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The effects of incubation environment, sex and pedigree on the hatchling phenotype in a natural population of loggerhead turtles

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ABSTRACT

Explaining environmental sex determination (when offspring sex is determined by a property of the embryonic environment) in reptiles remains one of the greatest problems in the field of sex allocation. We test Charnov and Bull's differential fitness hypothesis in a natural population of loggerhead sea turtles in the field. This hypothesis states that the embryonic environment affects a trait that has different fitness consequences for males and females. We experimentally manipulated the incubation environment experienced by each sex and measured the phenotypic variation observed in hatchlings from experimental clutches and additional natural nests. Sand temperature had a negative correlation and percent water content had a positive correlation on the size of hatchlings from natural nests, and there was a significant interaction between sex and sand temperature on mass. This suggests that females, who develop in warm temperatures, are larger than males at hatching. The Charnov and Bull hypothesis would explain this pattern of environmental sex determination if larger size at hatching leads to a greater increase in lifetime fitness for females than males.

Keywords: *Caretta caretta*, environmental sex determination, incubation temperature, loggerhead turtle, pedigree, phenotype.

INTRODUCTION

In some species, the sex of offspring is determined by the environment in which embryonic development occurs; this is known as environmental sex determination (Charnov and Bull, 1977). Charnov and Bull's (1977) differential fitness hypothesis provides an extremely general explanation of why environmental sex determination can be favoured over other methods of sex determination, such as genetic sex determination. Their hypothesis states that environmental sex determination is advantageous when the relationship between fitness and the embryonic environment is different for males and females. A clear case

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of the differential fitness hypothesis is found in *Gamarus dubeni*, where photoperiod is the environmental variable influencing sex determination (Bullheim and Bull, 1967). Competition for mates results in males gaining a greater fitness benefit than females from being large (McCabe and Dunn, 1997). Offspring produced at the start of the breeding season have the longest growth period and can reach a large size at the peak of the breeding season. As a short photoperiod is associated with the start of the breeding season, males are produced under conditions with a short photoperiod. This differential fitness concept has also been used to explain several cases of environmental sex determination, including that in nematodes (Caullery and Comas, 1928; Christie, 1929) and Atlantic silverside fish (Conover and Kynard, 1981).

However, attempts to explain environmental sex determination in reptiles have been less successful, and this remains one of the greatest problems for sex allocation theory (Janzen and Paukstis, 1988, 1991a; Shine, 1999; West *et al.*, 2002; Janzen and Krenz, in press; F.J. Janzen, submitted). In many reptiles, offspring sex is determined by incubation temperature, or temperature sex determination (Bull, 1980). The relationship between sex and incubation temperature is characterized by the 'pivotal' temperature, which produces an equal sex ratio, and the narrow, 'transitional' temperature range, which produces a mixed sex ratio (Bull, 1980). Across reptiles, three patterns of temperature sex determination have been observed: (1) male sea turtles are produced from nests with temperatures below the pivotal (29°C; Yntema and Mrosovsky, 1982; Mrosovsky and Pieau, 1991; Mrosovsky, 1994; Ackerman, 1997); (2) male lizards and alligators produced from nests above the pivotal (Charnier, 1966; Raynaud and Pieau, 1972; Wagner, 1980); and (3) male leopard geckos and crocodiles produced only from the middle range of nest temperatures (this pattern has two pivotal temperatures; Ferguson and Joanen, 1982; Gutzke and Paukstis, 1984). Unfortunately, because of the difficulty of experimentally separating the different effects of incubation temperature and sex on fitness, minimal progress has been made in determining the fitness advantages of temperature sex determination (Janzen, 1995; Shine, 1999). Ironically, species with genetic sex determination have provided some of the clearest evidence that temperature differentially affects the sexes (Burger and Zappalorti, 1988; Shine *et al.*, 1997; Elphick and Shine, 1999).

Here, we test Charnov and Bull's (1977) hypothesis in loggerhead turtles (*Caretta caretta*), a species with environmental sex determination. The temperature–sex function and the period when sex is determined are well documented in this species. (Yntema and Mrosovsky, 1980, 1982; Mrosovsky and Provancha, 1992). We were able to experimentally separate the effects of incubation environment and sex by manipulating incubation environment after sex had been determined. In long-lived species, such as sea turtles, it is very difficult to study lifetime reproductive success, especially with respect to juvenile traits. Several traits of hatchlings (e.g. size, mass, residual yolk content) may correlate with fitness, and incubation environment may affect these traits differently in males and females (see Shine *et al.*, 1997; Elphick and Shine, 1999). Similar instances, as well as their implications for sex allocation, are well documented in other organisms; for example, the size of parasitoid wasps at emergence has a strong influence on female survival and fecundity (Godfray, 1994; Visser, 1994; West *et al.*, 1996), but less of an effect on male mating success (Charnov *et al.*, 1981; Godfray, 1994). To provide a context for our experiment, we also documented phenotypic variation in hatchlings from natural nests within the population.

METHODS

Study site

We carried out both the experiment and natural nest study on the beaches of Northern Cyprus, eastern Mediterranean. We collected 18 whole clutches to use in the experiment, within 3 days of laying, from a 5 km stretch of beach (35°28′N, 32°94′E) that has a very low hatching success due to a high level of canine predation and regular inundation for the whole beach. We split each experimental clutch, containing 50–100 eggs, into two equal groups and buried them in artificial nests at a depth of 55 cm at two sites: (1) a warm site, a beach with female-producing incubation temperatures, at Alagadi (35°33′N, 33°47′E); and (2) a cool site, a beach with male-producing incubation temperatures, near Korucam (35°40′N, 32°92′E). Incubation temperatures are based on previously collected temperature and sex ratio data (Kaska *et al.*, 1998; Godley *et al.*, 2001a,b; Hays *et al.*, 2001). In addition, we examined phenotype from natural nests laid at these two sites.

Experimental design

At both of these sites, we buried split clutches in hatcheries approximately 8×5 m in size (rather than randomly on the beaches), as a concentrated area can be protected against disturbance by humans, nesting turtles, predation by dogs, foxes and crabs as well be efficiently monitored in the dark (when hatchlings emerge). This method means that replication was not carried out at the hatchery level, a potential problem that we return to in the Discussion. We assessed whether each hatchery was a good representative of the surrounding beach in terms of percent water content and temperature (the two main environmental variables known to affect phenotype). We calculated the mean of three temperature readings and three water content readings for each random sample site of each beach and each hatchery. Temperature was measured with a Hanna temperature probe (accurate to 0.3°C) and water content was calculated from the difference in weight of a 75 g sand sample after dehydration for 4 h at 250°C. Both temperature and percent water content data were collected for 20 beach and 20 hatchery sites at the cool hatchery. We measured temperature at 16 beach and 16 hatchery sites, and percent water content at 14 beach and 15 hatchery sites, on the warm beach.

Sex is determined during the middle third of the incubation period (Bull, 1981; Pieau, 1982; Yntema and Mrosovsky, 1982; Vogt and Bull, 1984), so we used the date each half clutch was laid in conjunction with temperature data from each hatchery to calculate when each half clutch had completed two-thirds of its incubation, based on previous temperature and incubation data from these sites (Kaska *et al.*, 1998; Godley *et al.*, 2001a,b). When each half clutch had reached this point, it was excavated and split into two new groups (making a total of four groups from each original clutch). One of these new groups was reburied in the same place and the other group moved to the hatchery on the other beach. This created a factorial design with four treatments: (1) females incubated at female-producing temperatures, (2) females incubated at male-producing temperatures for the final third of their incubation, (3) males incubated at male-producing temperatures and (4) males incubated at female-producing temperatures for the final third of their incubation. At each split, the identity of the original clutch that each group came from was maintained.

When each experimental clutch began to hatch, we collected all emerging hatchlings and took measurements from a random sample of up to 10 individuals. For each hatchling, the mean was calculated from each of three measurements of maximum straight carapace (dorsal shell) length, total body mass and total body fat. We made carapace measurements with callipers accurate to 0.1 mm and mass was measured using an Ohaus balance (accurate to 0.1 g). Body fat was measured using total body electrical conductivity (TOBEC), using an EM-SCAN model SA-3000 TOBEC meter for live animals. The TOBEC meter readings are relative values and require species-specific calibration for transformation to absolute values. Calibration involves calculating a regression equation relating TOBEC value to absolute fat content from a sample of dead hatchlings (which are hard to obtain outside the field). As TOBEC increases linearly with fat content, we were able to use relative TOBEC values in our analysis. We measured 238 hatchlings from 40 successful (produced live hatchlings) quarter clutch groups. Of the original 18 whole clutches, 3 were unsuccessful, 3 had one successful quarter clutch group, 3 had two successful quarter clutch groups, 5 had three successful quarter clutch groups and 4 had four successful quarter clutch groups.

We collected dead hatchlings for sexing by histology (Yntema, 1976, 1981; Yntema and Mrosovsky, 1980; Mirosovsky and Benabib, 1990; Mrosovsky *et al.*, 1999), to verify that the warm hatchery produced females and the cool hatchery produced males. We sexed 44 hatchlings from seven warm hatchery clutch groups and 16 cool hatchery quarter clutch groups.

Natural nest study

We also measured the phenotypic traits of up to 10 hatchlings from 12 nests in the same way as for the experimental clutches. At laying, we inserted Tinytalk data loggers (Orion components, accuracy 0.1 ± 0.3°C) into the centre of the clutch to give hourly nest temperature readings and calculated percent water content from a 3×75 g sample of sand taken from the egg chamber. In total, we measured 114 hatchlings from 11 nests at the warm beach and one nest at the cool beach. Percent water content was calculated for all 12 nests, but temperature data were only collected for eight nests, so we carried out analyses using temperature only on this subset.

Statistical analysis

We analysed all data in GLMstat, a program that uses generalized linear modelling (GLM; Crawley, 1993) techniques. For the sex ratio (proportion) data, we used binomial errors and a logit link function in an analysis of deviance, as proportion data often have non-normally distributed error variance and unequal sample size and this method retains maximum power (Crawley, 1993). A χ^2-test (proportion data) or *F*-test (parametric data) was used to assess whether the removal of a term caused a significant increase in deviance. The suitability of using binomial errors was assessed after fitting the full model by comparing the residual deviance and residual degrees of freedom. Relatively large values of residual deviance indicate overdispersion and a potential overestimation of significance. To account for this, the residual deviance is rescaled by the heterogeneity factor (ratio of residual deviance to degrees of freedom) and significance testing carried out with an *F*-test (Crawley, 1993). For each of the continuous variables (carapace length, mass and body fat), we

assumed normal errors and carried out a split-plot analysis (for nested data) to retain maximum power and avoid pseudoreplication at the within-clutch (block) level. To avoid problems associated with multiple analysis of the same data set, we only report *P*-values that were significant after Bonferroni correction.

RESULTS

Environmental characteristics of study sites

The warmer beach had a significantly higher mean ($\pm$ standard error) sand temperature (warm beach: 31.71 ± 0.11°C, $n = 32$; cool beach: 30.97 ± 0.84°C, $n = 40$: $F_{1,70} = 16.79$, $P < 0.01$) and a significantly lower mean percent water content (warm beach: 1.7 ± 0.10 g, $n = 29$; cool beach: 2.2 ± 0.10 g, $n = 40$: $F_{1,67} = 12.23$, $P < 0.01$) than the cooler beach. Within the beaches, each hatchery and its surrounding beach did not differ in mean sand temperature or mean percent water content (warm: temperature, $F_{1,30} = 3.18$, $P > 0.05$; percent water, $F_{1,27} = 0.08$, $P > 0.05$; cool: temperature, $F_{1,38} = 1.95$, $P > 0.05$; percent water, $F_{1,38} = 0.31$, $P > 0.05$).

Sex ratios of experimental clutches

We successfully produced female- and male-biased clutches from our two hatchery sites. First, the sex ratio of each experimental clutch was significantly influenced by the temperature of the hatchery in which the sex-determining period was spent (warm hatchery mean = 0.00 ± 0.0, $n = 7$ nests, 10 hatchlings; cool hatchery mean = 0.71 ± 0.08, $n = 16$ nests, 34 hatchlings: $F_{1,21} = 29.91$, $P < 0.01$, HF < 1). The warm hatchery produced entirely female clutches; a high percentage of males was produced from clutches in the cooler hatchery (Fig. 1). Second, the hatchery where the final third of incubation was spent had no effect on sex ratio ($F_{1,20} = 0.07$, $P > 0.05$, HF < 1).

Phenotype of experimental clutches

We carried out a nested analysis with clutch identity as the highest block, sex nested within clutch and final incubation environment nested within sex. Table 1 shows the relationships between each phenotypic trait and experimental treatment (see also Figs 2a–c). The four experimental treatments are represented as the two factors; sex, final environment and their interaction, each with two levels (female/male and warm/cool, respectively). For all phenotypic traits, neither the final environment nor sex had a significant effect. The interaction between sex and final incubation environment had a significant effect on hatchling mass (not length or body fat; Figs 2a,c). The mass of both male and female hatchlings that spent their final third of incubation in the warm environment was greater than that of same-sex hatchlings in the cool environment; this effect was more pronounced in male than female hatchlings. There was no significant effect of original clutch (i.e. block effect) on phenotypic traits. We also calculated narrow sense heritabilities for phenotypic traits (Roff, 1997). Heritabilities of male hatchlings were: carapace length = 0.02, mass = 0.03, body fat = 0.01. Those for female hatchlings were: carapace length = 0.01, mass = 0.03, body fat = 0.01.

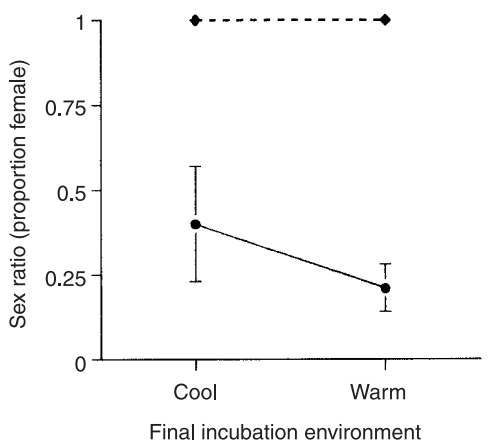

Fig. 1. Mean sex ratio of a sample of clutches used in the experiment. Sex was determined in the warm environment for females and the cool environment for males. From the female-producing treatment, we sexed samples from four clutches in the warm environment and three clutches from the cool environment. From the male-producing treatment, we sexed samples from seven clutches in the cool environment and nine clutches in the warm environment. Asymmetric standard error bars are shown for treatments where the standard error is greater than 0.0. ●, males; ◆, females.

Table 1. *F*-ratios and *P*-values (significant after Bonferroni correction) for each measure of phenotype and potential explanatory variables

	Sex–final environment	Sex	Final environment	Clutch identity
Carapace length	$F_{1,211} = 1.25$ $P = 0.27$	$F_{1,222} = 1.57$ $P = 0.21$	$F_{1,212} = 0.68$ $P = 0.41$	$F_{14,236} = 1.69$ $P = 0.06$
Mass	$F_{1,210} = 16.35$ $P < 0.0001$	$F_{1,222} = 0.18$ $P = 0.67$	$F_{1,211} = 0.62$ $P = 0.43$	$F_{13,235} = 1.45$ $P = 0.14$
Body fat	$F_{1,208} = 0.06$ $P = 0.81$	$F_{1,220} = 0.02$ $P = 0.89$	$F_{1,209} = 0.41$ $P = 0.52$	$F_{13,233} = 1.47$ $P = 0.13$

Note: Sex–final environment refers to the interaction between hatchling sex (determined by incubation temperature for two-thirds of incubation duration) and the incubation environment for the final third of incubation. Clutch identity refers to the original clutch each experimental group was from.

Phenotype of natural nests

Across nest means, temperature did not correlate significantly with any phenotypic traits (length, $F_{91,6} = 0.90$; mass, $F_{1,6} = 0.23$; body fat, $F_{91,6} = 1.19$). Water content had a significant positive correlation with body fat ($F_{1,10} = 7.72$, $P < 0.05$), but not with length ($F_{1,10} = 0.71$) or mass ($F_{1,10} = 1.27$). We also analysed these data using individuals as data points and clutch identity as a factor. Although these analyses were not independent from those above and involved pseudoreplication at the within-clutch level, they show the relative importance of environmental and clutch effects on phenotype. The analysis of individuals showed a strong effect of clutch identity with all three phenotypic traits (carapace length: $F_{11,102} = 22.39$, $P < 0.01$; mass: $F_{11,102} = 37.35$, $P < 0.01$; body fat: $F_{11,102} = 5.27$, $P < 0.05$). In this analysis,

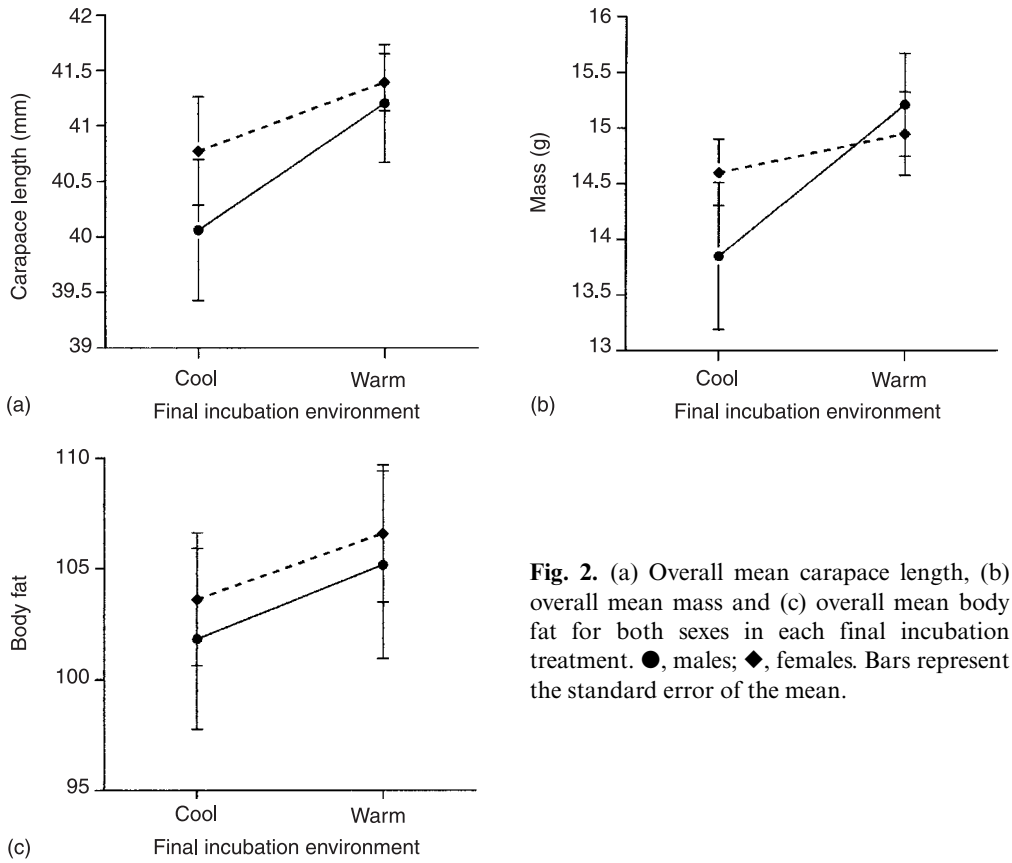

Fig. 2. (a) Overall mean carapace length, (b) overall mean mass and (c) overall mean body fat for both sexes in each final incubation treatment. ●, males; ◆, females. Bars represent the standard error of the mean.

temperature had a significant negative correlation and percent water content a significant positive correlation with carapace length (temperature: $F_{1,69} = 6.75$, $P < 0.05$, Fig. 3a; percent water: $F_{1,102} = 6.80$, $P < 0.05$, Fig. 3b), but not body mass (temperature, $F_{1,69} = 1.15$; percent water, $F_{1,101} = 1.00$) or body fat (temperature, $F_{1,69} = 0.04$; percent water, $F_{1,102} = 0.06$).

DISCUSSION

We successfully produced markedly different sex ratios in the different experimental hatcheries (warm = 100% female, cool = 70% male; Fig. 1). Manipulating incubation environment showed an interaction between environmental variables and the phenotype (mass) of male and female hatchlings ($P < 0.001$ after Bonferroni correction). The narrow sense heritabilities were low for males and females (<0.04), encompassing inheritance from both genes and maternal effects. The greater increase in mass (from the warm environment) gained by male than female hatchlings suggests that: (1) environmental temperature is less important in terms of body mass for female than male hatchlings and (2) to fit the Charnov and Bull (1977) hypothesis, it is less beneficial for males to be very heavy at hatching. For example, male turtles may metabolize too much of their yolk reserves if they develop in warm temperatures, whereas female fitness is enhanced or not adversely affected by warm temperatures. Conversely, in the analysis of individual hatchlings from natural nests,

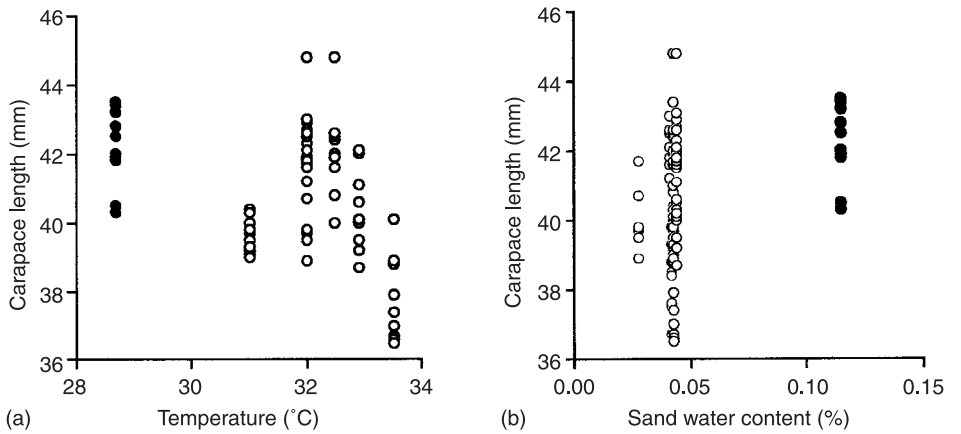

Fig. 3. (a) Plot of temperature and (b) plot of percent water content against carapace length of individual hatchlings from natural nests. There were significant correlations with this data set, but not when using mean carapace length for each nest. ○, data from warm beach; ●, data from cool beach.

temperature had a negative correlation and percent water content a positive correlation with carapace length (Figs 3a,b). In the analysis of nest means, the only significant result was a positive effect of percent water content on body fat, but this effect was not observed in individuals. We also found that it is possible to manipulate the incubation environment in the field after sex has been determined, and we hope this will provide a useful base for further field studies.

To our knowledge, this is one of only a few entirely field-based studies to address why the sex of reptiles is determined by an environmental variable. However, working on a field population of an endangered species constrained our experimental design; we could not replicate our two hatchery treatments and adequately protect hatchlings while minimizing the number of clutches used. This problem (Hurlbert, 1984) is common in studies of environmental sex determination; for example, laboratory studies commonly use data from multiple animals in a single incubator, as finances constrain the number of incubators per treatment. Although greater replication at the hatchery/block level is the best solution (e.g. by blocking the experiment and carrying out different treatments in the same incubator sequentially, or by repeating the experiment over multiple years), another approach has been to use indirect evidence to verify that treatment variables are the most important causal factors (e.g. Shine and Elphick, 2001). We found no significant difference between each hatchery and its surrounding beach in terms of water content and temperature, which are known to be important causal variables.

We have shown that the incubation environment can differentially influence the phenotype of male and female hatchling loggerhead turtles; if warm nests consistently produce large and heavy hatchlings, then these hatchlings are likely to be female and males (from cool nests) are likely to be smaller. The Charnov and Bull (1977) hypothesis could explain why environmental sex determination exists in this population if a larger size at hatching confers a greater lifetime fitness gain to females than males. However, there are several reasons to suspect that their hypothesis might not be important for this population (but see Rhen and Lang, 1995; Shine *et al.*, 1995; Janzen, 1996): (1) the difference in magnitude of

environmental and clutch effects on phenotype; (2) it is possible that carapace length, mass and body fat at hatching are traits that do not persist into adulthood – especially as females are the larger sex at maturity (Godley *et al.*, 2002); (3) also, it is possible that the small effects of temperature and humidity we detected indicate that the environment is important, but that Northern Cyprus may have a 'homogeneous incubation environment', and the environmental variation between nest sites is not great enough to generate large differences in phenotype. Stronger effects on phenotype may have been induced if we had manipulated the environment earlier in development (Shine and Elphick, 2001). Unfortunately, measuring lifetime reproductive success is currently impossible in long-lived species such as sea turtles, let alone associating variation in lifetime reproductive success with hatchling traits (Shine, 1999; but see C.L. Morjan and F.J. Janzen, submitted).

Current literature on environmentally induced variation in phenotype generally reports that large hatchling size correlates with: (1) water available to facilitate yolk metabolism (Morris *et al.*, 1983; Miller and Packard, 1992; Packard *et al.*, 1993; Packard, 1999); (2) cool temperatures, which are associated with humidity and longer incubation (Packard, 1999); (3) egg size and yolk provisioning (Packard *et al.*, 1993; Roosenburg, 1996; Steyermarker and Spotila, 2001; C.L. Morjan and F.J. Janzen, submitted). The relative contributions of the environment and maternal effects to phenotype are unclear and all possibilities, from equality to strong skews, have been documented in reptiles (Morris *et al.*, 1983; Janzen, 1993; Shine *et al.*, 1997; Packard, 1999). How these variables interact with fitness is unclear; for example, large hatchlings can run faster and escape predators (Miller *et al.*, 1987; Janzen, 1993; Packard, 1999), but have consumed more yolk and may need to feed sooner after emergence than small hatchlings that have larger yolk reserves.

Our experimental results contradict the sparse existing literature, because hatchlings spending the final third of their incubation period in the warm environment were heavier than their counterparts in the cool environment regardless of sex. We suggest two possible explanations for this observation. First, clutches in the cool hatchery may have experienced a detrimental level of sand water content. McGhee (1990) demonstrated that, in loggerhead turtle nests, the field mean for water content was 18% and levels exceeding 25% impaired growth. Second, there was an interaction between the environment and stage of development, so warm temperatures towards the end of incubation boosted hatchling size. Our paradoxical experimental results emphasize the need for interpreting the fitness consequences of phenotypic variation with caution and that further experimental investigation is merited.

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