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The Effect of Thermal Variance on the Phenotype of Marine Turtle Offspring

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ABSTRACT

Temperature can have a profound effect on the phenotype of reptilian offspring, yet the bulk of current research considers the effects of constant incubation temperatures on offspring morphology, with few studies examining the natural thermal variance that occurs in the wild. Over two consecutive nesting seasons, we placed temperature data loggers in 57 naturally incubating clutches of loggerhead sea turtles *Caretta caretta* and found that greater diel thermal variance during incubation significantly reduced offspring mass, potentially reducing survival of hatchlings during their journey from the nest to offshore waters and beyond. With predicted scenarios of climate change, behavioral plasticity in nest site selection may be key for the survival of ectothermic species, particularly those with temperature-dependent sex determination.

Introduction

Abiotic factors can have a profound influence on embryonic development in reptiles, notably, temperature (Booth 2006). Thermal environmental shifts have the power to influence numerous aspects of egg incubation, such as incubation du-

ration, offspring sex ratios, hatching success, morphology, and locomotor performance, in a vast number of reptile species (Godfrey et al. 1999; Brana and Ji 2000; Godley et al. 2001; Ashmore and Janzen 2003; Booth 2006; Witt et al. 2010; Refsnider 2013). Given climate change predictions and their associated risks to biodiversity (Sala et al. 2000; Thomas et al. 2004; Hansen et al. 2006; Goodess et al. 2011), it is becoming increasingly important that we seek to quantify and understand the potential environmental pressures that threaten the survival of vulnerable wild reptile populations, whose life strategies are inherently linked with climate.

In an attempt to understand the relationship between temperature and phenotype, a large number of studies have examined the effect of constant incubation temperatures on aspects of reptilian offspring morphology, performance, and survival (Ashmore and Janzen 2003; Du and Ji 2003; Schwanz and Janzen 2008; Micheli-Campbell et al. 2012; Refsnider 2013). The results of such studies are by no means universal, as highlighted in Booth (2006), in which contradictory findings from a range of investigations into the relationship between incubation temperature and offspring locomotor performance, as well as posthatch growth rates, are presented for freshwater turtles, lizards, snakes, and crocodilians.

One near-universal pattern that is typically observed in ectotherms is the temperature-size rule (TSR). This is a phenotypically plastic within-species response in which organisms reared in warm temperatures mature to a smaller adult body size than those reared in the cold (Atkinson 1994; Atkinson and Sibly 1997; Forster et al. 2011, 2012, 2013; Forster and Hirst 2012). Studies of the TSR focus predominantly on constant temperatures, comparing size at maturation in warmer environments versus colder environments. However, an increasing number of publications have also begun to emphasize the role of temperature variation in determining physiological and morphological attributes, with one recent study suggesting that increased temperature variation poses a greater risk to invertebrate species than climate warming (Vasseur et al. 2014). Similarly, a growing number of studies have begun to examine thermal variance during incubation and its effect on body size and other traits of reptilian offspring (Ashmore and Janzen 2003; Micheli-Campbell et al. 2012; Georges 2013; Li et al. 2013; Refsnider 2013), such as crawl and swim speed in freshwater turtles (Refsnider 2013) and hatching duration and success in lizards (Du and Feng 2008).

Despite the growing interest in thermal variance, relatively little focus has been given to marine turtles, perhaps because it is more challenging to successfully rear and study them in a temperature-controlled laboratory environment. As long-

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lived migratory reptiles, which exhibit temperature-dependent sex determination (TSD), natal philopatry, and high levels of site fidelity (Lutz et al. 2003), they have become the focus of extensive conservation efforts because of a magnitude of anthropogenic threats, such as coastal development, accidental capture in fishing gear, and illegal harvesting, as well as a rapidly changing abiotic environment (Polidoro et al. 2008). Marine turtles have been at a progressively greater risk from abiotic stress over the past 2 decades, particularly in the Mediterranean, where temperature extremes have continued to rise (Sala et al. 2000; Hansen et al. 2006; Goodess et al. 2011). While some may argue that, despite extensive climatic shifts, reptiles have persisted for millennia, such changes most likely occurred gradually, allowing species to adjust, adapt, and evolve to their changing environment over time (Booth 2006). The current anthropologically forced rate of change far exceeds that experienced in the past, and the Mediterranean region is predicted to undergo one of the greatest changes in biodiversity globally (Sala et al. 2000; Goodess et al. 2011).

In light of the observed and predicted temperature increases and their associated risks, many studies have looked to strengthen our knowledge of TSD in marine turtles, in which an incubation temperature above approximately 29°C produces a larger proportion of females and an incubation temperature below 29°C produces a larger proportion of males (Marcovaldi et al. 1997; Girondot 1999; Godfrey et al. 1999; Godley et al. 2001; Mrosovsky et al. 2009; Witt et al. 2010; LeBlanc et al. 2012). However, few studies have looked to quantify the effects of incubation temperature on other characteristics of offspring phenotype, such as size, with a handful having done so in natural nests without manipulation and the use of artificial incubation (Glen et al. 2003; Mickelson and Downie 2010; Read et al. 2013). Although artificial incubation can undoubtedly enhance our knowledge of the relationship between temperature and phenotype, such studies typically keep temperatures constant or cycled at a constant rate, failing to imitate the natural and unpredictable thermal variance that occurs in the wild. This thermal variation, and the rate at which it occurs, has the potential to significantly influence offspring development and is difficult to re-create in a laboratory environment. Thus, studies on in situ clutches are essential in order to understand the true nature of this relationship and how it is likely to change.

The primary objective of this study was to quantify the effects of thermal variance on offspring mass of the loggerhead turtle (*Caretta caretta*) in naturally incubating nests. The loggerhead is arguably the most ecologically generalized marine turtle, being globally distributed and having one of the least specialized diets of all marine turtle species, making it an ideal model species in which to assess the impact of global abiotic stress (Bolten and Witherington 2003). Given what has been presented in laboratory-based studies of freshwater turtles and other reptiles (Ashmore and Janzen 2003; Booth 2006; Micheli-Campbell et al. 2012), we hypothesize an inverse relationship between thermal variance and offspring mass, in which greater diel temperature fluctuations during

incubation will produce smaller offspring. Detecting such a relationship will bring us closer to understanding the link between thermal variance and phenotype in reptilian species. We also seek to understand and control for the role of maternal factors in influencing the amount of thermal variance experienced during incubation, as maternal effects and the extent to which females can exhibit behavioral plasticity may be key to the survival of the species in the face of rapid environmental shifts.

Material and Methods

Study Site

This investigation was conducted at an established study site on Alagadi Beach (35°33'N, 33°47'E), located in northern Cyprus in the eastern Mediterranean. This 2-km stretch of beach comprises two short coves, approximately 0.8 and 1.2 km long, divided by a rocky headland. Alagadi Beach is recognized as one of the most important turtle nesting beaches for loggerhead turtles in Cyprus and in the eastern Mediterranean. Consequently, it has been designated a Specially Protected Area because of the high number of marine turtles that frequent the beach each year during the breeding season (May–October). Although the beach is open to the general public during the day, it is closed at night, between 2000 and 0800 hours, for the duration of the breeding season to avert any possible disturbance to nesting turtles and hatching nests. Average daily air temperatures throughout the breeding season generally range between 27° and 36°C, and there is little or no rainfall during the summer months. No nests experience shading on Alagadi Beach.

Data Collection

We recorded intranest temperature for 57 *Caretta caretta* clutches laid by 29 females during the 2011 and 2012 nesting seasons. Temperature loggers (Tinytag, Gemini Dataloggers, Chichester, UK; resolution $\pm 0.1^\circ\text{C}$, accuracy $\pm 0.3^\circ\text{C}$), which had been previously calibrated and programmed to record at hourly intervals, were placed approximately in the center of the clutch during laying, at which point the female was measured (curved carapace length [CCL]), tagged if no existing tags were present, and allowed to cover her eggs naturally. After 40 d of incubation, a circular wire mesh ring cage (height = 30 cm, diameter = 2 m) was placed around the nest to capture emerging hatchlings. Hatchlings were then weighed using electronic scales (accuracy ± 0.1 g), measured using digital callipers (straight carapace length; accuracy 0.1 mm), and released. We sampled the first 10 hatchlings to emerge or a randomly selected subsample when >10 emerged in a group on the initial night of hatchling emergence. Following hatchling emergence, the clutch remains were excavated, the depth of the top and bottom of the clutch and of the temperature data logger were recorded to the nearest centimeter using a measuring tape (calculated as an average of

three repeat measurements), and the data logger was retrieved. Clutch success was assessed through counts of hatched and unhatched egg shell fragments.

Data Analysis

Mean incubation temperature was calculated as the average of all temperature readings taken between the time of laying and midnight on the initial night of hatchling emergence. Diel thermal variance was calculated as the average daily temperature fluctuation experienced during incubation (i.e., the mean of the difference between the minimum temperature and the maximum temperature for each day of incubation).

All statistical analyses were conducted using the statistical software package R (ver. 2.13.2). Three generalized linear mixed models (GLMMs) were used to analyze the effect of both maternal (female size, lay date, clutch size, clutch number, total number of clutches laid by the female, nest depth) and abiotic (mean incubation temperature, mean diel thermal variance) factors on mean offspring mass and hatching success, as well as to determine the significant drivers of thermal variance. Female ID and year were included as random factors in all instances. Final models were generated using stepwise elimination of nonsignificant terms ($P > 0.05$). Significance of all fixed variables was calculated using maximum likelihoods and Wald statistics (χ^2). In addition, regression analysis was used to assess whether a significant relationship ($P < 0.05$) existed between mean female CCL and (a) mean nest depth and (b) mean diel thermal variance.

Results

A total of 57 clutches were monitored across two consecutive nesting seasons (2011: $n = 30$; 2012: $n = 27$), during which a total of 864 loggerhead turtle hatchlings were measured (2011: $n = 410$; 2012: $n = 454$). Table 1 shows maximum, minimum, and mean values for all relevant data used in this investigation. The distribution of nest depths (midpoint depth of clutch) in both years was very similar (fig. A1a). Mean offspring mass varied markedly, however, being approximately 11.3% lower in 2012 than 2011 (fig. A1b). Mean diel thermal variance during incubation ranged from 0.17° to 1.41°C. This

variation decreased toward the middle of the nesting season, before gradually increasing again. This increase was particularly great in 2012 from mid-July onward (fig. 1). Mean incubation temperature increased initially, gradually decreasing toward the middle of August, reflecting expected seasonal surface temperature changes and peaking at 33.3°C in 2011 and 34.0°C in 2012 (fig. 1).

Of the maternal effects (female size, lay date, clutch size, clutch order, total number of clutches, nest depth) and abiotic factors (mean incubation temperature, diel thermal variance) included in our model, only mean diel thermal variance had a significant influence on mean offspring mass (GLMM; $\chi^2_{4,5} = 19.23$, $P < 0.001$; fig. 2a); for every 0.5°C increase in mean diel thermal variance during incubation, mean offspring mass decreased by approximately 0.74 g, which is equivalent to 4.6% of body mass.

Consequently, a second GLMM was used to determine the drivers of change in mean diel thermal variance (mean nest depth, mean female CCL). Mean diel thermal variance was significantly influenced only by mean nest depth (GLMM; $\chi^2_{4,5} = 8.77$, $P = 0.003$; fig. 2b); for every 10-cm increase in mean nest depth, mean diel thermal variance decreased by 0.15°C. We also found a significant positive relationship between mean female CCL and mean nest depth (ANOVA; $F_{1,55} = 10.49$, $P = 0.002$; fig. 2c) and a corresponding negative relationship between mean female CCL and mean diel thermal variance (ANOVA; $F_{1,47} = 9.99$, $P = 0.003$).

Having established how both maternal and abiotic factors influenced offspring phenotype, a final model (incorporating mean diel thermal variance, mean incubation temperature, and mean nest depth) was used to determine which of these factors, if any, significantly influenced hatching success. Only mean incubation temperature had a significant influence on hatching success (GLMM; $\chi^2_{4,5} = 17.77$, $P < 0.001$). Hatching success appeared to be greatest between 31° and 32°C, and our data suggest that clutches incubated at a mean temperature exceeding 35.1°C will result in 0% hatching success (fig. 2d).

Discussion

Having collected data over two consecutive nesting seasons, we show that thermal variance during incubation does sig-

Table 1: Descriptive statistics of 2011 and 2012 data collected for female curved carapace length (CCL), offspring mass, nest depth, mean diel thermal variance per nest, and mean incubation temperature per nest

	Female CCL (cm)		Offspring mass (g)		Nest depth (cm)		Mean diel thermal variance (°C)		Mean incubation temperature (°C)	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
Maximum	81.6	80.2	18.2	17.8	55.8	54.3	1.1	1.4	33.3	34.0
Minimum	65.3	68.5	11.1	11.4	19.8	28.3	.2	.2	30.7	29.0
Mean	75.8	75.0	16.3	14.5	42.3	42.8	.5	.5	32.1	32.3
±SE	1.1	1.0	.3	.3	1.5	1.1	.1	.1	.1	.3

Note. Number of clutches: 2011, $n = 30$; 2012, $n = 27$. Temperature readings were taken from data loggers placed in the approximate center of the clutch at the time of laying.

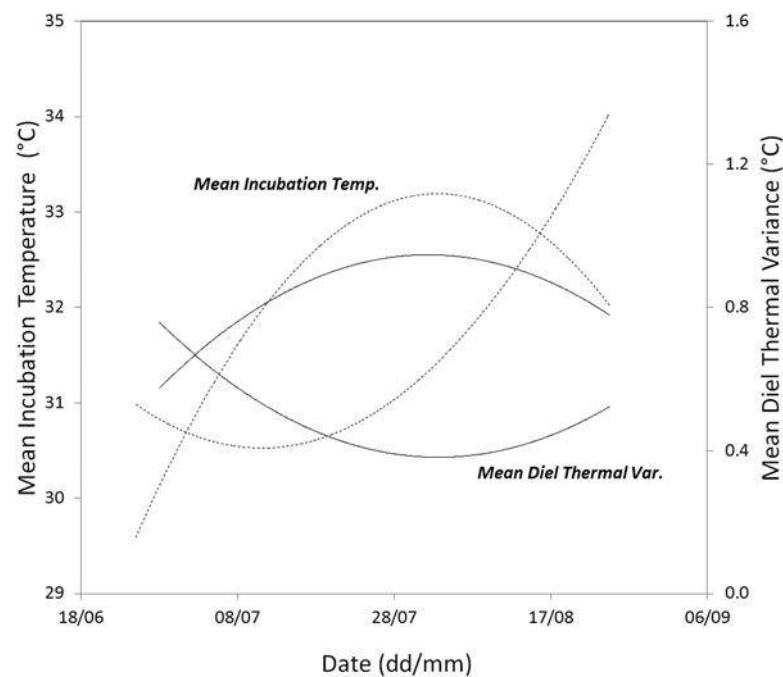


Figure 1. Change in mean incubation temperature (n-shaped curve) and mean diel thermal variance during the nesting season for 2011 (solid line) and 2012 (dotted line).

nificantly influence offspring mass in loggerhead turtles. The apparent inverse relationship between the extent of diel thermal variance experienced during nest incubation and loggerhead turtle offspring mass supports our hypothesis, complementing the findings of previous laboratory-based studies on reptiles (Ashmore and Janzen 2003; Booth 2006; Micheli-Campbell et al. 2012) and strengthening their reliability by verifying that a similar relationship does exist in the wild.

Besides the direct influence of abiotic factors, because of seasonal variability in environmental conditions, thermal variance was significantly influenced by nest depth, not surprisingly, as shallower nests have an increased proximity to the sand surface and a greater vulnerability to abiotic change (Booth 2006). Perhaps more interestingly, nest depth was significantly coupled with female size, suggesting that this is an important maternal factor in determining the extent of temperature variation experienced during incubation and consequently offspring mass. The results suggest that, although female size does not appear to directly affect offspring mass via a genetic hereditary component, it does have an environmental maternal influence.

But why does offspring mass matter? Direct links between turtle hatchling morphology and fitness have been previously proposed (Ashmore and Janzen 2003; Booth 2006; Burgess et al. 2006; Mickelson and Downie 2010; Micheli-Campbell et al. 2012; Read et al. 2013; Refsnider 2013), and any change in mass as a result of abiotic stress has the potential to affect hatchling survival during their initial journey from the nest to the sea, a stage in which they face a substantial predatory

threat from canids, feral/domestic dogs, birds, and ghost crabs. A negative change in marine turtle hatchling mass has been shown to be accompanied by a decrease in flipper size and a decline in terrestrial locomotion performance (Mickelson and Downie 2010). Conversely, Refsnider (2013) showed that an increase in diel thermal variation increased hatchling speed in freshwater turtles. In addition, higher incubation temperatures have been revealed to reduce swimming speed in green turtles *Chelonia mydas* (Burgess et al. 2006). Turtle hatchlings face a considerable predatory threat once in the water, and a reduction in swimming speed would increase the amount of time spent in the predator zone. Indeed, Gyuris (2000) found that larger freshwater turtle hatchlings had significantly greater survivorship because of the limited gape size of the fish that prey on them, suggesting that there is a fitness advantage to being bigger. Any significant increase in diel thermal variance experienced during incubation is likely, therefore, to have consequences for hatchling mass, locomotion performance, predation risk, and ultimately survival.

Clearly, the proximate mechanisms that cause marine turtle offspring to be smaller under greater thermal variance still need to be addressed. Although the effect of mean incubation temperature on hatchling mass was found to be nonsignificant in our model, it should not be overlooked. For instance, higher mean incubation temperatures have been shown to produce significantly smaller hatchlings in the green turtle (Burgess et al. 2006). It may be that the effect of mean incubation temperature on hatchling mass was not strong enough to detect from our data. Sand water content has also been shown to be

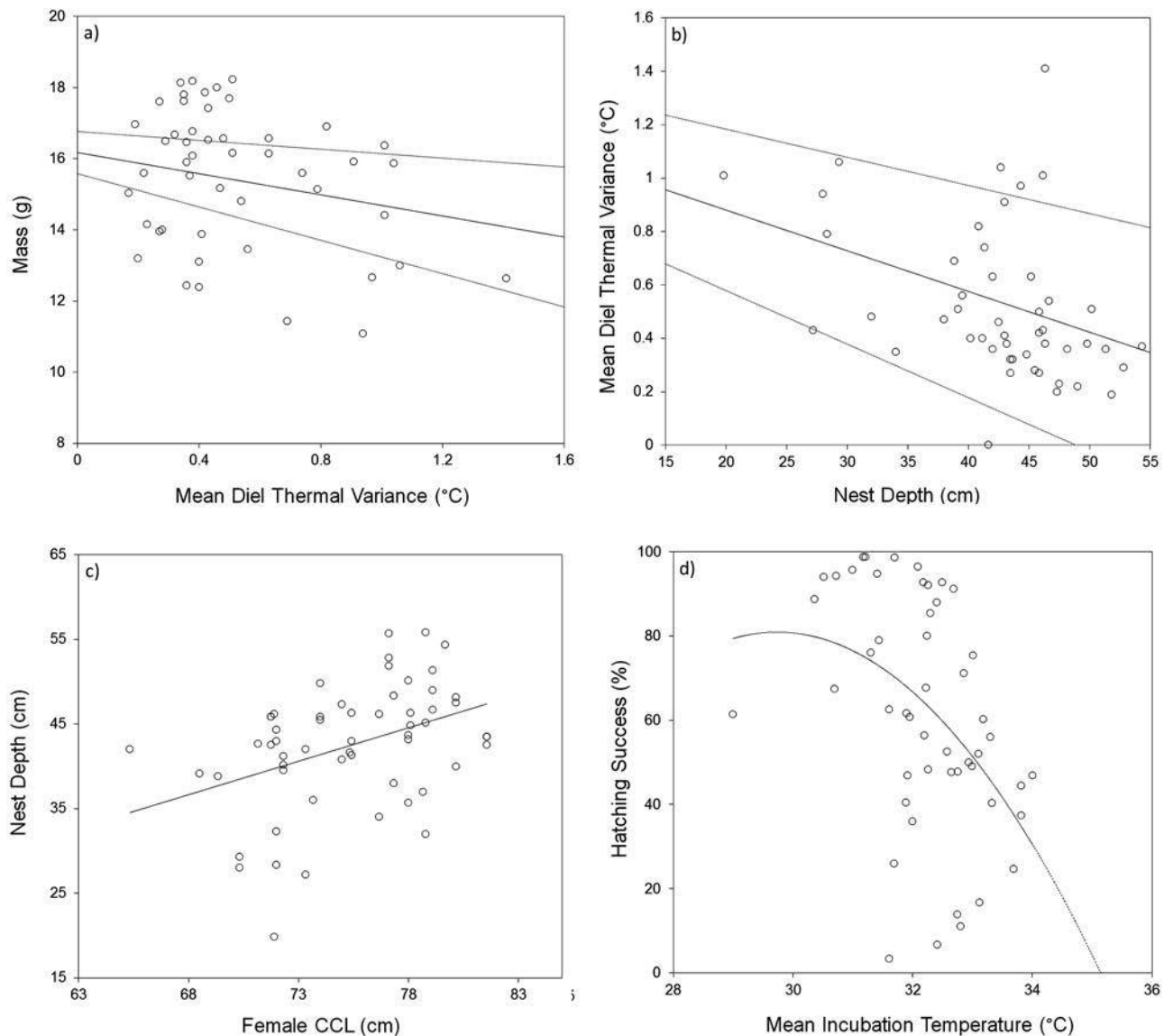


Figure 2. *a*, Effect of mean diel thermal variance on offspring mass. Solid line represents model output. Dotted lines represent the standard error (± 0.86 g). *b*, Effect of nest depth on mean diel thermal variance. Solid line represents model output. Dotted lines represent standard error ($\pm 0.005^{\circ}\text{C}$). *c*, Relationship between female curved carapace length (CCL) and nest depth. *d*, Relationship between mean incubation temperature and hatching success over 2011 and 2012. Dotted line represents extrapolated line of best fit.

positively correlated with hatchling size in loggerheads (McGehee 1990; Reece et al. 2002). Addressing these concerns would undoubtedly help improve our understanding of the mechanisms governing this relationship.

In contrast to the findings of a recent laboratory-based study on freshwater turtles (Micheli-Campbell et al. 2012), thermal variance appeared to have no significant influence on hatching success. Although focused on different species, this disparity may be explained by the relatively large difference in diel thermal variance experienced. Whereas the maximum mean diel temperature fluctuation recorded during our study was only 1.41°C , diel variation during artificial incubation in

the laboratory study reached 12°C . Additionally, artificially incubated eggs were repeatedly exposed to a constant level of diel variation during development, unlike those incubated naturally in the wild. If mean diel thermal variance increased substantially in the Mediterranean and conditions became less stable, we may expect a detectable relationship between diel temperature fluctuation and hatching success to arise in marine turtles.

Nests with higher mean incubation temperatures had lower hatch success on average. By extrapolating these data, we estimate that a mean incubation temperature exceeding approximately 35.1°C will result in 0% hatching success. This

figure is in line with recently predicted figures of $\sim 35^{\circ}\text{C}$ (Hawkes et al. 2007; Valverde et al. 2010). The absolute maximum incubation temperature recorded during this study was 35.9°C . If these mounting data are accurate, these figures appear extremely concerning when viewed alongside predicted surface temperature increases of up to 4°C by 2100 (Hansen et al. 2006), suggesting that TSD and thermal variance may become irrelevant if mean incubation temperatures exceed the predicted threshold for successful hatching.

There has been much discussion of late regarding the potential for maternal adjustment of nest depth in model species of freshwater turtles, more specifically, whether the manipulation of nest depth is likely to deliver benefits in the face of a rapidly changing climate (Georges 2013; Refsnider et al. 2013a, 2013b; Rödder and Ihlow 2013; Schwanz 2013). In a small-bodied freshwater species, Refsnider et al. (2013b) concluded that maternal adjustment of nest depth is unlikely to significantly improve offspring sex ratio skews, suggesting instead that shifts in components of nest site choice, rather than nest depth, may be a more profitable maternal response. Indeed, in comparing painted turtle (*Chrysemys picta*) neonates from maternally selected nest sites with those from randomly selected nest sites, Mitchell et al. (2013) recently presented evidence that sex ratio selection appears to be an important component of nest site choice in reptiles with TSD, supporting theories that behavioral plasticity will be a key factor in ensuring the reproductive success of reptiles. Given that marine turtles exhibit natal philopatry and site fidelity, the choices available to a nesting female when choosing a nest site are limited (Lutz et al. 2003; Hawkes et al. 2007). Deeper nests may help to negate the impacts of abiotic stress, but as shown in this study, nest depth is positively correlated with female size. Assuming that fertility is constant with age and size, this potentially means that the reproductive success of recently matured smaller females may be most at risk from less thermally stable conditions.

In several areas, beach development and coastal squeeze have begun to reduce the amount of cooler shaded habitat available at nesting sites, such as dunes and dense vegetation, that may otherwise provide preferable incubation conditions and improve both hatching success and sex ratios (Hawkes et al. 2007; Schlacher et al. 2007; Refsnider and Janzen 2012). Recent publications have begun to recognize the value of nest shading that natural vegetation provides, highlighting its importance in maintaining thermal heterogeneity and facilitating behavioral plasticity in nest site choice (Kamel 2013; Refsnider et al. 2013c; Woods et al. 2014). Perhaps most im-

portant, then, if females are to behave plastically when choosing a nest site and maximize their reproductive success, conservation efforts need to focus on maintaining heterogeneity at nesting sites, providing microhabitats that allow females to do so. Hence, protecting nesting beaches that support sufficient beach vegetation for clutch shading should be a conservation priority. Additionally, this means that for populations that are heavily conservation dependent, options are available to relocate clutches at risk to a more suitable area of the beach, without having to transport them long distances. Where vegetation has been lost and is unlikely to recover, artificial shading could be a solution. Shading existing nests would remove the need to relocate clutches; however, this does require substantial manpower.

Marine turtle life history and a lack of heterogeneity may limit nest site choice for females. Consequently, rather than adapt spatially, females may adapt temporally. Based on the findings in this study and the predictions of others (Pike et al. 2006; Hawkes et al. 2007), we may expect to see turtles nesting earlier or later in the season, when temperatures are less extreme. However, diel thermal variance appears greater during these periods. Thus, although nesting earlier may avoid detrimental mean incubation temperatures, the effects of thermal variance, if independent of the high thermal limits experienced at Alagadi, may persist. Whether hatchlings will be faster, as suggested by Refsnider (2013), or suffer costs to terrestrial locomotion, as suggested by Mickelson and Downie (2010), it is evident that the mechanisms governing this relationship need to be explored in more depth and the fitness consequences and potential adaptive benefits of such a response should be investigated in more detail. This study highlights the need for further research into how different patterns of temperature variation affect reptilian offspring and the mechanisms driving these changes.

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APPENDIX

Supplemental Figure

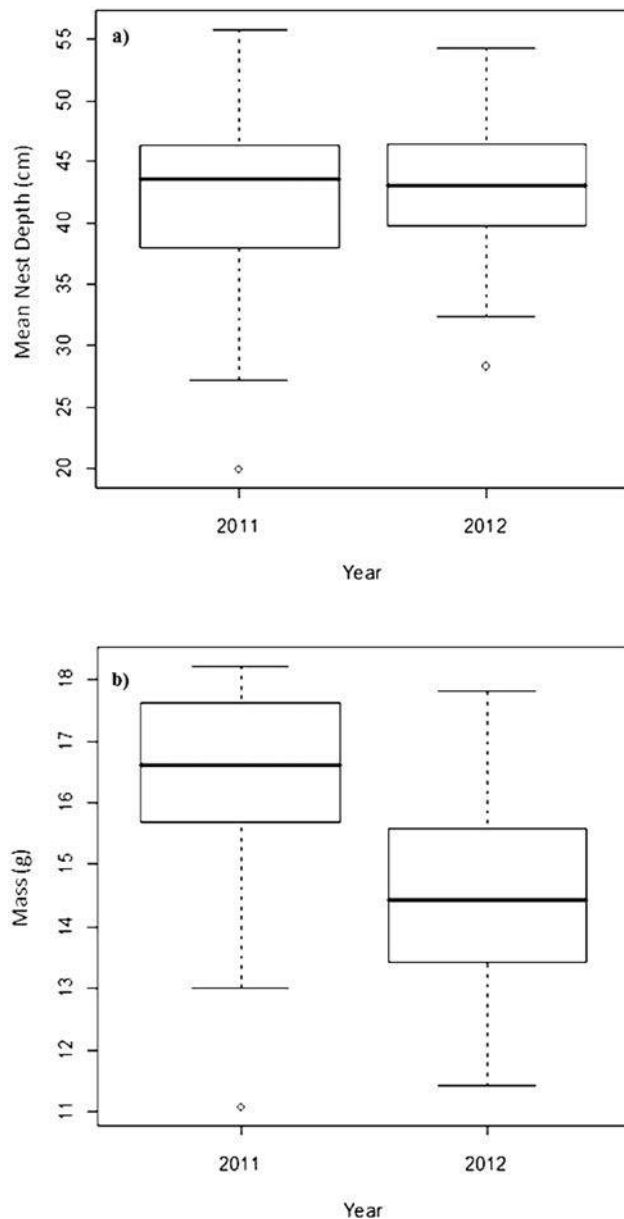


Figure A1. *a*, Distribution of mean nest depth for 2011 and 2012. *b*, Distribution of mean offspring mass per nest for 2011 and 2012 (whiskers represent data within 1.5 interquartile range of the lower and upper quartiles).

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