.4
	Combined	244	175.5	25.8	
GW (mm)	Female	85	156.0	19.1	102.7-190.0
	Male	157	145.6	18.4	94.8-175.6
	Combined	242	149.3	19.3	
CPA (mm)	Female	85	23.2	4.0	13.7-31.0
	Male	157	23.0	3.0	13.0-29.9
	Combined	241	23.1	3.4	
PTT (mm)	Female	85	45.4	7.0	23.4-59.2
	Male	156	61.0	8.7	22.5-81.4
	Combined	241	55.5	11.0	
PTC (mm)	Female	85	14.1	4.2	4.6-21.9
	Male	156	29.2	5.2	15.6-45.0
	Combined	241	23.8	8.7	
LMC (mm)	Female	84	10.6	1.9	6.7-18.9
	Male	157	15.5	2.3	9.1-22.3
	Combined	241	13.8	3.2	

Abbreviations: SCL=straight carapace length, SPL=straight plastron length, GW=greatest width, CPA=carapace plastron aperture, PTT=plastron to tail tip, PTC=plastron to cloaca, LMC=left middle claw

Table 2. Descriptive statistics of *T. scripta* blood chemistry and DXA analysis from turtles collected in Payne County, OK. These values include repeated measures of individuals over a 3.5 year period (April 2004-October 2007).

Metric	Sex	N	Mean	SD	Range
Ht	Female	85	15.3	7.8	1-34
	Male	167	16.6	8.0	0-34
	Combined	255	16.2	7.9	
Ca (mg/dL)	Female	86	5.9	3.9	0.8-14.9
	Male	177	5.2	3.1	0.2-11.4
	Combined	266	5.5	3.4	
Mg (mEq/L)	Female	87	1.6	1.2	0.0-4.7
	Male	177	1.7	1.1	0.0-4.6
	Combined	267	1.7	1.1	
Phos (mg/dL)	Female	87	2.1	1.2	0.8-6.5
	Male	177	2.3	0.7	0.3-3.9
	Combined	267	2.3	0.9	
TP (g/dL)	Female	87	2.8	1.1	0.3-5.6
	Male	177	3.1	1.1	0.6-6.8
	Combined	267	3.0	1.1	
ALP (U/L)	Female	87	55.84	47.71	8.71-307.09
	Male	177	60.54	40.50	0.00-238.96
	Combined	267	59.79	44.30	
Area (cm^2)	Female	61	125.79	32.07	50.06-191.79
	Male	121	107.33	31.0	41.11-165.48
	Combined	182	113.52	32.54	
Corrected BMC (g)	Female	61	184.41	71.73	35.25-358.80
	Male	121	142.77	66.10	19.18-309.04
	Combined	182	156.73	70.64	
Corrected BMD (g/cm^2)	Female	61	1.400	0.276	0.698-1.871
	Male	121	1.255	0.283	0.464-1.890
	Combined	182	1.303	0.288	
FM (g)	Female	22	92.6	215.2	0.0-1007.2
	Male	30	52.2	72.0	0.0-329.9
	Combined	52	69.3	149.8	
$Mass_{DXA}$ (g)	Female	22	958	478	290-1764
	Male	30	777	313	236-1523
	Combined	52	854	398	

Abbreviations: Ht = hematocrit, Ca=calcium, Mg=magnesium, Phos=phosphorus, TP=total protein, ALP=alkaline phosphatase, BMC=bone mineral content, BMD=bone mineral density, FM=fat mass.

Table 3. Results of curvilinear regression analysis of relative bone mass against straight carapace length in male and female *T. scripta* collected in Payne County, OK. Results are presented both with and without the inclusion of juveniles.

Sex	n	Coefficient	Estimate	SE	t	P	R^2
Male	68	a	0.2313	0.0801	2.89	0.005	0.48
		b	76.6086	58.5033	1.31	0.195	
		x_o	112.4345	43.3265	2.60	0.012	
Male w/ Juvenile	70	a	0.1811	0.0068	26.81	<0.001	0.64
		b	27.7475	6.3245	4.39	<0.001	
		x_o	111.5277	4.6637	23.91	<0.001	
Female	35	a	0.1800	0.0180	9.99	<0.001	0.42
		b	48.2568	31.0731	1.55	0.130	
		x_o	89.8920	21.1311	4.25	<0.001	
Female w/ Juvenile	37	a	0.1690	0.0045	37.18	<0.001	0.76
		b	23.7486	5.6345	4.21	<0.001	
		x_o	112.9443	5.0321	22.44	<0.001	

Figure Legends

Figure 1. Plasma biochemicals calcium (A), magnesium (B), phosphorus (C), total protein (D), and alkaline phosphorus (E) regressed on hematocrit for *T. scripta* collected in Payne County, OK (n=135).

Figure 2. Monthly changes in plasma calcium (A), phosphorus (B), magnesium (C), total protein (D), alkaline phosphatase (E), and hematocrit (F) for male (closed triangles) and female (open circles) *T. scripta* collected in Payne County, OK. Values are given as mean ±1SD. Different letters indicate significant differences among months (sex data pooled).

Figure 3. Relationships between bone mineral content and SCL (A), and between relative bone mass and SCL (B), for male (open circles) and female (closed circles) *Trachemys scripta* collected in Payne County, OK. Lines shown for male (dashed) and female (solid) turtles are fitted to a power and sigmoidal function for the BMC to SCL and the relative bone mass to SCL relationships, respectively. Plot C represents inclusion of juvenile turtles in the relationship between relative bone mass and SCL for both sexes.

Figure 4. Monthly changes in corrected bone mineral content (A) and corrected bone mineral density (B) for male (open circles) and female (closed circles) *T. scripta*. Values are given as least square means ± 1SE.

Figure 1

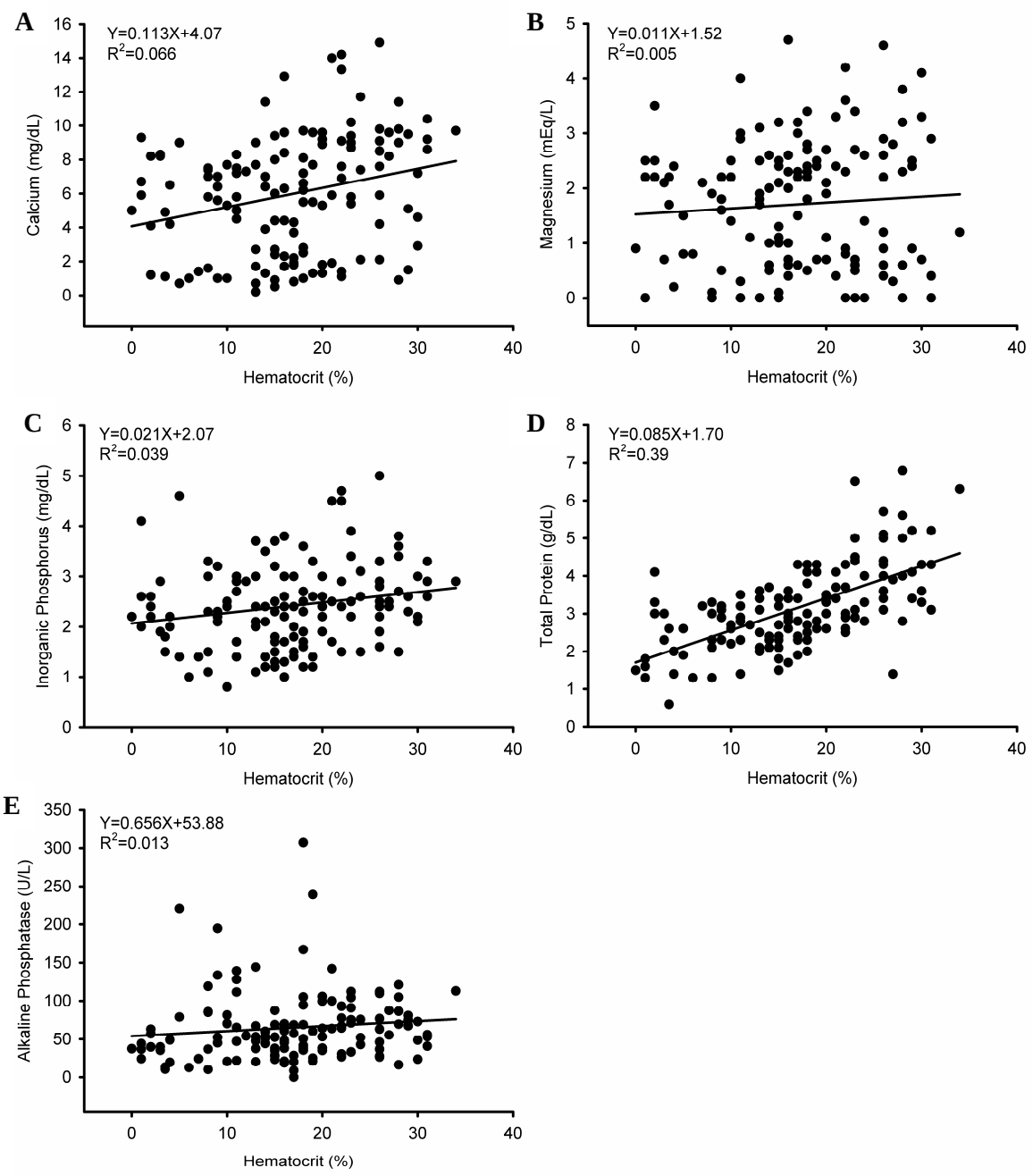

A
Y=0.113X+4.07
R2=0.066
Calcium (mg/dL)
Hematocrit (%)
B
Y=0.011X+1.52
R2=0.005
Magnesium (mEq/L)
Hematocrit (%)
C
Y=0.021X+2.07
R2=0.039
Inorganic Phosphorus (mg/dL)
Hematocrit (%)
D
Y=0.085X+1.70
R2=0.39
Total Protein (g/dL)
Hematocrit (%)
E
Y=0.656X+53.88
R2=0.013
Alkaline Phosphatase (U/L)
Hematocrit (%)

Figure 2

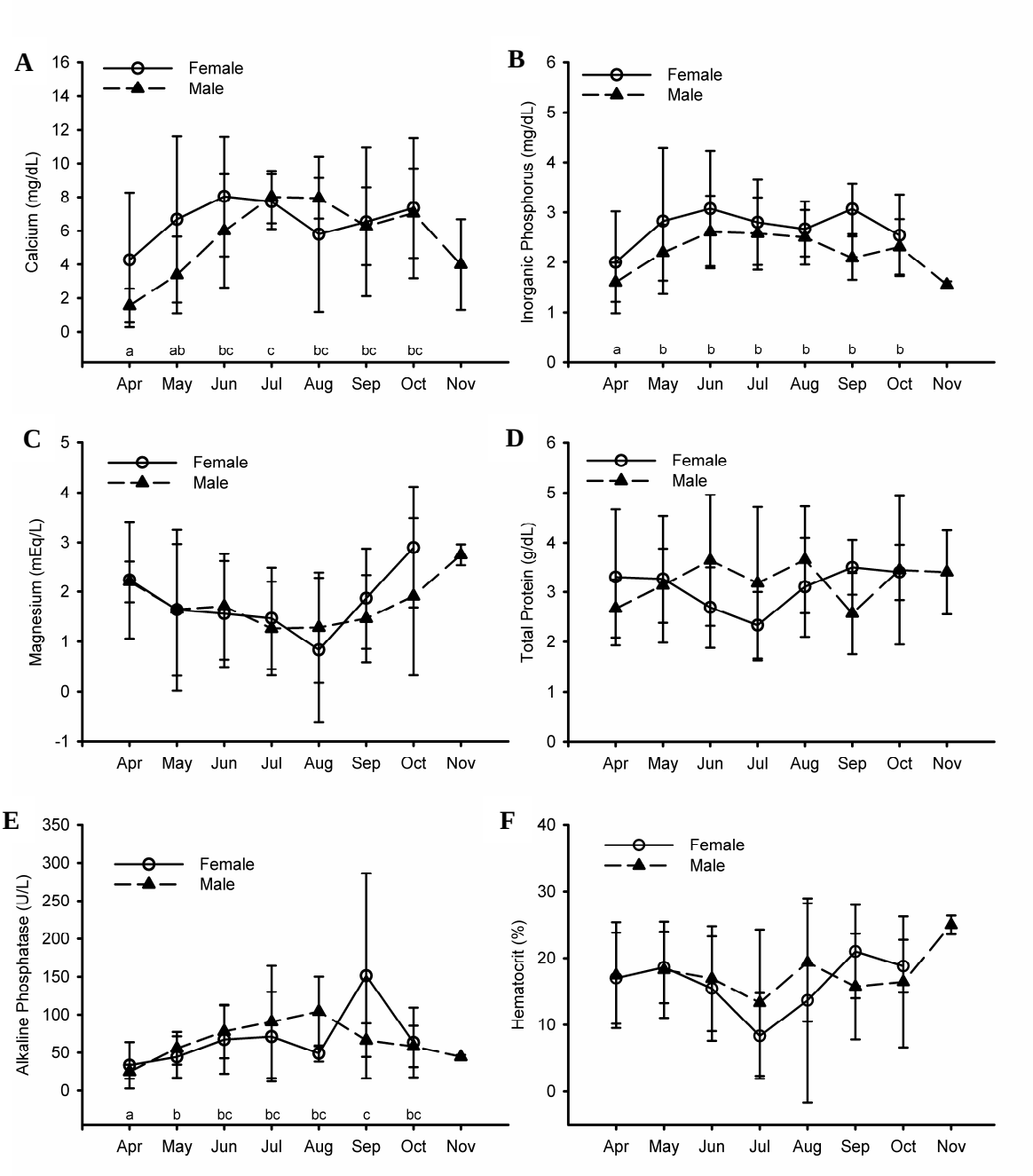

A
Female
Male
Calcium (mg/dL)
a ab bc c bc bc bc
Apr May Jun Jul Aug Sep Oct Nov
B
Female
Male
Inorganic Phosphorus (mg/dL)
a b b b b b b
Apr May Jun Jul Aug Sep Oct Nov
C
Female
Male
Magnesium (mEq/L)
Apr May Jun Jul Aug Sep Oct Nov
D
Female
Male
Total Protein (g/dL)
Apr May Jun Jul Aug Sep Oct Nov
E
Female
Male
Alkaline Phosphatase (U/L)
a b bc bc bc c bc
Apr May Jun Jul Aug Sep Oct Nov
F
Female
Male
Hematocrit (%)
Apr May Jun Jul Aug Sep Oct Nov

Figure 3

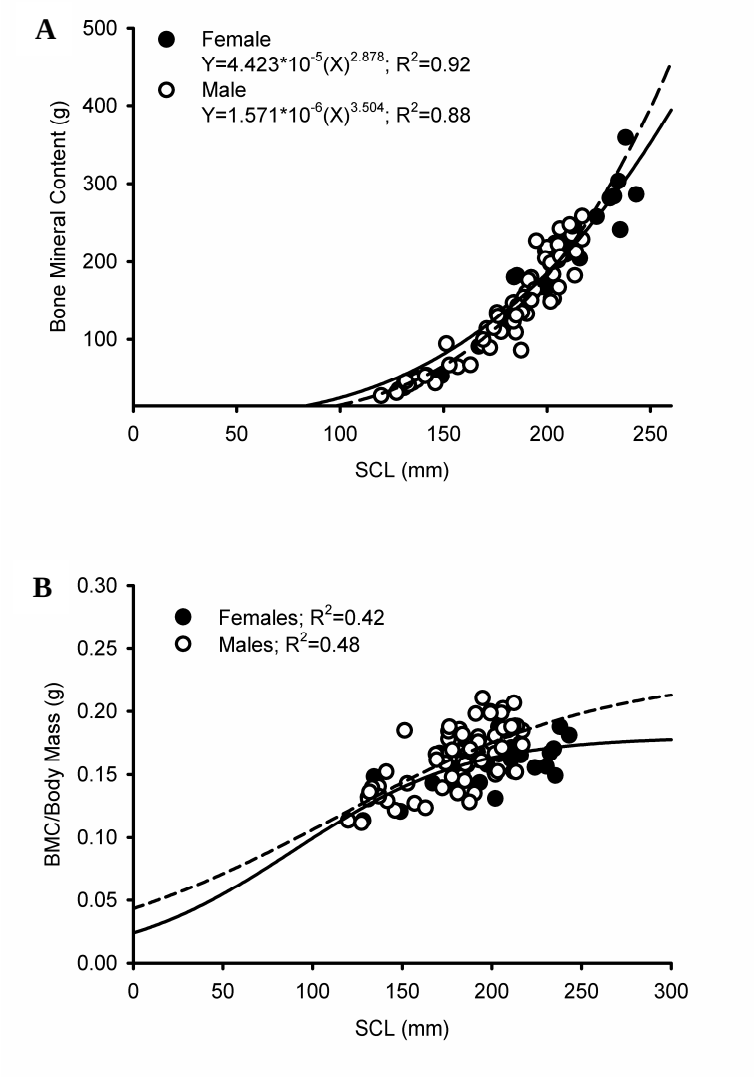
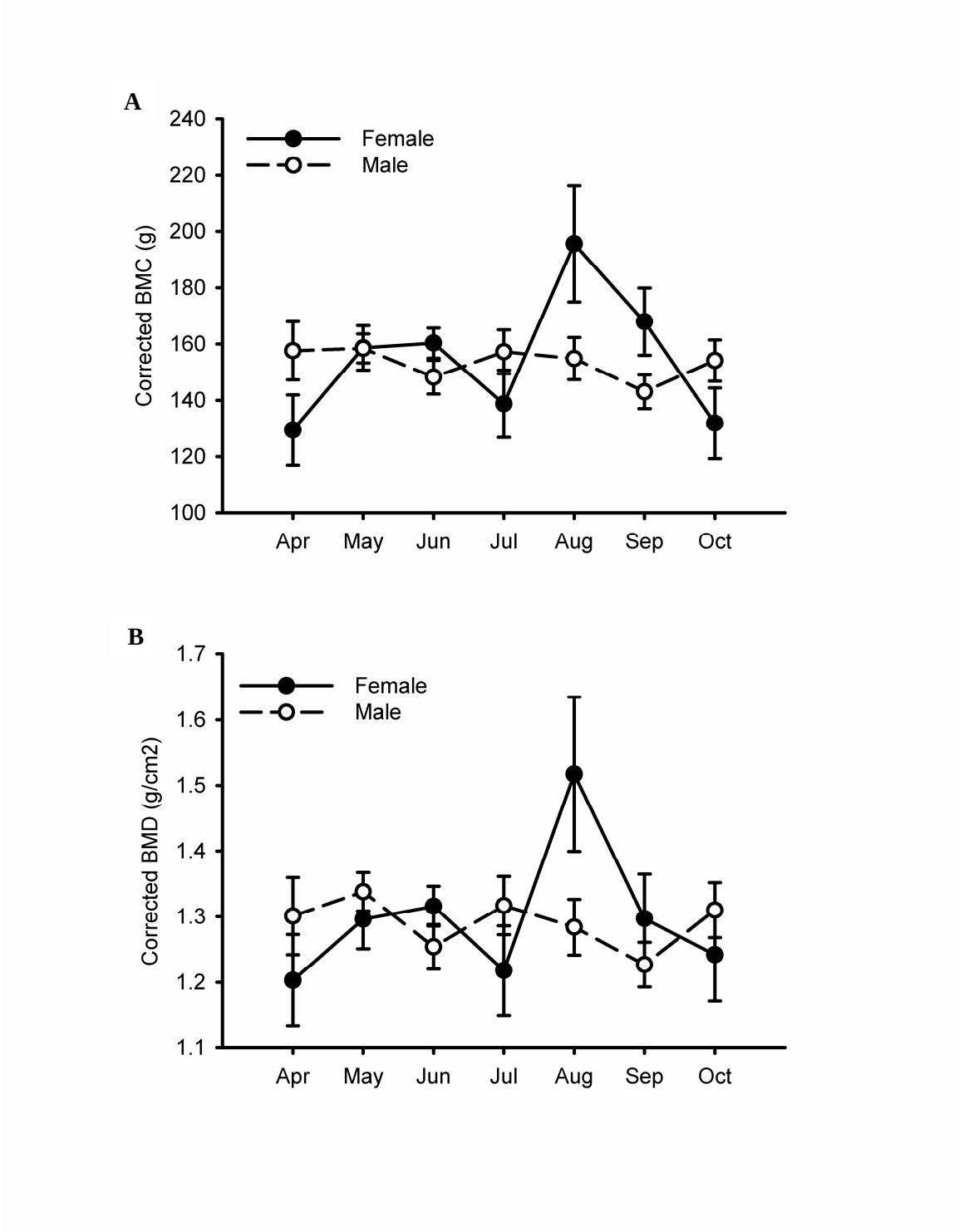

A
Female
Y=4.423*10-5(X)2.878; R2=0.92
Male
Y=1.571*10-6(X)3.504; R2=0.88
Bone Mineral Content (g)
SCL (mm)
B
Females; R2=0.42
Males; R2=0.48
BMC/Body Mass (g)
SCL (mm)
C
Female; R2=0.76
Male; R2=0.64
Juvenile
BMC/Body Mass (g)
SCL (mm)

Figure 4


A
240
220
200
180
160
140
120
100
Corrected BMC (g)
Female
Male
Apr
May
Jun
Jul
Aug
Sep
Oct
B
1.7
1.6
1.5
1.4
1.3
1.2
1.1
Corrected BMD (g/cm2)
Female
Male
Apr
May
Jun
Jul
Aug
Sep
Oct

VITA

Matthew David Stone

Candidate for the Degree of

Doctor of Philosophy

Dissertation: EFFECTS OF SEASON, SEX, AND AGE ON THE CALCIUM PHYSIOLOGY AND BONE DYNAMICS OF TURTLES

Major Field: Zoology

Biographical:

Personal Data: Born in Columbia, Maryland, on February 18, 1978, the son of Robert and Karen Stone

Education: Graduated from Centennial High School, Ellicott City, Maryland, in May 1996; received Bachelor of Science degree in Biology from Eckerd College, St. Petersburg, Florida, in May 2000; received Master of Science degree in Biology from Southwest Missouri State University (now Missouri State University), Springfield, Missouri, in December 2002. Completed the requirements for the Doctor of Philosophy degree in Zoology at Oklahoma State University, Stillwater, Oklahoma, in May 2009.

Experience: Teaching assistant at Missouri State University for General Biology and Comparative Vertebrate Anatomy. Employed by Southwest Baptist University as adjunct faculty for Anatomy and Physiology. Employed as an instructor for Anatomy & Physiology and Nutrition at Ozarks Technical Community College. Teaching assistant at Oklahoma State University for General Biology, Ornithology, General Physiology, and Mammalian Physiology. Employed by OSU as an instructor for Mammalian Physiology and General Physiology

Professional Memberships: Zoology Graduate Student Society, Southwestern Association of Naturalists, Society of Integrative and Comparative Biology, Oklahoma Academy of Science.

Name: Matthew David Stone Date of Degree: May, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECTS OF SEASON, SEX, AND AGE ON THE CALCIUM PHYSIOLOGY AND BONE DYNAMICS OF TURTLES

Pages in Study: 105 Candidate for the Degree of Doctor of Philosophy

Major Field: Zoology

Scope and Method of Study: This study investigated the potential tradeoffs between maternal bone density and the investment of calcium into offspring in the Texas tortoise (*Gopherus berlandieri*) and red-eared slider (*Trachemys scripta*). Specifically, this study examined the effects of season, sex, and age on bone density and plasma biochemicals associated with calcium physiology. Additionally, this study investigated the efficacy of dual-energy x-ray absorptiometry as a tool in the prediction of body composition in turtles.

Findings and Conclusions: Findings from this study suggest seasonal changes in calcium physiology that are associated with reproduction in female *G. berlandieri*. These physiological changes might have important implications for the maintenance of maternal bone density in this species. In the case of *T. scripta*, I did not document any reproductive influences on calcium physiology or bone density due solely to the production of the egg. This disparity is likely due to differing maternal investment of calcium between *G. berlandieri* and *T. scripta*. Additionally, I found that dual-energy x-ray absorptiometry is an effective predictor of bone mass in turtles.

ADVISER'S APPROVAL: Dr. Matt Lovern