

EFFECTS OF TEMPERATURE ON HATCHING SUCCESS IN FIELD INCUBATING NESTS OF SPUR-THIGHED TORTOISES, *TESTUDO GRAECA*

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Spur-thighed tortoises, *Testudo graeca*, in south-western Spain lay 3-4 clutches in shallow nests from April to June. In the present study the incubation temperature of nests laid in field enclosures in April, May and June was monitored over four years. Mean daily temperature throughout incubation averaged 27.9°C, but displayed a wide daily range, with average maximum values around 41°C (also in nests where hatching success was >0), and an absolute maximum of almost 50°C. Early (April) nests displayed lower mean daily temperatures than intermediate (May) and late (June) nests, although all nests reached similar high temperatures during the hottest month (July). Incubation temperatures were affected by nest vegetation cover. Incubation length varied from 67-129 days. Because the length of incubation was negatively correlated with nest temperature, early nests had longer incubation periods than intermediate and late nests. Hatching success averaged 61% and was mainly affected by variables related to maximum temperatures. Thus unsuccessful nests (i.e. no eggs hatching) were associated with higher temperatures or longer exposure to higher temperatures. Differences in hatching or nest success were not related to the nesting month, but might have been influenced by the location of the nest. Lethal temperatures for embryo development were frequently reached during July, therefore vegetation cover of the nest is likely to play an important role in avoiding deleterious nest environments.

Key words: chelonia, incubation temperature, nesting, reproductive success

INTRODUCTION

Reptile eggs are highly influenced by their nest environment, mainly soil moisture and temperature. While flexible shelled eggs require the absorption of water from the surroundings to complete development, embryos of hard shelled eggs are relatively independent of variation in substrate moisture, relying mostly on the water supplied by the female at oviposition (Packard, 1999; Tracy & Snell, 1985; Congdon & Gibbons, 1990). Temperature, however, significantly affects both types of eggs. Thermal tolerance limits of embryos are known to range between 22 and 35°C in most reptiles for incubation at constant temperatures in the laboratory. However, field incubating embryos may withstand short periods of temperatures below and above these limits (Ewert, 1979; Congdon & Gibbons, 1990). Incubation temperature strongly influences embryo development and growth rates, thus conditioning the length of the incubation period (Gutzke *et al.*, 1987; Packard & Packard, 1988; Deeming & Ferguson, 1991). Fast embryonic development is associated with a less efficient metabolism, producing large residual yolks (Deeming & Ferguson, 1991). Incubation temperature of eggs kept under laboratory conditions also affects sex determination (e.g. Pieau, 1972, 1982; Janzen & Paukstis, 1991; Bull, 1980), as well as other hatchling phenotypic traits, behaviour and survival (e.g. Deeming & Ferguson, 1991; Janzen, 1993; Cagle *et al.*, 1993; Bobyne & Brooks, 1994;

Spotila *et al.*, 1994; Shine *et al.*, 1997; Elphick & Shine, 1998; Wilson, 1998; Packard *et al.*, 1999; Rhen & Lang 1999). The variation of temperature in natural nests has been described in some species of reptiles [e.g. *Emys orbicularis* (Pieau, 1982), *Chelydra serpentina* (Packard *et al.*, 1985), *Emydura macquarii* (Thompson, 1988), *Chrysemys picta* (Cagle *et al.*, 1993), *Sphenodon punctatus* (Thompson *et al.*, 1996), *Chelodina expansa* (Booth, 1998), *Chelonia mydas* and *C. caretta* (Kaska *et al.*, 1998), *Amphibolorus muricatus* (Harlow & Taylor, 2000), *Bassiana duperreyi* (Shine, 2004), *Cyclura cychlura* (Iverson, 2004)]. Natural incubation temperatures are known to affect embryo survival (Thompson *et al.*, 1996), as well as sexual differentiation of embryos (Pieau, 1982, Harlow & Taylor, 2000), and may be used to predict sex-ratio at hatching (Hanson *et al.*, 1998, Marcovaldi *et al.*, 1996). Although temperature may frequently reach lethal levels for embryos in wild nests, only a few studies have analysed the effect of incubation temperature on hatching success in field incubating nests (Shine & Elphick, 2001; Shine, 2004, Thompson, 1996, Congdon *et al.*, 1987). Nest temperatures in the field may vary throughout the nesting season, as well as among localities and years. Differences in incubation temperature throughout the nesting season affect the timing of hatching and the developmental trajectories of embryos, eventually affecting hatchling phenotype (Shine, 2004).

In this study we describe the variation of incubation conditions in field nests of *Testudo graeca* in a population of south-western Spain. Each year females in this

population produce 1-4 clutches of 1-7 hard shelled eggs, laid in shallow nests of about 8 cm depth, usually under some degree of vegetation cover (Díaz-Paniagua *et al.*, 1996). A demographic analysis indicated that egg and/or juvenile survivorship are usually low, while occasional episodes of successful recruitment significantly contribute to revert otherwise declining population growth tendencies (Díaz-Paniagua *et al.*, 2001). Within this framework, the assessment of the factors that influence hatching success is paramount to the understanding of the mechanisms affecting the dynamics of the population and the determination of effective management measures.

Previous studies on egg incubation of this species under laboratory conditions have showed that sex differentiation is temperature dependent, with a pivotal temperature of $30.5 \pm 0.5^\circ\text{C}$ (Pieau, 1975). Hatching success, the period from hatching to emergence, and hatchling morphology have been described from field incubating nests of *T. graeca* in a previous study (Díaz-Paniagua *et al.*, 1997). Because rigid egg shells prevent the loss of large amounts of water to dry surroundings (Packard & Packard, 1988), the eggs of *T. graeca* are assumed to be mostly influenced by incubation temperature, and therefore this study is centred on the variation of the thermal environment of field incubating nests. Our objectives were (1) to describe the variation of incubation temperature for clutches laid at different moments of the nesting season and in different years, and (2) to analyze the relation between the profile of incubation temperature and hatching success.

MATERIAL AND METHODS

We monitored hatching success and incubation conditions in 56 *Testudo graeca* nests in four different years: 1996, 1997, 1999 and 2000 (Table 1). Every year we captured female tortoises during the nesting season, from mid-March or early April to June. Female capture was random, thus some of the clutches both among years as well as among nesting periods within the same year were from the same female. The presence of oviductal eggs was determined through X-raying (see Díaz-Paniagua *et al.* (1996) for details on the X-raying procedure). Egg-bearing females were kept in individual field enclosures of approximately 10 m² located within the natural nesting area of *Testudo graeca* in Doñana National Park (SW Spain). All enclosures included natural vegetation (mainly shrubs of *Stauracanthus genistoides*, and *Halimium halimifolium*), under which free ranging tortoises usually lay their eggs (Díaz-Paniagua *et al.*, 1996). Vegetation cover was not measured in the enclosures, but every female had the choice between shaded and unshaded spots in its enclosure for egg-laying. All females were weighed daily after the end of their activity period. Egg laying was assumed when a weight loss occurred approximately equivalent to the total mass of the eggs detected on radiography (mean egg mass from Díaz-Paniagua *et al.*, 1996). The enclosure was then

TABLE 1. Number of nests (*n*), mean hatching rate and nest success for each nesting period of *Testudo graeca* in the four study years.

Year	Nesting period	<i>n</i>	Hatching Rate (%)	Nest success (%)
1996	early	1	50.0	100
	intermediate	5	95.0	100
	late	6	75.0	83.3
	all nests	12	81.3	91.7
1997	early	7	72.4	100
	intermediate			
	late	8	74.6	87.5
1999	all nests	15	73.6	93.3
	early	6	55.5	83.0
	intermediate	5	50.0	80.0
2000	late	1	33.0	100
	all nests	12	49.7	83.0
	early	5	48.4	80.0
	intermediate	9	45.9	55.6
All years	late	3	33.3	33.3
	all nests	17	44.4	58.8
	early	19	60.1	89.0
	intermediate	19	59.4	74.0
	late	18	64.4	77.8
	all nests	56	61.3	80.0

thoroughly searched for the nest site through palpation by one or two persons. All females were released at their original capture sites after oviposition.

Forty-five nests were fitted with a temperature data-logger placed among the eggs (11 in 1996, 7 in 1997, 11 in 1999 and 16 in 2000), usually 2-3 days after egg-laying. Incubation temperature was recorded at 30-minute intervals using Onset Stowaway data-loggers in 1996-1997 and Onset Tidbit data-loggers in 1999-2000. All data loggers were checked for consistency in temperature recording prior to use in the field and after data launching. After introduction of the data-logger nests were covered with a wire grid of about 15cm x 20 cm, to prevent other females from digging at the same point. Vegetation cover above each nest was classified in a gradient from 0-100%, considering the percentage of shade vertically projected over a 50 cm diameter circle centred on the nest. Temperature in nests with vegetation cover >0.5 was compared with that of nests with lower or no cover.

In the first week of July, after all females had oviposited, nests were manipulated after Díaz-Paniagua *et al.* (1997) in order to control egg pipping and hatchling emergence. From 1st August onwards we monitored the nests daily by lifting the sand bag in order to record the date of egg pipping.

We defined the hatching date of each nest as the day when pipping of the first egg was observed. Accordingly, the incubation period was here defined as the number of days elapsed between the day of egg-laying and the day of first egg pipping.

To determine the temperature profile of each nest we used the temperature records from nest detection (usually 1-2 days after oviposition) to the day of first egg pipping. For nests in which no egg hatched, we considered the data recorded up to 15 September. For 12 nests detected 7-16 days after the estimated nesting date, mean, maximum and minimum daily nest temperatures during these first days were predicted through multiple regression, using complete temperature data sets from other simultaneously incubating nests as independent variables. In all cases regressions had $R^2 > 90\%$.

For each nest we calculated the following parameters: (1) mean ($\text{mean}T_d$), maximum ($\text{max}T_d$) and minimum ($\text{min}T_d$) daily temperature; (2) the average of $\text{mean}T_d$ ($\text{xmean}T_d$), $\text{max}T_d$ ($\text{xmax}T_d$) and $\text{min}T_d$ ($\text{xmin}T_d$) for the whole incubation period; (3) minimum (T_{min}), and maximum (T_{max}) temperatures for the whole incubation period; (4) the number of days in which the mean daily temperature was over 20, 25, 30 and 35°C ($nT_{\text{mean}>20}$, $nT_{\text{mean}>25}$, $nT_{\text{mean}>30}$, $nT_{\text{mean}>35}$, respectively) and the corresponding average mean temperatures for these days ($\text{xmean}T_{\text{mean}>20}$, $\text{xmean}T_{\text{mean}>25}$, etc.); (5) the number of days with maximum temperature over 30, 35, 40 and 45°C ($nT_{\text{max}>30}$, $nT_{\text{max}>35}$, $nT_{\text{max}>40}$, $nT_{\text{max}>45}$, respectively) and the corresponding mean maximum temperatures for these days ($\text{xmax}T_{\text{max}>30}$, $\text{xmax}T_{\text{max}>35}$, etc.); (6) the number of days with minimum temperature over 20 and 25°C ($nT_{\text{min}>20}$ and $nT_{\text{min}>25}$, respectively) and the corresponding mean minimum temperatures for these days ($\text{xmin}T_{\text{min}>20}$, $\text{xmin}T_{\text{min}>25}$).

Nesting dates were grouped in three categories (hereafter referred to as nesting periods): (1) early nests (eggs laid in April); (2) intermediate nests (eggs laid in May); and (3) late nests (eggs laid in June). The hatching rate per nest was calculated as the number of eggs hatched divided by the total number of eggs. Nest success was defined as a discrete variable (0 = no eggs hatched in the nest; 1 = at least one egg hatched in the nest) indicative of viable incubation conditions, apart from other factors that could affect hatching rate (e.g. egg infertility).

The correlation of incubation length with temperature variables was analyzed individually and using multiple regression. We compared temperature variables (groups 2 and 3, see above) among nesting periods and years using ANOVA. Nest success was compared among years and nesting periods using the χ^2 test. In order to assess which temperature variables mainly affected hatching success, we analyzed the individual correlation of temperature variables (groups 2 to 6, see above) with hatching rate, and then compared the significantly correlated variables among successful and unsuccessful nests using ANOVA. The significance level for comparisons was adjusted to $P=0.0031$ following a Bonferroni correction.

To assess the influence of incubation temperature variables on hatching rates we carried out a logistic regression analysis in which nest success was the response variable, and temperature parameters were predictor

variables. In a first approach, the regression was calculated for only one temperature parameter at a time, and in a second step we calculated regressions for two combined parameters among those presenting significant individual relations with the response variable. Only uncorrelated variables were combined in the second analysis.

RESULTS

VARIATION OF INCUBATION TEMPERATURE

Tortoises nested from early April to the end of June. The yearly nesting season, as represented by the sequence of recorded nesting dates of monitored tortoises, differed significantly among years ($F_{3,52}=3.96$, $P=0.0129$). The sample size for each nesting period (Table 1) reflects the actual monthly availability of females in the field in each year and was related to climatic differences among years. For example, in 1996, exceptionally low early spring temperatures generally delayed the onset of the nesting season to mid-May.

Tortoise nests experienced a wide variation in daily as well as whole-period temperature progression (Fig. 1). Incubation temperature averaged 27.9°C. Very high

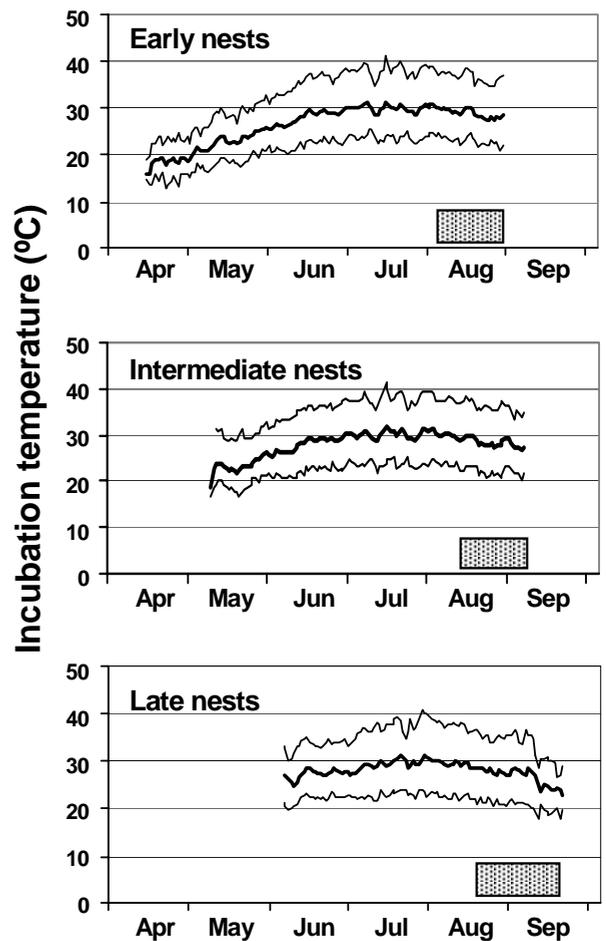


FIG. 1. Evolution of mean, maximum and minimum temperature in field incubating nests of *Testudo graeca* in Doñana National Park (pooled data for nests monitored in 1996, 1997, 1999 and 2000). The grey horizontal bar at the bottom indicates the span of hatching dates.

TABLE 2. Incubation period (in days) and mean incubation temperature ($x\text{mean}T_d$, in °C) for nests monitored over different nesting periods (early, intermediate, late and overall) and years. Numbers are the arithmetic mean \pm SD, followed by the range (in parenthesis).

Year	Overall	Early	Intermediate	Late
INCUBATION PERIOD				
1996	95.3 \pm 10.8 (79-117)	117.0	99.2 \pm 1.6 (98-102)	87.0 \pm 8.3 (79-100)
1997	100.0 \pm 22.6 (67-129)	118.6 \pm 14.5 (89-129)	—	81.4 \pm 9.7 (67-90)
1999	110.0 \pm 12.9 (93-128)	121.6 \pm 5.4 (114-128)	99.8 \pm 1.7 (98-102)	93
2000	107.6 \pm 19.3 (76-131)	117.8 \pm 16.3 (90-131)	103.8 \pm 16.2 (87-126)	76
All years	102.9 \pm 18.6 (67-131)	119.1 \pm 12.1 (89-131)	101.0 \pm 9.3 (87-126)	83.9 \pm 9.1 (67-100)
INCUBATION TEMPERATURE				
1996	28.0 \pm 1.3 (26.3-30.5)	26.4	28.1 \pm 0.9 (27.3-29.8)	28.3 \pm 1.5 (26.3-30.5)
1997	27.8 \pm 1.4 (25.3-29.1)	26.0 \pm 1.0 (25.3-26.7)	—	28.5 \pm 0.5 (27.7-29.1)
1999	28.0 \pm 2.0 (23.5-30.4)	27.8 \pm 1.6 (26.2-30.0)	29.1 \pm 1.3 (27.5-30.4)	23.5
2000	27.7 \pm 1.6 (24.9-29.2)	26.5 \pm 1.5 (24.9-29.2)	28.2 \pm 1.3 (25.2-29.2)	29.0 \pm 0.3 (28.8-29.2)
All years	27.9 \pm 1.6 (23.5-30.5)	27.5 \pm 1.6 (24.3-30.3)	28.4 \pm 1.2 (25.2-30.4)	28.1 \pm 1.7 (26.3-30.5)

temperatures were reached in the nests during the hottest month (July). In 10 nests we recorded T_{max} values over 45°C. The highest recorded temperature was 49.8°C, in July in a nest where no egg hatched. However, $T_{\text{max}} = 47.4^\circ\text{C}$ was recorded in another nest where hatching success was >0 . Nest temperature exhibited a wide daily range, with a mean range of 13.4 °C. T_{min} never dropped below 10°C. In general, early, intermediate and late nests were characterized by different incubation temperature regimes, although all of them were exposed to similarly high temperatures during the hottest month (July). Overall early nests displayed lower temperatures than later nests (Table 2). Considering the whole incubation period, nests from different nesting periods differed significantly in $x\text{mean}T_d$ ($F_{2,42}=6.20$, $P=0.0044$), T_{min} ($F_{2,41}=10.29$, $P<0.0002$) and $x\text{min}T_d$ ($F_{2,41}=5.20$, $P<0.01$), early nests exhibiting lower values than intermediate and late nests for all three parameters. In contrast, all nests reached similar maximum temperatures. Considering only incubation during July, the only month in which nests from all nesting periods were incubating during the whole month, no significant difference was found in $x\text{mean}T_d$ among nests from different nesting periods. The daily average of $x\text{mean}T_d$ for all nests varied significantly among years ($F_{3,120}=10.87$, $P<0.0005$), which was mainly due to higher mean daily temperatures in nests in 1999.

Until the first half of May $\text{mean}T_d$ stayed under 24°C. During this period mostly early nests were incubating. Early nests started incubation around 19°C (Fig. 1). In the second half of May, when incubation of most intermediate nests started, $\text{mean}T_d$ values climbed to around 26°C. Until the end of May $\text{min}T_d$ values stayed between 16 and 20°C, while $\text{max}T_d$ only rarely surpassed 30°C. In June, when incubation of late nests began, $\text{mean}T_d$ climbed from 26-27°C to 27-29°C in the first and second halves, respectively. In July and August $\text{mean}T_d$ stayed between 29-30°C, while $\text{min}T_d$ and $\text{max}T_d$ values were steadily over 20°C and 33°C, respec-

tively. In July $\text{max}T_d$ frequently reached over 40°C (highest $\text{max}T_d=49.8^\circ\text{C}$). By the end of August, when eggs of most early and intermediate nests had already hatched, incubation temperatures started to decrease, keeping levels similar to June throughout the remaining incubation time of most late nests.

Tortoise nests were frequently located close to or under the cover of shrubs, which partially shaded them. Vegetation cover of the nests was negatively related to $x\text{mean}T_d$ ($r=-0.409$, $P=0.0079$). Nests where vegetation cover was less than 50% shade (46% of the nests) reached significantly higher temperatures than nests with more than 50% shade ($F_{1,39}=9.26$, $P=0.0042$). Only 10% of the nests had no cover at all (0% shade), while 15 % were completely covered (100% shade) under a dense shrub.

HATCHING DATE AND INCUBATION LENGTH

The hatching date differed significantly among nesting periods ($F_{2,37}=15.24$, $P=0.0001$), but not among years. The earliest hatchings occurred in the first half of August, and were from early and intermediate nests (which did not differ significantly), while eggs from late nests started to hatch significantly later, towards the end of August (Tukey post-hoc test). Most early and intermediate eggs hatched until the end of August, while late eggs hatched in the second half of September (Fig. 1).

Incubation periods ranged between 67 (a late nest in 1997) and 129 days (an early nest in 1997) and decreased significantly from early to late nests ($F_{2,43}=45.07$, $P<0.0001$) (Table 2). No significant differences were observed among years.

The length of the incubation period was significantly and negatively correlated with T_{min} ($r=0.580$, $P=0.0003$), $x\text{min}T_d$ ($r=-0.461$, $P=0.006$), $x\text{mean}T_d$ ($r=-0.375$, $P=0.02$) and positively correlated with $nT_{\text{mean}>20}$ ($r=0.681$, $P<0.0001$) and $nT_{\text{min}>20}$ ($r=0.388$, $P=0.02$). A similarly good predictive value was obtained for the two functions: Incubation length = 46.03 -

TABLE 3. Values of temperature variables that differed significantly between 33 successful and 11 unsuccessful *Testudo graeca* nests monitored in 1996, 1997, 1999 and 2000 in Doñana National Park. Numbers are the arithmetic mean \pm SD, followed by the range, and ANOVA results of comparisons between successful and unsuccessful nests (df = 1,39). Pearson correlation coefficients and corresponding significance levels are for the relation between temperature variables and hatching rates (significant results following the Bonferroni corrected significance level = 0.0031).

Incubation temperature variables	Successful nests	Unsuccessful nests	F (p)	Correlation with hatching rate
Average of maximum daily temperatures (xmaxT _d)	34.7 \pm 2.4 (29.8-39.1)	37.7 \pm 1.7 (34.7-40.5)	13.7 (0.0006)	-0.555 (0.0001)
Absolute maximum temperature (T _{max})	41.5 \pm 2.4 (37.1-47.4)	46.5 \pm 1.9 (43.4-49.8)	37.7 (<0.0001)	-0.681 (0.0001)
Number of days with mean temperature >20°C (nT _{mean>20})	92.9 \pm 16.4 (29-120)	111.9 \pm 13.0 (88-132)	12.2 (0.0011)	-0.419 (0.0042)
Number of days with mean temperature >30°C (nT _{mean>30})	21.9 \pm 15.4 (0-59)	38.3 \pm 19.2 (16-79)	14.5 (0.0004)	-0.536 (0.0002)
Number of days with mean temperature >25°C (nT _{mean>25})	79.6 \pm 11.7 (29-95)	101.5 \pm 12.6 (75-122)	28.1 (<0.0001)	-0.600 (<0.0001)
Average temperature of days with mean temperature >30°C (xmeanT _{mean>30})	30.9 \pm 0.4 (30.2-31.9)	31.5 \pm 0.52 (30.7-32.6)	8.3 (0.0062)	-0.524 (0.0002)
Number of days with minimum temperature >20°C (nT _{min>20})	97.0 \pm 15.2 (69-115)	81.3 \pm 9.5 (66-111)	16.4 (0.0002)	-0.492 (0.0007)
Number of days with maximum temperature >35°C (nT _{max>35})	51.5 \pm 24.7 (1-90)	85.3 \pm 18.0 (59-114)	16.0 (0.0003)	-0.574 (0.0001)
Number of days with maximum temperature >40°C (nT _{max>40})	14.2 \pm 15.3 (0-60)	40.5 \pm 21.8 (21-83)	16.4 (0.0003)	-0.614 (0.0001)
Mean maximum temperature in days with maximum temp >30°C (xmaxT _{max>30})	35.8 \pm 1.7 (32.3-39.1)	38.7 \pm 1.4 (37.4-41.1)	21.7 (<0.0001)	-0.679 (<0.0001)

1.67 xmeanT_d + 1.09 nT_{mean>20} (R²=0.669) and Incubation length = 58.88 - 2.627xminT_d + 1.08nT_{mean>20} (R²=(0.669).

INFLUENCE OF INCUBATION TEMPERATURE ON HATCHING AND NEST SUCCESS

Overall hatching rate was 61%, and although a lower percentage was registered in nests from 1999 and 2000, we did not find significant differences among years nor nesting periods, despite the lower values registered for 1999 and 2000 (Table 1). Nest success did not differ significantly among years or nesting seasons. We recorded values over 80% in all seasons of the four study years except in intermediate and late nests in 2000, when no eggs hatched in six of 12 nests.

The comparison of successful and unsuccessful nests mostly revealed significant differences in variables related with maximum temperatures (xmaxT_d, T_{max} and xmaxT_{max>30}) and with the number of days with high temperatures (nT_{mean>20}, nT_{mean>25}, nT_{mean>30}, nT_{min>20}, nT_{max>35}, nT_{max>40}), suggesting that egg mortality was associated with the higher temperatures experienced by unsuccessful nests (see Table 3). Similarly those variables related to maximum temperatures were significantly and negatively correlated with hatching rates (Table 3). The highest correlation coefficients were obtained for T_{max}, x_{max}T_{max>30}, nT_{max>40} and nT_{max>35}, and all had significantly higher values in unsuccessful nests compared to successful ones. The number of days with maximum temperature >40°C was almost three times higher in unsuccessful nests than in successful ones.

Among the logistic regression equations for prediction of successful and unsuccessful nests using individual variables only nT_{mean>25} and T_{max} obtained correct classification >90% (Table 4). The predictive ability of these two variables increases to up to 95% in combination with nT_{mean>35}, T_{min} and x_{min}T_{min>20}.

TABLE 4. Results of logistic regression analysis using nest success rate as response variable and incubation temperature variables as explanatory variables for nests of *Testudo graeca* monitored in Doñana National Park (see Material and Methods for definitions of variable names).

	R ²	Correct predictions (%)	Variable coefficients	
			Intercept	V1 V2
ONE VARIABLE				
nT _{mean>25}	0.435	91.4	20.08	-0.22
T _{max}	0.490	90.3	45.70	-1.02
TWO VARIABLES				
nT _{mean>25} * nT _{mean>35}	0.377	95.5	20.99	-0.22 2.19
nT _{mean>25} * T _{min}	0.386	95.5	21.97	-0.22 -0.07
T _{max} * x _{min} T _{min>20}	0.412	93.0	9.92	-1.01 1.56
T _{max} * x _{min} T _d	0.403	90.7	31.48	-1.04 0.70
T _{max} * nT _{min>25}	0.406	90.5	45.95	-1.03 0.07

DISCUSSION

VARIATION OF INCUBATION TEMPERATURE

The main period of egg-laying of *Testudo graeca* in Doñana occurs from the beginning of April until the first half of June, with only a few nests recorded at the end of March and during the second half of June (Díaz-Paniagua *et al.*, 2001). This protracted nesting season allows female tortoises to lay up to four clutches in a year (Díaz-Paniagua *et al.*, 1996).

Ambient temperature gradually increases throughout the nesting season of *T. graeca*, so that eggs laid in different months are exposed to different temperature regimes. Notably, early nests were incubated at lower temperatures than intermediate and late nests during approximately the first third of their incubation period, while during the two remaining thirds of incubation nests of different months are exposed to similar temperatures. This adaptation to a wide range of thermal incubation environments enables tortoises in south-western Spain to attain a high clutch frequency before the summer inactivity period (Díaz-Paniagua *et al.*, 1995).

Reptiles excavate their nests in the soil, and depending on the depth, incubation temperature may be more or less influenced by the diurnal cycle of sun radiance and heating/cooling cycles of the ground (Packard & Packard, 1988). Therefore, species with shallow nests have a wider daily variation in temperature than species nesting in deep holes (Ewert, 1979). The location of the nests on bare or vegetated ground also affects the variation of incubation temperature (Ewert, 1979; Congdon *et al.*, 1987; Janzen, 1994; Wilson, 1998; Weisrock & Janzen, 1999). *T. graeca* in Doñana National Park lay eggs in shallow nests with an average depth of 8 cm. Frequently the nests are partially shaded by a shrub, but they are also dug in bare ground (Díaz-Paniagua *et al.*, 1996). Nest temperature experienced a wide variation throughout the day, as well as throughout the whole incubation period. Mean incubation temperature in the field was about 28°C, a value frequently recorded for development of reptile eggs (see e.g. Ewert, 1979; Packard & Packard, 1988), but nest temperatures frequently reached values above the reported tolerance limits for reptile eggs (33-35°C, according to Ewert, 1979; Congdon & Gibbons, 1990; Packard & Packard, 1988). However, thermal tolerance limits have been described for constant incubation conditions in the laboratory, while embryos developing in the field are known to withstand short periods of temperatures above or below thermal limits (Congdon & Gibbons, 1990; Packard & Packard, 1988; Ewert, 1979). Similar high and fluctuating incubation temperatures are also likely to be found for other *Testudo* species around the Mediterranean, although detailed description of field incubation temperature in other populations has not yet been reported.

HATCHING DATE AND INCUBATION LENGTH

Embryo development is accelerated by increasing temperature (Packard & Packard, 1988; Congdon & Gibbons, 1990; Deeming & Ferguson, 1991). Embryos from early *T. graeca* nests were exposed to relatively low temperatures during the initial phase of incubation, and are likely to have had very slow or even no development during this period, which is supported by the fact that hatching dates of eggs from early nests did not differ significantly from those of nests laid in May. In contrast, late nests were exposed to relatively higher temperatures during the whole incubation period, which resulted in faster embryo development.

We have found no evidence that incubation conditions of early, intermediate and late nests affect hatching rates in different ways. However, eggs from early nests had slower early development and longer incubation time, which may be expected to influence hatchling phenotype. In a previous study we detected that hatchlings from latest nests had better physical condition than hatchlings from earlier nests (Díaz-Paniagua *et al.*, 1997), probably because their higher mean incubation temperature was related to higher metabolic efficiency during development (Packard & Packard, 1988).

As a consequence of decreasing incubation length from early to late nests the hatching season was much shorter (45 days) and synchronized than the nesting season (80 days). This may be adaptive in south-western Spain, for it prevents hatchling emergence to extend into autumn. On the other hand, emergence from the nest may be delayed for several days (Díaz-Paniagua *et al.*, 1997), which probably enables hatchlings to overcome periods of harsher summer climate.

INFLUENCE OF INCUBATION TEMPERATURE ON HATCHING RATE AND NEST SUCCESS

Overall, 39% of eggs did not hatch in this study, while 20% of nests were total failures (no egg hatched). Hatching and nest success rates were unrelated to nesting period and years, and are thus unlikely to be associated with a particular tendency in thermal incubation regime. Unsuccessful nests had significantly higher values for all variables related to maximum temperature, indicating that total nest failure was caused by excessively high temperatures during incubation. This result suggests that lethal temperatures for embryo development may frequently be reached in the soil in Doñana, mainly during July. Some chelonians locate their nests under canopy vegetation cover, close to standing water or in deep holes to avoid extreme temperatures during incubation (Wilson, 1998; Morjan, 2003; Weisrock & Janzen, 1999; Kolbe & Janzen, 2002). For *Kinosternon baurii*, a small aquatic turtle with shallow nests, a similar influence of maximum temperature on embryo mortality was described. *K.*

baurii apparently selected nest sites close to vegetation and avoided open sites. Embryo mortality was higher in nests located in open sites, which reached higher maximum temperatures over longer periods than covered nests (Wilson, 1998).

In Doñana National Park protection from lethal temperature peaks in shallow nests can be provided mainly by locating the nests under the shade of vegetation, where nest temperature was significantly lower than in unshaded nests. This is probably why most tortoises in Doñana locate their nests at the base of shrubs (Díaz-Paniagua *et al.*, 1996). Nest success and hatching rate decreased from the first to the last study year. Even though these differences were not statistically significant, they suggest that the study design – females confined each year in the same small enclosures – might have artificially influenced hatching success. The deterioration of vegetation within the enclosures during the four study years due to natural drying of shrubs and cumulative tortoise burying activity probably constrained the availability of adequate nesting sites.

The association of nest failure with higher maximum temperatures and the relation among temperature and vegetation cover suggests that microhabitat structure is important for the successful incubation of *T. graeca* in Doñana. Juvenile survival is generally low in this population (Díaz-Paniagua *et al.*, 2001), but high hatching success has also been observed (Díaz-Paniagua *et al.*, 1997). The stability of the *T. graeca* population in Doñana National Park depends on high adult survival but also on sporadic high juvenile recruitment bouts (Díaz-Paniagua *et al.*, 2001). In this sense, the persistence of high hatching success rates may enhance population stability and this may be achieved by conserving a habitat microstructure that enables that a sufficiently high proportion of nests do not reach lethal incubation temperatures. The same might be applicable to other *T. graeca* populations, which inhabit regions of very hot and dry late spring and summer climate around the Mediterranean. The Doñana National Park population is effectively protected, as is its habitat. However, many other *T. graeca* populations have been suffering severe habitat loss or deterioration (Andreu *et al.*, 2004; Bertolero & Cheylan, 2004; Zwartepoorte, 2004; Bour, 2004a,b,c; Leontyeva, 2004; Shacham, 2004). A consequence of that may be that, even where populations are still able to subsist, ideal conditions for egg incubation may have been compromised.

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