Herpetological Conservation and Biology 12(2):548–555.
Submitted: 11 July 2015; Accepted: 21 February 2017; Published: 31 August 2017.

Differences in Fluctuating Asymmetry Among Four Populations of Gopher Tortoises (*Gopherus polyphemus*)

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Abstract.—**Fluctuating asymmetry (FA) has been used to measure population fitness as a result of genetic and environmental processes. We compared FA in carapace shape, using a novel geometric morphometric method, among four populations of Gopher Tortoises (*Gopherus polyphemus*), a long-lived species of conservation concern endemic to the southeastern U.S. Coastal Plain. Populations used in this study included: 1) the Wade Tract, an old-growth Longleaf Pine (*Pinus palustris*) forest located in southwestern Georgia, USA; 2) Gopher Island, located within the Chattahoochee River basin at the Alabama-Georgia border, USA; 3) Conecuh National Forest, located in southcentral Alabama, USA; and 4) a mitigation-recipient site in southeastern Mississippi, USA. Based on prior studies of the genetic structure of *G. polyphemus* populations, we predicted that the Mississippi population would have the highest FA, while the Gopher Island population would have the lowest FA. We found significant differences in FA among populations of Gopher Tortoises ($H = 11.15$, df = 3, $P = 0.011$), with the highest FA in the tortoises from Mississippi and the lowest FA in tortoises from Conecuh National Forest. We did not find a significant difference in FA between sexes ($F_{2,111} = 0.389$, $P = 0.679$). Regression analyses indicated that across populations, size (as measured by straight-line carapace length) did not influence FA, although there was a significantly negative effect of size on FA within the Gopher Island population ($t = -2.039$, df = 33, $P = 0.050$). These results highlight the need for continued study of Gopher Tortoises and FA, especially in Mississippi, where fitness may be reduced.**

Key Words.—endangered species; geometric; morphometric; population fitness; turtle

Introduction

Fluctuating asymmetry (FA) is defined as the deviation of the symmetry of an individual from perfect bilateral symmetry and is considered a negative indicator of the ability to resist random and small developmental accidents (Van Valen 1962). These developmental accidents are generally the result of genetic or environmental stress (Graham et al. 2010). It has thus been hypothesized that relative differences in the mean FA between populations indicate fitness differences among them, which includes survival and reproduction (Graham et al. 2010). In general, FA is quantified in structural traits of an organism that are theoretically bilaterally symmetric but possess minor deviations from perfect symmetry. Thus, mechanisms that increase FA among these structural traits are the results of genetic processes and/or direct environmental effects. Genetic causes of increased FA are driven by detrimental allelic combinations that may render individuals more vulnerable to environmental perturbations (Palmer and Strobeck 1986), while direct environmental effects on FA are due to higher incidence of environmental perturbations (such as contaminants) that weaken developmental stability (Wright and Zamudio 2002; Lazic et al. 2013). Populations with low heterozygosities or allelic richness are predicted to have higher FA than genetically diverse populations (Palmer and Strobeck 1986).

Comparisons of FA among populations remain controversial, as differences remain between the degree and direction of predicted and observed responses (Leung et al. 2000). Differences between predictions and observations of FA among populations may be rooted in the specific trait studied (Dongen 2006). Ideally studies of FA should use combinations of traits, rather than single traits, when inferring fitness using FA scores (Dongen 2006). Also, traits under direct selection (such as anti-predator traits) are less likely to show meaningful differences in FA than traits that do not confer a direct fitness advantage (such as color pattern asymmetries; Lazic et al. 2013).

The few available studies of FA in tortoises have generated inconsistent results. Buica and Cogalniceanu (2013) found no differences in FA among populations (including between sexes or age classes) of Eurasian

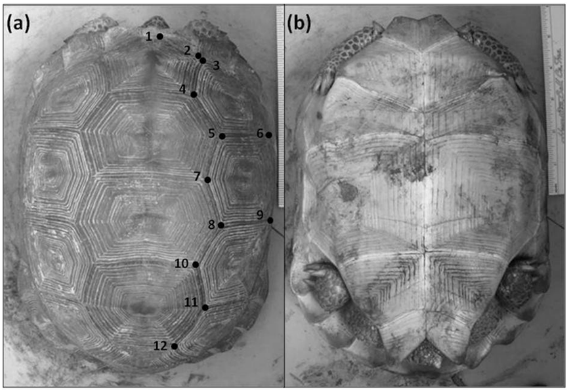

Figure 1. Sample digital photographs of a Gopher Tortoise (*Gopherus polyphemus*) carapace (a) and plastron (b) used in this study to measure fluctuating asymmetry. Because of tilting, plastron photographs were not used in this analysis. The twelve carapace landmarks used are indicated on this individual, and are numbered 1–12. The precise locations of landmarks used in this analysis are the following: (1) the posterior junction of the nuchal and nearest right hand marginal scute; (2) the posterior junction of the most anterior right hand marginal and second most anterior marginal scute; (3) the most anterior point on the right anterior-most pleural scute; (4) the most proximal point of the anterior-most pleural scute; (5) the proximal junction of the first two anterior pleural scutes; (6) the distal junction of the first two anterior pleural scutes; (7) the most proximal point on the second anterior-most pleural scute; (8) the proximal junction of the second and third anterior-most pleural scutes; (9) the distal junction of the second and third anterior-most pleural scutes; (10) the most proximal point on the third anterior-most pleural scute; (11) the proximal junction between the two posterior-most pleural scutes; (12) the most proximal point of the most posterior pleural scute. (Photographed by Jeffrey Goessling)

Spur-thighed Tortoises (*Testudo graeca*). However, Băncilă et al. (2012) found that in *T. graeca*, FA varied as predicted between populations and sexes. Specifically, Băncilă et al. (2012) found that males had greater plastral FA than females, and that this effect was independent of carapace length. While these studies present conflicting results, Buica and Cogalniceanu (2013) conclude that all of the populations in their study might represent high-fitness populations, where population-level differences in FA may not be expected.

Past studies underscore the value of using structural traits (such as the turtle shell) of long-lived vertebrates for evaluating FA. Turtle shells contain suitable biological landmarks for measuring FA. In addition, a turtle shell represents the genotype by environment interactions of an individual throughout its life, and the shell itself is susceptible to both minor and major environmental perturbations (Băncilă et al. 2012). While the shell as an overall structure is under selection, specific landmarks on the shell (such as the location of scute sutures), may be under less direct selection, therefore bilateral differences in location of these points could serve as landmarks for comparing FA in turtles.

Gopher Tortoises (*Gopherus polyphemus*) are a long-lived specialist of open-canopy forests of the southeastern U.S. Coastal Plain. Across their distribution, Gopher Tortoises have suffered severe population declines due to a range of factors that include direct mortality from human consumption or road mortality, as well as significant loss of suitable habitat (Auffenberg and Franz 1982). Moreover, the U.S. Fish and Wildlife Service (USFWS; 2011) concluded that Gopher Tortoises throughout their range warranted federal protection but such protection was precluded. Demographic models suggest that many populations of Gopher Tortoises will continue to decline (Bruggeman and Jones 2014). Currently, Gopher Tortoises are federally listed as threatened in their range west of the Mobile and Tombigbee Rivers in Alabama and provided additional state protection in the remainder of the range of the species (USFWS 1987).

We explored whether there were differences in FA among four populations of Gopher Tortoises. In addition, we compared these FA scores to published genetic data on this species that indicated relatively low genetic diversity in the western populations of Gopher Tortoises (Clostio et al. 2012), including those from Mississippi (Ennen et al. 2010) and relatively high genetic diversity in the Gopher Island population of tortoises (Ennen et al. 2011). We predict that if carapace FA is a negative indicator of population fitness in Gopher Tortoises specifically due to genetic mechanisms, the population from Mississippi would have the highest FA score. Additionally, if genetic processes account for population-level differences in FA, we predict that the Gopher Island population would have the lowest FA. Alternatively, if FA is mainly affected by environmental factors, we predict that the Wade Tract tortoises would have the lowest FA, as this site has largely been unaffected by anthropogenic factors and is in high-quality habitat (Guyer et al. 2013).

Materials and Methods

We collected digital photographs from tortoises in four populations across three states: Conecuh National Forest (Alabama, USA), Gopher Island (Georgia, USA), Wade Tract (Georgia, USA), and a mitigation bank recipient site (Mississippi, USA). Because we collected digital pictures in the field, we standardized the method by which images were obtained. We held the camera approximately 1 m above each tortoise, with the camera focused on the medial aspect of the tortoise (Fig. 1). We collected multiple digital photographs of the plastron and carapace of each tortoise. We took digital photographs without using the zoom to prevent distortion and reduce parallax errors. Images were downloaded to a computer, and we subsequently selected the best of the replicated photographs of the plastron and carapace for analyses. We identified individual tortoises to sex based on the presence of male secondary sex characteristics, including enlarged gular projections and a pronounced plastron concavity (Tuberville et al. 2014). We did not determine sex of individuals if secondary sex characteristics were not clear, or if an individual was too immature to demonstrate secondary sex characteristics.

Site descriptions.—The population in Conecuh National Forest (Craig Guyer, et al., unpubl. report) is located in a stand of secondary growth mixed pine-oak uplands on both suitable and priority soils for Gopher Tortoises. This population also included individuals from a nearby private property. Historically Conecuh National Forest was farmed for cotton; however, the U.S. Forest Service initiated a Longleaf Pine (*Pinus palustris*) restoration effort in the late 1980s. Restoration at this site included thinning of canopy trees and applying prescribed burns to return fire to the landscape. The site is maintained on a mainly dormant-season fire regime approximately once every 3–5 y.

The Gopher Island population is on an 8.45 ha island located within Lake Eufaula, Georgia. Soils on the island are deep sands, and are considered ideal for Gopher Tortoises. The habitat is characterized as having a Longleaf Pine-Turkey Oak (*Quercus laevis*) overstory, a midstory of scrub oaks (*Quercus* sp.) and hawthorns (*Crataegus* sp.) and ground covers dominated by Wiregrass (*Aristida stricta*), broomsedges (*Andropogon* sp.), and Prickly Pear Cactus (*Opuntia humifusa*). There is currently no habitat management at this site. Although the plant species composition indicates the potential for high quality habitat, the lack of prescribed fire and the presence of a hardwood midstory indicate that this is currently a degraded habitat.

The Mississippi site, a recipient-site mitigation bank, is located just southeast of the boundary of the northern compartment of DeSoto National Forest. For this study, we used only the existing resident Gopher Tortoises. The site was typical of a fire-suppressed Loblolly Pine (*Pinus taeda*) plantation, with thick underbrush and high-density stands of secondary-growth Loblolly Pine. Intensive restoration efforts have been underway for the past 5 y including thinning to open the canopy, prescribed burning approximately once every 3 y, and low-density planting of Longleaf Pine. The soil type at this site is considered priority for Gopher Tortoises.

The Wade Tract is a private quail plantation in southwest Georgia, and is considered a high-quality Gopher Tortoise site (Guyer et al. 2013) because of its desirable habitat structure and plant species composition; however, much of the soil is classified as acceptable and not priority for tortoises. Tortoises at this site occur in high density, and the habitat is characterized as old-growth Longleaf Pine, with the native groundcover vegetation never having been destroyed for agricultural use. The habitat at this site has been maintained with a regular prescribed fire regime on a 1–2 y burn interval (Guyer et al. 2013) with many fires occurring in the growing season.

Quantification of fluctuating asymmetry.—Previously used analytical procedures that incorporate geometric morphometrics in testing FA require direction from numerous citations and the tedious synthesis of steps (Klingenberg and McIntyre 1998; Klingenberg et al. 2002; Klingenberg 2011; Adams and Otárol-Castillo 2013). Here we test a novel method of FA quantification requiring only the TPS geometric morphometric packages and MorphoJ (Klingenberg 2011) and adequately avoids pseudoreplication in error assessment. Such methodology is outlined below. We aligned photographs horizontally such that the orientation of each tortoise was standardized in Adobe Photoshop (version 7.0). We saved all images and then mirrored across the horizontal plane and we saved the mirrored image. For each image, we assigned 12 landmarks and digitized on the top half (right side) of the carapace (Fig. 1). The same landmarks were quantified on the mirrored image of each tortoise using TPS Dig2 (Rohlf 2004). While we initially obtained FA scores for both the carapace and the plastron, we removed the plastron scores from our analyses. We only used the carapacial scores because the carapacial photographs were less susceptible to tilting while taking the photograph (because the animal is able to rest flat and inside its shell). Taking the plastron photographs required restraining the animal on its back, which often caused the photographs to become skewed in the x, y, and z planes, thus not representing a reliable measure of FA.

We used the program TPS Regr (Rohlf 2009) to compile TPS reports and save a weight matrix file. From the weight matrix file, we saved relative warp scores, which we standardized by adding the lowest relative warp score to every relative warp score to maintain relative difference in numbers while standardizing to a minimum of zero. We calculated the absolute value of the difference in relative warp score for each axis of each landmark within individual tortoises to quantify the difference in landmark position between the right and left side of the specimen. We calculated the final FA scores as the mean of all absolute value differences, thus generating one overall FA score for each individual.

Statistical analyses.—To compare population-level differences in FA, we used Analyses of Variance (ANOVA) to test for differences in FA among populations. When analyzed by site, and even after log transforming

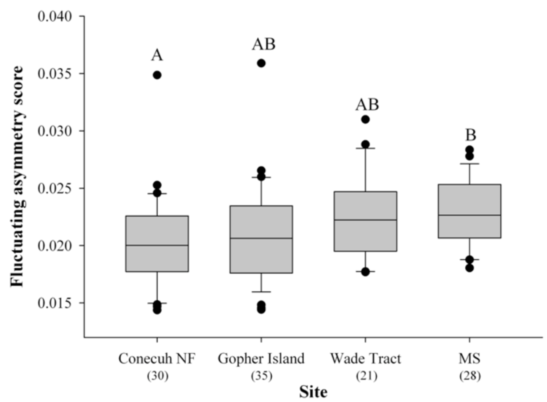

Figure 2. Fluctuating asymmetry (FA) values among four populations of Gopher Tortoises (*Gopherus polyphemus*). Grouping variables are noted above box plots, and sample sizes for each sampled population are in parentheses below site names.

Table 1. The median FA Score (MFA), number of males (No.M), number of females (No.F), number of tortoises for which sex could not be determined (No.U), and straight-line carapace size range (CL) in four populations of Gopher Tortoises (*Gopherus polyphemus*) in southeastern North America. The abbreviation N.F. = National Forest.

Site	MFA	No.M	No.F	No.U	CL (mm)
Wade Tract	0.0222	8	12	1	262–339
Gopher Island	0.0206	12	13	10	57–334
Conecuh N.F.	0.0200	11	11	8	60–290
Mississippi	0.0226	12	15	1	163–254

FA scores, the data were not normally distributed. Thus, we used a Kruskal-Wallis non parametric ANOVA to compare untransformed FA scores among sites and we made post-hoc site comparisons using Dunn's Method. When analyzed by sex, FA scores were normally distributed, thus we used a one-way ANOVA to compare males, females, and animals for which sex could not be diagnosed (e.g., immature and young adult tortoises). Lastly, we were interested in the effect of size on FA. To identify the effects of size on FA, we regressed FA verses straight-line carapace length within and among all of the populations. For all tests, $\alpha = 0.05$.

Results

We obtained photographs sufficient to calculate FA scores from 114 Gopher Tortoises, which included 31 individuals from Conecuh National Forest, 35 from Gopher Island, 21 from the Wade Tract, and 27 from Mississippi. Of the 114 individuals, we identified 51 as females, 43 as males, and 20 as juveniles, which were small individuals lacking secondary sexual characteristics. We found that there were significant differences in FA among the four sites sampled (Table 1; $H = 11.15$, df = 3, $P = 0.011$). There was a significant difference in FA between the Conecuh National Forest population and Mississippi population of *G. polyphemus* (Fig. 2). The Gopher Island and Wade Tract populations were intermediate between the Mississippi and Conecuh National Forest populations. There were no differences in FA scores between the sexes ($F_{2,102} = 0.389$, $P = 0.679$). When analyzed across all populations, we found that size did not affect FA ($t = -0.315$, df = 83, $P = 0.710$; Fig. 3a); however, when tested within populations, FA was affected by size in the Gopher Island population ($t = -2.039$, df = 33, $P = 0.050$; Fig. 3b). In the other three populations, size did not affect FA (Conecuh NF: $t = 0.973$, df = 27, $P = 0.339$; Wade Tract: $t = -1.361$, df = 19, $P = 0.189$; Mississippi: $t = -0.399$, df = 25, $P = 0.694$). However unlike three of the other sites, data from the Wade Tract did not include tortoises < 260 mm carapace length (Fig. 3a).

Discussion

Population genetics, environmental stress, and the combination of these factors can cause increased population FA (Lens et al. 2000). Our analysis indicated differences are present in FA among populations of Gopher Tortoises. We identified high mean FA in the Mississippi population of Gopher Tortoises, which partly confirmed our prediction that genetic diversity is negatively related to population-level FA. We predicted that the Gopher Island tortoise population would have the lowest mean FA, as this site has been shown to have surprisingly high genetic diversity (Ennen et al. 2011). While the Wade Tract site is considered high-quality habitat for Gopher Tortoises (Guyer et al. 2013), Ennen et al. (2011) found that this site had lower genetic diversity relative to the Gopher Island tortoises. Thus, based on genetic diversity alone, we predicted that tortoises at the Wade Tract site would have higher FA than the Gopher Island site. While not significantly different, the median FA scores for these populations supported this prediction.

Following our prediction, we found that the Mississippi population of Gopher Tortoise had the greatest degree of FA. Our prediction was based on a study that identified this population as having unusually low genetic diversity (Ennen et al. 2010). Further support that the Mississippi population of Gopher Tortoises is suffering from low fitness, and potentially inbreeding depression, has been indicated by these animals having reduced egg viability (Epperson and Heise 2003; Noel et al. 2012) and high predator-independent juvenile mortality (Matt Hinderliter pers. comm.). All of these population-level parameters (e.g., high FA, low egg viability, and high juvenile mortality) may be caused by the underlying low genetic diversity (Ennen et al. 2010). Future study should continue on these

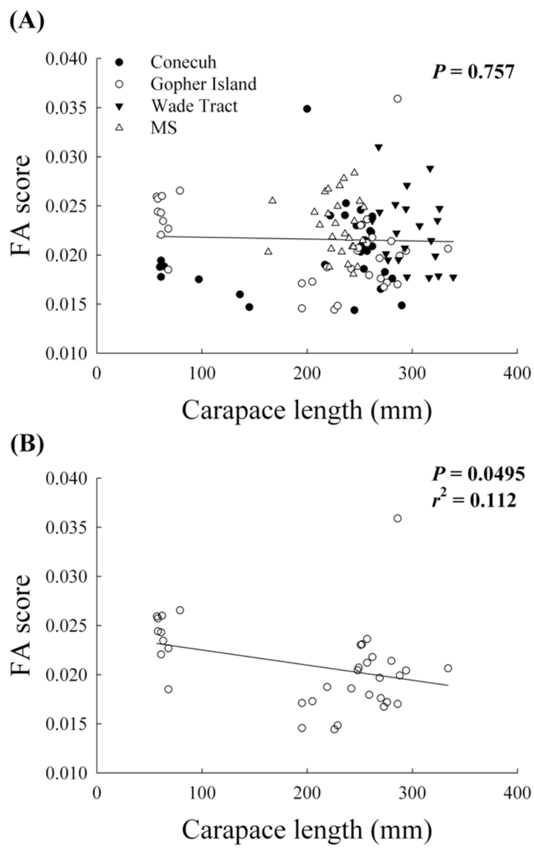

Figure 3. Relationship of fluctuating asymmetry (FA) scores by straight-line carapace length among four populations (A) and for the Gopher Island population only (B) of Gopher Tortoises (*Gopherus polyphemus*).

populations of Gopher Tortoises that may be nearly or completely isolated. If FA serves as a relevant indicator of population fitness, these data suggest this population is suffering low fitness, and ultimately management decisions (such as translocation for genetic rescue; Weeks et al. 2011) may become important to mitigate the low fitness in the federally protected populations of Gopher Tortoises.

The populations included in this study spanned a range of habitat quality, with the Wade Tract representing the highest-quality forest cover and vegetation structure. However, soil features may also be of great importance to the presence and viability of Gopher Tortoise populations (Hermann et al. 2002). Gopher Tortoises require deep, well-drained, sandy soils for adequate burrowing (Hermann et al. 2002), and thus soil type may also influence population FA scores, either by directly impacting carapacial growth patterns, or through indirectly affecting individual condition, and thus FA. Of the four sites sampled in this study, the Mississippi population of tortoises existed on soils with the lowest clay content (Table 2) and generally the highest sand content. While the Wade Tract is considered high-quality habitat, the soils at this site may be considered intermediate in quality, as clay content is generally higher and sand content is generally lower than other sites with Gopher Tortoises. The Conecuh National Forest tortoises existed on sites with relatively high sand content, and relatively low clay content, while the Gopher Island population existed on soils with slightly less sand and slightly more clay. Given that the overall highest quality soil type for Gopher Tortoises used in this study was present at the Mississippi site, it is clear that soil type is not the determining factor of increased FA in this population. If poor quality soils were the determining factor in carapacial FA, we would have expected the lowest FA scores in Mississippi tortoises, and the highest FA scores in Wade Tract tortoises. These patterns further support that hypothesis that poor population genetics of Gopher Tortoises from Mississippi may be reducing their fitness.

Because tortoises from Conecuh National Forest are exceptionally small (Tuberville et al. 2014), and our FA data indicated that these animals were the most symmetrical, we were interested in further testing for a relationship between size and FA. Regression analyses between size and FA failed to identify a significant effect across all populations or within the Conecuh National Forest, Wade Tract, or Mississippi tortoise populations. However, we did find a significant negative effect of size on FA in the Gopher Island population. Failing to find a consistent relationship across all populations in this study may be at least partly due to low sample sizes of animals sampled across all sizes among all populations. The negative relationship that we identified between

Table 2. Key physical components of the predominant soil types at each of four populations of Gopher Tortoises (*Gopherus polyphemus*) in southeastern North America sampled in this study (data extracted from: http://websoilsurvey.sc.egov.usda.gov/). Abbreviations are NLS = Norfolk loamy sand; LLS = Lucy loamy sand; OSL = Orangeburg sandy loam; FSL = Faceville sandy loam; FLFS = Fuquay loamy fine sand; BLFS = Bonifay loamy fine sand; ELS = Eustice loamy sand; BFSL = Benndale fine sandy loam; WLFS = Wadley loamy fine sand.

Site	Gopher Island	Wade Tract			Conecuh National Forest		Mississippi		
Soil type	NLS	LLS	OSL	FSL	FLFS	BLFS	ELS	BFSL	WLFS
Percentage sand in top 2 m	67	72	61	57	72	73	86	54	77
Percentage clay in top 2 m	22	16	21	29	15	12	6	10	5
Moist bulk density (g/cm^3)	1.52	1.62	1.59	1.48	1.62	1.60	1.63	1.58	1.61

size and FA in the Gopher Island population was the opposite of what has been shown between size and FA in prior studies of turtle FA, which showed a positive relationship between size and FA (Davis and Grosse 2002).

Our analysis represents the first study of FA in Gopher Tortoises, and conclusions from this study suggest that there are likely important differences in fitness among populations of this species. The interpretation of among-population differences in FA remains difficult as we still lack a thorough understanding of the importance of genetic verses environmental factors in affecting FA. However, our data generally support a prediction that FA in Gopher Tortoises is related to genetic diversity. Furthermore, Velo-Anton et al. (2011) found that carapacial scute anomalies were negatively related to genetic diversity in European Pond Turtles (*Emys orbicularis*), further supporting the importance of genetic richness in our measures of FA. Together, these results highlight the need for increased conservation and management of Gopher Tortoises in populations (e.g., Mississippi) that are likely experiencing reduced fitness.

We are confident that the carapace landmarks used in our study suitable landmarks for measuring FA in turtles. Because the FA scores were derived from multiple landmarks per side, this methodology is free from one of the major issues of many FA studies that fail to use multiple points for parameterization (Graham et al. 2010). This methodology of measuring FA in turtles may be of great use to biologists interested in comparing FA among turtle populations, as the sampling is relatively quick, inexpensive, and non-destructive to individuals in the population. Additionally, further studies of FA among populations of turtles will further our understanding of the degree and nature to which this measure can indicate fitness in such long-lived species.

Acknowledgments.—We thank Westervelt Ecological services and John McGuire for providing data on the Mississippi population of tortoises, the U.S. Army Corps of Engineers for access to the Gopher Island property, Tim Mersmann and the U.S. Forest Service for access to Conecuh National Forest, and Tall Timbers Research Station and the Jeptha Wade Family for preserving the Wade Tract and providing access to tortoises at the site. This research was conducted with the approval of the Auburn University Institutional Animal Care and Use Committee (# 2013-2226). We are grateful to Suzi Mersmann, Sierra Stiles, and James Stiles for help with fieldwork. We are also very grateful to Craig Guyer, Brian Folt, David Steen, Helen Tripp, Melissa Miller, Rebecca John, and Scott Goetz for offering thorough discussion and helpful insight on this project and manuscript preparation.

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