The effects of constant and fluctuating incubation temperatures on sex determination, growth, and performance in the tortoise Gopherus polyphemus

Jeffery P. Demuth

Abstract: Temperature-dependent sex determination is one of the best documented yet evolutionarily enigmatic sex-determining systems. The classical theoretical framework suggests that temperature-dependent sex determination will be adaptive when males and females benefit differentially from development at certain temperatures. Empirical evidence has not provided convincing support for this "differential-fitness" hypothesis. Furthermore, since most experiments utilize constant temperature incubation treatments to explore phenotypic response to temperature, few studies have addressed the consequences of incubation under natural conditions. In this study I utilized constant-temperature laboratory incubations and natural-nest incubations to determine the effects of temperature on sex, size, growth, and locomotor performance in the tortoise Gopherus polyphemus. Constant-temperature incubations do induce substantial growth and performance variation in these tortoises. However, the data do not clearly support the differential-fitness hypothesis because (i) growth variation does not result in adult size dimorphism, (ii) performance differences are confined to a very short period after hatching, and (iii) natural incubation temperatures do not vary sufficiently to produce significant phenotypic variation in traits other than sex.

Résumé : La détermination du sexe en fonction de la température (TSD) est l’un des systèmes évolutifs de détermination du sexe les plus étudiés et, malgré cela, l’un des plus énigmatiques. Le cadre théorique classique laisse croire que la TSD est adaptative lorsque les mâles et les femelles bénéficient différemment du développement à des températures données. Les données empiriques ne supportent pas de façon convaincante l’hypothèse du « fitness différentiel ». De plus, comme dans la plupart des expériences destinées à explorer la réponse phénotypique à la température l’incubation se fait à température constante, peu de chercheurs ont examiné les conséquences de l’incubation dans des conditions naturelles. Dans cette recherche, j’étudie l’incubation dans des conditions de laboratoire à température constante et dans des conditions naturelles d’incubation au nid pour déterminer les effets de la température sur le sexe, la taille, la croissance et la performance locomotrice de la tortue Gopherus polyphemus. Les incubations à température constante donnent lieu à des variations importantes de la croissance et de la performance chez ces tortues. Cependant, les données ne supportent pas vraiment l’hypothèse du fitness différentiel puisque (i) la variation dans la croissance ne résulte pas en un dimorphisme sexuel de la taille chez les adultes, (ii) les différences de performance sont limitées à une très courte période après l’éclosion et (iii) les températures d’incubation dans des conditions naturelles ne varient pas suffisamment pour produire une variation phénotypique significative des caractéristiques autres que le sexe.

Introduction

Organisms with separate sexes have evolved numerous mechanisms for determining the sex of an individual. Although the majority of sex-determining systems involve direct genetic control, systems in which sex is determined by environmental factors experienced some time after conception are common in some groups and are phylogenetically widespread (Bull 1983). These environmental sex determination (ESD) systems are evolutionarily intriguing because they can result in sex-ratio biases that do not fit well within classical Fisherian theory (Bull and Charnov 1989). Much of the empirical effort directed toward explaining various forms of ESD is motivated by theory suggesting that it is adaptive to allow environment to determine sex when the sexes benefit differentially from certain environments (Charnov and Bull 1977). Therefore, most ESD studies have sought, at least initially, to determine whether some environments differentially benefit the fitness of one sex over that of the other (Bull 1983; Shine 1999). In many instances, data have corroborated differential-fitness hypotheses (Bull 1983; Conover 1984; Naylor et al. 1988; Blackmore and Charnov 1989); however, their ability to explain temperature-dependent sex determination (TSD) in reptiles remains a point of contention (Bull and Charnov 1989; Ewert and Nelson 1991; Janzen and Paukstis 1991a, 1991b; Burke 1993; Shine 1999).

Reptilian TSD is one of the best documented yet evolutionarily enigmatic forms of ESD (Ewert et al. 1994; Lang and Andrews 1984; Viets et al. 1994). In species with TSD, sex is a threshold trait where the proportion of embryonic development that occurs above or below a pivotal temperature during the middle third of development determines the individual’s sex (Georges 1989). An explanation of the
evolution of TSD in reptiles has been elusive for three reasons. First, TSD takes three patterns. Two of the patterns have a single transition from males to females or vice versa. The third pattern produces males at intermediate temperatures and females at the extremes.

The second difficulty in using the differential-fitness hypothesis to explain TSD is that the effects of temperature on fitness are difficult to measure, therefore we must study surrogates for fitness. Incubation temperature has been demonstrated to affect the duration of the incubation period (Sexton and Marion 1974; Webb and Cooper-Preston 1989; Van Damme et al. 1992), hatching size (Brooks et al. 1991; Allstead and Lang 1995), the growth rate (McKnight and Gutzke 1993; O’Stein 1998; Rhen and Lang 1999), behavior (Gutzke and Crews 1988; Burger 1990), thermoregulation (Joanen et al. 1987; O’Stein 1998), and locomotor performance (Janzien 1995; Doody 1999). Of these traits, only locomotor performance is known to directly affect survivorship (Jayne and Benett 1990; Janzen 1995), although all of the listed traits have been proposed to indirectly affect lifetime reproductive success (reviewed in Shine 1999). No empirical study of the evolution of TSD has rejected the differential-fitness hypothesis based on the above traits. However, there are few generalizations and at least eight mechanisms have been proposed to explain how sex, temperature, and other traits might interact to make TSD adaptive (Ewert and Nelson 1991; Roosenburg and Niewiarowski 1998; Shine 1999).

Finally, the third shortcoming of most TSD studies is that they have not addressed the consequences of fluctuating incubation conditions. The only direct comparison of constant and natural incubation effects used a species with genetic sex determination (GSD) and concluded that fluctuating temperatures may affect embryos differently than constant temperatures (Doody 1999). Georges (1989) proposed a model to translate fluctuating temperatures into constant temperature equivalents (CTEs) by accounting for disproportionate changes in development rate relative to temperature changes. In laboratory experiments, the CTE was a better predictor of sex ratio than the mean incubation temperature (Georges et al. 1994); however, the only study to test CTEs with natural nests found that the CTE did not satisfactorily predict sex ratio (De Souza and Vogt 1994).

In this study I ask whether sex, size, growth, and locomotor performance in the tortoise Gopherus polyphemus are affected by incubation temperature, and whether constant-temperature incubations and natural-nest incubations affect traits differently. Finally, results from this and previous studies are reviewed in light of their evidence for differential fitness.

Materials and methods

Study organism

Gopherus polyphemus is a long-lived, relatively slow growing turtle endemic to the southeastern coastal plain of the United States. Gopher tortoises are unique among turtles of the eastern United States in their adaptation to xeric habitats and construction of extensive subterranean burrows (Ernst et al. 1994). Adult females produce one clutch per year. Clutch sizes range, on average, from 5.18 to 8.9 and are negatively correlated with latitude. Nesting generally occurs in May and June, with hatching beginning in August and running through October (Iverson 1980; Butler and Hull 1996; Smith et al. 1997). Incubation periods in nature vary latitudinally from 110 days in South Carolina to 80–90 days in northern Florida (Diemer 1986). In most studies, the mounds of excavated sand at the burrow opening (or burrow apron) contain the majority of reported nests (reviewed in Butler and Hull 1996).

Study site

The Kennedy Space Center, Merritt Island National Wildlife Refuge, and Canaveral National Seashore (hereafter referred to jointly as KSC) are located on the Atlantic coast of central Florida in Brevard and Volusia counties. The National Aeronautics and Space Administration (NASA), the U.S. Fish and Wildlife Service, and the National Park Service jointly manage KSC, which is the largest area of protected habitat for tortoises on Florida’s Atlantic coast. Of 593 tortoises captured and marked from 1992 through 1997, only 80 were recaptured even once, indicating a large population (J.P. Demuth and R. Seigel, unpublished data).

The densest colonies of tortoises at KSC are located in coastal-strand habitat, which lies immediately behind the coastal dunes and is characterized by dense vegetation of saw palmetto (Serenoa repens), rapanea (Rapanea punctata), nakedwood (Myrcianthes fragrans), tough buckthorn (Rumelia tenax), Hercules’ club (Zanthoxylum clava-herculis), bay (Persea borbonia), and snowberry (Chionodora alba) with small scattered areas of open sand. Tortoise colonies are typically associated with the more open sandy areas.

Egg collection and incubation

Most female tortoises were captured while they foraged along roadsides. Others were either picked up during surveys of tortoise colonies or trapped by placing large buckets near burrow openings. Adults suspected to be gravid were X-rayed to verify their reproductive status and determine clutch size. Gravid females were injected with 0.5 cc/kg of Arg-vasotocin intramuscularly to induce oviposition. As eggs were laid, I measured (maximum and minimum diameter ± 0.1 mm), weighed (± 0.01 g; Fisher Scientific XT top-loading electronic balance), and assigned them randomly but uniformly across incubation treatments so that no female had 2 eggs per treatment unless she had more than 9 eggs. I chose constant-temperature laboratory incubations at 26, 28, 29, 30, 31, and 34°C because Burke et al.’s results (1996) suggest a pivotal temperature near 30°C for this species. Although this broad range of 7 constant temperatures necessarily decreases the number of eggs per treatment, it ensures a comprehensive sample of potential phenotypic responses to temperature.

Incubators were calibrated using Hobo-Temp data loggers for 1 week prior to initiation of incubations. Probes were placed in the center and at the edge of substrate-filled egg boxes and outside the egg box. Air temperatures within incubators (Hova-Bator, G.Q.F. Manufacturing Co., Savannah, Georgia) fluctuated a maximum of ±0.8°C but substrate temperatures did not deviate from the nominal temperature. The substrate consisted of 1:1 vermiculite:water by mass. To control for potential effects of hydric conditions, I misted inside each box every second day to maintain constant mass.

Field incubation treatments consisted of constructing artificial nests at two localities where recent predation on the original nest had occurred. I placed eggs at the average depth of and in a typical orientation for eggs in other gopher tortoise nests found at KSC (personal observation; see Results). Additionally, I monitored two completely natural nests. Based on previous searches of each locality, natural nests contained eggs that were not more than 5 days old. Each egg in the experimental and natural nests was given a unique identification letter and its position from the top of the egg to the ground surface was measured (±1.0 cm). I enclosed each nest with metal screens (0.634 cm mesh) and placed Hobo-Temp data loggers at the top and bottom to record thermal profiles at 1.6-h intervals. After 70–75 days (>75% of the total incubation period), eggs were brought back to the laboratory and maintained at 34°C until hatching. Since the temperature-sensitive period for sex deter-
mination is confined to the middle third of development, and 30°C is near mean nest temperatures and CTEs, bringing eggs into the laboratory during the final days of incubation should not have affected sex determination, and likely influenced hatching time only slightly.

After 70 days, incubators were checked daily for signs of hatching. For the purpose of standardization, I considered an egg hatched as soon as the hatching was visible. Hatchlings remained in their compartments until they had absorbed all yolk. When it was removed, each hatching was given an identification number by clipping its marginal scutes and writing the number on its plastron.

Hatchlings were housed in individual plastic boxes without lids. Full-spectrum heat lamps were set on a 12 h light:12 h dark cycle and the temperature fluctuated from 31 ± 2°C during light hours to 22 ± 2°C during dark hours. The small water dish initially provided to each hatching was rarely used for purposes other than hiding under, so the dishes were removed when hatchlings were between 3 and 5 months of age. After that time, all tortoises were soaked weekly for 1 h in large tubs containing 4–6 cm of water. Feeding began immediately following the first locomotor-performance trial (7 days after hatching); food consisted of a mixture of carrots, broccoli, spinach, and commercial iguana pellets fed ad libitum. I replaced uneaten food with fresh food every other day.

Individuals were sexed using the laparoscopy technique described by Rostal et al. (1994). Hatchlings were starved for 2 days before their procedure. They were also soaked in a water bath for 1 h on the day preceding laparoscopy. These steps were taken as further precautions against infection in case the intestine or bladder was nicked during the initial incision. The gonads of each individual were examined independently by at least two investigators with no knowledge of the incubation temperature. In cases where investigators did not agree on the sex, those hatchlings were eliminated from pertinent analyses.

At 3–5 months of age, hatchlings were transported to Southeastern Louisiana University in Hammond, where they were held under conditions as close to the KSC conditions as possible. Temperature fluctuated approximately 0.5°C more in Louisiana, but diet, water availability, and light cycles were identical. At 6–8 months of age, hatchlings were transported back to KSC and held under the original conditions until their release at the site of their mother’s burrow. The age variation at time of transport reflects variation in hatching dates. Although hatchlings experienced the Louisiana and KSC environments at different ages, growth and performance data should be unaffected, owing to the similarity of housing conditions.

During the subsequent year’s nesting season, I attempted to record temperatures from five additional nests and four arbitrary locations in nesting habitat to find out whether my field treatments produced typical natural environments. The arbitrary locations were in small open areas near clusters of tortoise burrows. A single probe was placed at mid-nest depth at each location. No eggs were manipulated in this 2nd year, so sex ratios can only be estimated.

**Size and performance measures**

Growth was measured for the following traits at 2, 14, 28, 42, and 271 days using dial calipers (±0.1 mm): straight-line carapace length, plastron length, carapace width at the pectoral–abdominal junction, and height at the pectoral–abdominal junction. Hatchlings were also weighed to the nearest 0.01 g using a Fisher Scientific XT top-loading electronic balance.

Locomotor performance was measured as the time required to traverse a 1 m long track constructed from a 5 × 5 cm fiber-glass pipe with the top side cut out and containing a 1 cm deep sand substrate that was changed before each trial. Performance was measured at 7, 14, 28, and 42 days. At each age, three trials separated by 45 min were conducted. All performance data were collected at a consistent time (17:30–20:00) and temperature (31 ± 2°C). To control for motivation, I continuously tapped each hatching on the carapace with a pencil eraser. Tapping was necessary because without subsequent contact the typical response of tortoises set on the track was to remain stationary.

**Data analyses**

The effect of incubation temperature on hatching success was analyzed using contingency-table analysis. Logistic regression of the numbers of males and females produced at each constant temperature was used to predict sex ratios based on the CTE and mean nest temperature. Results obtained at 34°C were not included in the regression for reasons noted below. Principal component analysis was used to determine whether all size measures were informative for subsequent analyses. Differences in size and growth were tested using two-way ANOVA with incubation treatment and gender as fixed effects. Locomotor performance was analyzed using various ANOVA models for effects of incubation treatment, gender, and age (see Results for details). Only the second two trials were used in analyses of performance because they were consistently faster and more uniform than the first run. Repeatabilities were calculated using the equations of Lessels and Boag (1987). For repeatability within treatment across ages, I used individual means.

Where body-size measures or egg size were covariates of growth or performance, regression residuals were used in ANOVA. Tukey’s post-hoc multiple-comparisons test was used to distinguish among means of significantly affected traits. Appropriate assumptions of parametric statistics were verified for all tests, and differences were considered significant at α = 0.05. Locomotor-performance data were log-transformed to meet the assumption of normality in ANOVA models. Test statistics were computed using SYSTAT (SYSTAT Inc. 1996) and means are reported ±1 SD unless otherwise specified.

To estimate the minimum development temperature (T₀) for CTE calculations, I regressed the inverse of incubation time against incubation temperature (y intercept = T₀; Georges et al. 1994). Since the temperature-sensitive period for gonadogenesis in turtles occurs in the middle third of development (Bull and Vogt 1981), CTEs for evaluating the sex ratio were calculated only for the middle third of the total incubation period. CTEs used for comparing traits other than sex ratio use the entire incubation profile.

**Results**

I assigned 180 eggs from 24 females to the various treatments. The clutch size was 7.46 ± 2.61 (mean ± SD) (range = 3–15) eggs. The maximum egg diameter was 41.70 ± 2.43 mm (range = 32.5–49.3 mm) and the minimum egg diameter was 38.62 ± 2.26 mm (range = 31.2–43.0 mm), respectively. The egg mass was 38.11 ± 5.46 g (range = 20.40–48.75 g). Of the 180 incubated eggs, 88 hatched (48.9%) and 1 individual from 34°C was accidentally removed from the egg at 85 days because it appeared that no eggs had survived that treatment. Incubation treatment significantly affects hatching success (χ² = 28.689, df = 7, P < 0.001; Table 1) because of the low success at 34°C and relatively high success from field treatments (78%). The single hatching from 34°C required special care to survive, therefore the treatment is excluded from the phenotypic analyses below.

Incubation time is inversely related to incubation temperature (time = 254.8–5.662 × temperature; F[1,70] = 215.89, P < 0.001, r² = 0.755). For every 1°C change in constant-temperature incubation (or CTE), incubation time changes approximately 5.7 days (Table 1). T₀ for G. polyphemus at KSC is approximately 16.5°C. CTEs are slightly better than
Table 1. Effects of incubation environment on incubation time, hatching success, and sex determination.

<table>
<thead>
<tr>
<th>Incubation treatment</th>
<th>No. of eggs</th>
<th>Incubation period (days)</th>
<th>Hatching success (%)</th>
<th>No. of hatchlings</th>
<th>Predicted % males&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CTE</td>
<td>Mean</td>
</tr>
<tr>
<td>26°C</td>
<td>23</td>
<td>112.57 ± 5.50</td>
<td>34.78</td>
<td>8</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>28°C</td>
<td>22</td>
<td>94.93 ± 4.92</td>
<td>63.63</td>
<td>11</td>
<td>3</td>
<td>78.6</td>
</tr>
<tr>
<td>29°C</td>
<td>24</td>
<td>88.64 ± 7.03</td>
<td>58.33</td>
<td>9</td>
<td>5</td>
<td>64.3</td>
</tr>
<tr>
<td>30°C</td>
<td>24</td>
<td>83.07 ± 4.73</td>
<td>62.50</td>
<td>5</td>
<td>10</td>
<td>33.3</td>
</tr>
<tr>
<td>31°C</td>
<td>23</td>
<td>79.86 ± 4.67</td>
<td>60.87</td>
<td>1</td>
<td>13</td>
<td>7.2</td>
</tr>
<tr>
<td>32°C</td>
<td>22</td>
<td>77.22 ± 2.86</td>
<td>40.90</td>
<td>0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>34°C</td>
<td>24</td>
<td>85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.15</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Experimental nest 1</td>
<td>6</td>
<td>87.75 ± 0.96</td>
<td>83.33</td>
<td>2</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>Experimental nest 2</td>
<td>6</td>
<td>90.5 ± 2.38</td>
<td>66.66</td>
<td>2</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Natural nest</td>
<td>6</td>
<td>na&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.33</td>
<td>1</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Nest 4</td>
<td>7</td>
<td>10.1</td>
<td>49.1</td>
<td>30.94</td>
<td>29.33</td>
<td>4.74</td>
</tr>
<tr>
<td>Nest 5</td>
<td>6</td>
<td>95.3</td>
<td>96.6</td>
<td>27.05</td>
<td>26.80</td>
<td>1.61</td>
</tr>
<tr>
<td>Arbitrary location</td>
<td>6</td>
<td>90.5</td>
<td>95.6</td>
<td>27.61</td>
<td>27.00</td>
<td>2.58</td>
</tr>
</tbody>
</table>

Note: For nests where both top and bottom temperatures are known, the midpoint is used. The incubation temperature for nests only includes the temperature-sensitive period for sex determination (i.e., the middle third). Fluctuation is one-half of the average daily difference between maximum and minimum temperatures. Incubation treatment significantly affects hatching success ($\chi^2 = 28.689, P < 0.001$), incubation time ($t = 254.8 - 5.662 \times \text{temperature}; F_{1,110} = 215.89, P < 0.001$), and sex. Nests 4 and 5 and the “Arbitrary location” were only monitored through the first three-quarters of the incubation period, therefore the duration of the incubation period and hatching success are unknown. The overall predicted sex ratio from CTE of natural conditions is 1.08 females:1.0 male.

<sup>a</sup>Predicted % males = $\text{exp}(39.0356 - 1.332174 \times \text{temperature}) / [1 + \text{exp}(39.0356 - 1.332174 \times \text{temperature})] \ (\chi^2 = 41.521, P < 0.001)$.

<sup>b</sup>The only hatchling from 34°C was removed from the egg at 85 days.

<sup>c</sup>The exact laying date is unknown for the natural nest.
mean temperature at explaining variance in incubation time ($r_{\text{mean}} = 0.951$, $r_{\text{CTE}} = 0.999$).

**Sex determination**

Hatchlings at 3–5 months retained yolk and lacked obvious gonadal differentiation. Therefore, hatchlings were sexed at 7–9 months of age, when the testes appeared as yellowish, ribbonlike, well-vascularized structures. The ovaries appeared opaque white, without vascularization, and in a few cases with silica-like primordial follicles. Because incompletely regressed Müllerian ducts were present in several males, the presence of oviducts was not considered conclusive evidence of sex. Sex was successfully determined in 83 of 89 (93.3%) hatchlings. In six cases investigators did not agree on the individual’s sex. Of these six unknowns, two were from 30°C, two from 28°C, one from 32°C, and one from 29°C. All six are excluded from determination of the pivotal temperature. There was no mortality due to this sexing technique.

Sex is determined by incubation temperature: % male = \[\frac{\exp(39.0356 - 1.332174 \times \text{temperature})}{1 + \exp(39.0356 - 1.332174 \times \text{temperature})}\] ($\chi^2 = 41.521, P < 0.001$). None of the constant-temperature laboratory incubations produced a 1:1 sex ratio, but solving the regression for the temperature at which 50% males are produced yields the pivotal temperature of 29.3°C (Fig. 1). Higher temperatures produce primarily females, whereas lower temperatures produce primarily males. Both sexes are obtained at constant temperatures of 28, 29, 30, and 31°C. CTEs are more tightly correlated with sex ratio than mean temperature in field nests ($r_{\text{mean}} = 0.139$, $r_{\text{CTE}} = 0.76$; Fig. 1).

**Growth and performance**

Principal component analysis of morphometric measures shows that at each stage a single factor explains hatching size. I therefore use carapace length as a representative measure of size because it consistently explains the largest
Table 2. Carapace lengths (mean ± SD) of hatchlings at 2, 14, 28, 42, and 271 days.

<table>
<thead>
<tr>
<th>Incubation treatment</th>
<th>Carapace length (mm)</th>
<th>2 days</th>
<th>14 days</th>
<th>28 days</th>
<th>42 days</th>
<th>271 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>26°C</td>
<td>n=7</td>
<td>45.50 ± 2.68</td>
<td>47.43 ± 2.65</td>
<td>47.42 ± 2.63</td>
<td>48.67 ± 3.08</td>
<td>53.04 ± 4.69</td>
</tr>
<tr>
<td>28°C</td>
<td>n=14</td>
<td>46.00 ± 2.58</td>
<td>48.07 ± 2.85</td>
<td>48.96 ± 3.07</td>
<td>49.64 ± 2.96</td>
<td>56.44 ± 4.62</td>
</tr>
<tr>
<td>29°C</td>
<td>n=14</td>
<td>45.89 ± 2.41</td>
<td>47.75 ± 2.70</td>
<td>48.39 ± 2.84</td>
<td>48.75 ± 2.85</td>
<td>53.71 ± 4.87</td>
</tr>
<tr>
<td>30°C</td>
<td>n=15</td>
<td>45.53 ± 2.24</td>
<td>47.77 ± 2.74</td>
<td>48.83 ± 3.00</td>
<td>49.33 ± 3.03</td>
<td>55.33 ± 5.68</td>
</tr>
<tr>
<td>31°C</td>
<td>n=14</td>
<td>45.18 ± 3.20</td>
<td>47.36 ± 3.20</td>
<td>48.32 ± 3.26</td>
<td>48.83 ± 3.04</td>
<td>55.36 ± 5.01</td>
</tr>
<tr>
<td>32°C</td>
<td>n=9</td>
<td>44.06 ± 2.52</td>
<td>46.72 ± 1.99</td>
<td>47.39 ± 2.03</td>
<td>48.39 ± 2.57</td>
<td>55.52 ± 3.92</td>
</tr>
<tr>
<td>Experimental nest 1</td>
<td>n=4</td>
<td>45.80 ± 1.03</td>
<td>47.63 ± 0.48</td>
<td>48.63 ± 0.48</td>
<td>48.63 ± 0.48</td>
<td>54.79 ± 2.26</td>
</tr>
<tr>
<td>Experimental nest 2</td>
<td>n=5</td>
<td>45.60 ± 1.82</td>
<td>47.80 ± 1.60</td>
<td>49.50 ± 1.41</td>
<td>49.70 ± 1.52</td>
<td>53.91 ± 1.04</td>
</tr>
<tr>
<td>Natural nest</td>
<td>n=5</td>
<td>45.88 ± 1.10</td>
<td>47.20 ± 1.25</td>
<td>48.10 ± 1.34</td>
<td>48.60 ± 1.52</td>
<td>53.94 ± 1.40</td>
</tr>
</tbody>
</table>

Note: Egg mass had a significant influence on hatching size at each age. Hatchlings from warmer incubations tended to be smaller initially but grew faster (see the text), so the pattern was reversed by 271 days of age. However, incubation effects were not significant at any age.

- *P < 0.01
- **P < 0.005

Discussion

Burke et al. (1996) first described TSD in *G. polyphemus* based on eggs incubated at 26, 29, and 32°C. The present study corroborates their findings and more precisely defines the pivotal temperature to be 29.3°C (Fig. 1). This finding is
consistent with the prediction of Burke et al. (1996) that the pivotal temperature would be at the lower end of the 29–30°C range. However, the South Carolina tortoises from their study may have slightly higher pivotal temperatures because of the trend toward decreasing pivotal temperatures in more southerly and easterly ranges of some species (Ewert and Nelson 1991). The 4°C range of temperatures that produced some individuals of both sexes was greater than that typically found in TSD studies. Although the design of my study is ill-suited to determining the cause of the spread, the distribution of sexes from within incubators suggests that it is not due to within-incubator temperature variation. I feel that it is
Time (s; mean ± SD) required to crawl 1 m for hatchlings 7, 14, 28, and 42 days after hatching.

Table 3.

<table>
<thead>
<tr>
<th>Incubation treatment</th>
<th>7 days old</th>
<th>14 days old</th>
<th>28 days old</th>
<th>42 days old</th>
<th>All ages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental nest 1</td>
<td>38.6 ± 20.4 (0.35)</td>
<td>27.6 ± 21.3 (0.60)</td>
<td>23.4 ± 19.2 (0.97)</td>
<td>25.6 ± 17.8 (0.87)</td>
<td>32.6 ± 17.2 (0.56)</td>
</tr>
<tr>
<td>Experimental nest 2</td>
<td>44.9 ± 21.9 (0.98)</td>
<td>30.4 ± 21.2 (0.57)</td>
<td>22.3 ± 20.6 (0.65)</td>
<td>22.3 ± 20.6 (0.65)</td>
<td>22.3 ± 20.6 (0.65)</td>
</tr>
<tr>
<td>Natural nest</td>
<td>44.9 ± 21.9 (0.98)</td>
<td>30.4 ± 21.2 (0.57)</td>
<td>22.3 ± 20.6 (0.65)</td>
<td>22.3 ± 20.6 (0.65)</td>
<td>22.3 ± 20.6 (0.65)</td>
</tr>
</tbody>
</table>

Note: Values in parentheses are repeatabilities. Two-way ANOVA indicates a significant main effect of age but not incubation treatment or interaction. Hatchlings were significantly faster at 14 days than at 7 days of age (Tukey's HSD test, P = 0.001) and were significantly faster at 28 days than at 14 days of age (P = 0.005).

Temperature effects on growth

Incubation temperature did not significantly affect hatching size, but did affect subsequent growth. The slight trend for hatchlings from higher temperatures to be smaller was overcome by the growth advantage at female-producing temperatures (31 and 32°C; Fig. 2B). The overall trend for females to grow more than males was not significant because at one primarily male producing temperature (28°C), growth also increased relative to incubations near the pivotal temperature. Incubation at 26°C produced all males, but hatching success was low, and did not follow trends in hatching size or growth. Therefore, I suspect that 26°C is too low a temperature for normal embryonic development.

The growth data from my study differ from those from a study of Gopherus agassizii incubated at 28.1, 30.6, 32.8 and 35.3°C. Spotila et al. (1994) found that incubation temperature affected G. agassizii hatching size, but not growth up to 120 days. In contrast to findings of the present study, Spotila et al. (1994) concluded that “All hatching tortoises grow at similar rates when housed under constant conditions. Therefore, small hatchlings do not catch up to their larger siblings.” The apparent difference between their conclusion and results from the present study may only be a matter of duration, since I may not have detected growth differences at 120 days that became apparent at 271 days.

Other studies have shown temperature effects on hatching growth in snapping turtles (Chelydra serpentina; Brooks et al. 1991; McKnight and Gutzke 1993; O’Steen 1998; and Rhen and Lang 1999), diamondback terrapins (Malaclemys terrapin; Rosenberg and Kelley 1996), and alligators (Alligator mississippiensis; Joanen et al. 1987). Since differences in thermoregulatory behavior may obscure direct effects of temperature on growth (O’Steen 1998), it is possible that the growth differences I found would be obscured if hatchlings were able to thermoregulate. However, Spotila et al. (1994) showed no difference in G. agassizii thermoregulation caused by different incubation temperatures, and Rhen and Lang (1999) demonstrated that in C. serpentina, incubation temper
Fig. 3. Thermal profiles from the top and bottom of one natural and two experimental tortoise nests. The thick line denotes the bottom of the nest and the thin line denotes the top. (A) Natural nest. (B) Experimental nest 1. (C) Experimental nest 2. Differences in fluctuation between the top and bottom of nests magnify the temperature difference experienced by embryos in the same nest.

Temperature has an effect on growth rate that is independent of sex or the ability to thermoregulate.

The ability to generalize among studies of the effect of temperature on growth is impeded by different TSD patterns (i.e., patterns Ia, Ib, and II; Ewert and Nelson 1991), variation among incubation protocols, and conditions in which hatchlings are raised. An emerging consistency among some studies is that the sex which is larger at adulthood grows
faster (Roosenburg and Kelley 1996; Rhen and Lang 1999). This fits well with a form of the differential-fitness hypothesis, termed the sexual size dimorphism (SSD) hypothesis, which proposes that the sex whose lifetime reproductive success benefits most from large size will be produced at temperatures that confer the most rapid growth (Head et al. 1987; Ewert and Nelson 1991; Shine 1999). The increased growth of males incubated at primarily male producing temperatures and females incubated at primarily female producing temperatures in the present study is consistent with predictions of the SSD hypothesis; however, adult tortoises from KSC do not exhibit SSD. The carapace lengths of adult males (261.48 ± 25.35 mm (mean ± SD; n = 219) and females (261.06 ± 37.05 mm; n = 208) are virtually identical (J.P. Demuth and R. Seigel, unpublished data). Therefore, the SSD hypothesis cannot explain the maintenance of TSD in G. polyphemus from KSC.

Effects of temperature on performance

Several studies have demonstrated an influence of incubation environment on reptilian performance and behavior in both TSD and GSD species (Lang 1987; Gutzke and Crews 1988; Burger 1989, 1990; Van Damme et al. 1992; Janzen 1993, 1995; Doody 1999; Elphick and Shine 1999; O’Steen 1998; Rhen and Lang 1999). Thus far the strongest support for the differential-fitness hypothesis, based on performance in a TSD species, was a demonstration that incubation temperature and sex interact to affect C. serpentina survivorship in a seminatural setting (Janzen 1995). Janzen’s (1995) results are difficult to relate to those from other studies of performance because the hatchlings that performed worst from the perspective of speed and propensity to move were the ones that survived best. It is relevant to the present study that differences in survivorship were manifest at an early age (56 months).

In the present study, incubation temperature influenced performance indirectly through sex. The differences in performance were not persistent and by 28 days there were no differences in performance among treatments or between the sexes. Repeatability scores show that most performance variation occurs among individuals within a given age, but across all four ages, there is as much variation within as among individuals (Table 3). Although uniform rearing conditions mean that cautious interpretation is warranted, performance trends suggest that the opportunity for differential-fitness consequences due to crawl speed must be realized in a relatively short period after hatching. Butler et al. (1995) found that hatchling gopher tortoises often construct burrows within 1 day of hatching. Since burrows offer protection against predation and desiccation, hatchling tortoises might experience strong selection for locomotor performance within the time during which this study demonstrates the greatest influence of incubation environment. The potential for strictly sex specific differences in speed and perhaps propensity to move is intriguing but does not support the differential-fitness hypothesis because it is not clear that incubation temperature is important within either sex.

Nest incubations

Previous studies have shown that nesting microhabitat can influence sex ratio in TSD species (Vogt and Bull 1984; Janzen 1994b). Vogt and Bull (1984) found a bimodal distribution of nest sex ratios, with all male clutches coming from vegetated locations and all females from open areas. Janzen (1994b) showed that in “typical” years painted turtle (Chrysemys picta) nesting beaches with less southern and western solar exposure produced male-biased sex ratios. In the present study, all monitored nests were in qualitatively similar microhabitats. Since my sample of natural nest temperatures does not include non-apron nests, estimates of primary sex ratio may be biased toward females unless non-apron nests are located in areas like the arbitrary location (Table 1) monitored in the second year.

In most studies that have estimated primary sex ratios from field incubations of TSD reptiles, it was found that few nests produce mixed-sex clutches (reviewed in Janzen 1994b). Overall sex-ratio biases are also commonly predicted (Ewert and Nelson 1991). Data from my study do not follow these patterns. However, a problem with interpreting field studies of TSD reptiles (particularly turtles) is that the nesting conditions and primary sex ratios measured in one or a few years of study are only a small fraction of what a population will experience even within one female’s reproductive lifetime. Janzen (1994a) demonstrated this difficulty by modeling C. picta sex ratios over 50 years using historical ambient-temperature data. Although within-year sex ratios ranged from nearly 100% male to 100% female, the sex ratio across all years was 46% male.

Sex ratios of tortoises at KSC old enough to sex via secondary sex characteristics is 1.07:1.0 (227 males:213 females; J.P. Demuth and R. Seigel, unpublished data). The sex ratio is nearly the same if only recaptures are counted (1.0:1.08, or 37 males:40 females). These adult ratios are identical with those predicted for hatchlings in the 2 years of this study (1.0 males:1.08 females; Table 1). However, the results of loggerhead sea turtle (Caretta caretta) studies conducted from 1986 to 1990 suggest that temperatures were higher in the recent past. Mosrovsly and Provancha (1989, 1992) report that mean temperatures of C. caretta nests within a few hundred metres of the tortoise nests I monitored were between 30 and 31°C. The proximity of the nesting locales and similarity of nesting microhabitats likely result in similar solar exposure for nest sites of both species. Since G. polyphemus nests are shallower and fluctuate in temperature more than those of C. caretta, one may infer that tortoise nests were warmer in the late 1980s than during the current study. Together, the data for primary and adult tortoise sex ratios and sea turtle nesting suggest that either there are sufficient cool years to offset warmer ones or that females are retained in the population at a lower rate than males. Movement data suggest that the latter is not the case, since males have larger ranges and move longer distances to find mates (Diemer 1992), but differential mortality cannot be ruled out.

The small number of temperature profiles obtained in this study limits the strength of generalizations about nest characteristics and primary sex ratios of KSC tortoises. However, both the estimate of how many female tortoises laid nests in burrow aprons and the position of those nests are similar to the results from studies summarized by Butler and Hull (1996). Furthermore, the demonstration that differences in temperature fluctuation within nests magnify the difference in CTE is important and should be true of most shallow-
nesting TSD species. Within-nest temperature variation has largely been unrecognized as important in the literature. The only previous study to distinguish sex ratios within nests also showed significant effects of egg position (Georges 1992). It is noteworthy that although within-nest temperature variation was sufficient to differentiate a threshold trait such as sex, it is unlikely to affect continuously variable traits such as growth and performance to a biologically meaningful extent.

Conclusions
The best evidence in support of differential fitness comes from growth patterns (Rhen and Lang 1999) and performance (Janzen 1995). However, the uniform adult size and limited duration of differences in performance demonstrated in this study belies the common explanations for how these traits might contribute to the adaptive significance of TSD in G. polyphemus. Additionally, there was insufficient temperature variation within or among nests in this study to differentiate traits other than sex.

The variability in reptilian sex ratios has long been perplexing (Bull and Charnov 1989). It has long been recognized that the deviations from a 1:1 sex ratio that potentially result from TSD may favor GSD. However, the limited data on adult sex ratios for reptiles with TSD do not differ substantially from those that lack TSD (Ewert and Nelson 1991). Furthermore, TSD will select for life-history characteristics that minimize sex-ratio deviations (Bull 1980). Perhaps the ability to maintain viable populations even with relatively labile sex ratios is a consequence of TSD that also makes it hard to lose. Although some potential costs of TSD have been examined theoretically (Girondot and Pieau 1996), little attention has been paid to the lack of empirical data to confirm a cost of TSD.

The role of evolutionary constraint is downplayed in most studies of TSD. The argument against constraint has been that TSD is “phylogenetically plastic” (Ewert and Nelson 1991; Shine 1999). However, this is an entirely subjective determination. Evidence suggests that the kinds of variation produced in this and other constant-temperature studies are ubiquitous among reptiles with both GSD and TSD (Burger 1990; Doody 1999; Elphick and Shine 1999). Unfortunately, most TSD research has been done in species that do not have close GSD relatives, which would allow the results of incubation experiments to be compared. Since the phylogenetic distribution of TSD suggests that most changes are from TSD to GSD, studying closely related taxa that have different sex-determining mechanisms will allow a shift in the traditional question from “is TSD adaptive?” to “what changes favor the evolution of GSD?”

Acknowledgements
I thank R. Seigel, M. Plummer, and B. Crother for assistance in various aspects of the study design, analysis, and reporting. Thanks are also extended to M. Ewert, M. Wade, and B. Freedberg for comments on the manuscript and R. Smith for help in the field. This work was done under U.S. Fish and Wildlife Service (USFWS) permit 90353. NASA, USFWS, and the National Park Service provided housing at KSC. The J.L. Landers Student Research Award and Society for the Study of Amphibians and Reptiles Grants in Herpetology also provided funding.

References

© 2001 NRC Canada


© 2001 NRC Canada