Biological Journal of the Linnean Society, 2004, **81**, 1–16. With 8 figures

Determinants of reproductive success and offspring sex in a turtle with environmental sex determination

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Received 7 October 2002; accepted for publication 22 May 2003

Despite the importance of maternal effects in evolution, and knowledge of links among nest site choice, timing of nesting, offspring sex, and reproductive success in animals with environmental sex determination, these attributes have not been rigorously studied in a combined and natural context. To address this need we studied the relationships between three maternal traits (nest site choice, lay date, and nest depth) and two fitness-related attributes of offspring (hatchling sex and embryonic survival) in the riverine turtle *Carettochelys insculpta*, a species with temperature-dependent sex determination, for four years. Predation and flooding were the major sources of embryonic mortality in 191 nests. Embryonic survival was influenced by both lay date and nest site choice: in one year when nesting began later than average, nests laid later and at lower elevations were destroyed by early wet season river rises. In other years early nesting precluded flood mortality. However, turtles did not nest at the highest available elevations, and a field experiment confirmed that turtles were constrained to nest at lower elevations where they could construct a nest chamber. The principal determinant of hatchling sex in 140 nests was lay date, which in turn was apparently related to the magnitude of the previous wet season(s). Clutches laid earlier in the season (a female's first clutch) produced mainly males, while later clutches (her second clutch) yielded mostly females, due to seasonal increases in air temperatures. Accordingly, later nesting produced female-biased hatchling sex ratios in 1996, while earlier nesting resulted in sex ratios near unity in the other years. However, all-female nests were more likely to be flooded than mixed-sex or all-male nests in years when nesting was late. In conclusion, we found evidence that the position of two maternal trait distributions (elevation of the nest site and lay date), associated with the reproductive strategy of *C. insculpta,* reflect a combination of natural selection, physical constraints, and phenotypic plasticity. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2004, **81**, 1–16.

ADDITIONAL KEYWORDS: *Carettochelys insculpta* – embryonic survival – nest site choice – reptile – timing of reproduction.

INTRODUCTION

A central goal in evolutionary ecology is to identify and explain phenotypic variation, and to infer how natural selection stabilizes the distributions of phenotypes. Traditionally, trait distributions were explained by genotype and environmental effects, but recently the adaptive significance of maternal effects has become increasingly recognized (Rossiter, 1996; Mousseau & Fox, 1998a, 1998b). Phenotypes can be dramatically influenced by 'inherited environmental effects', particularly between mothers and their offspring (Mousseau & Fox, 1998b).

Oviparous mothers lacking parental care can invest in their offspring in two ways: by providing material to the egg to meet the needs of the developing embryo, and by influencing the incubation environment through nest site choice (Bernardo, 1996; Roosenberg, 1996). In choosing a nest site there are two primary considerations. First, natural selection should favour mothers that choose nests sites that maximize offspring survival. Second, in species with environmental sex determination (ESD), natural selection should also favour mothers whose nest site choice tends to produce optimal offspring sex ratios (Fisher, 1930; Bull, 1980).

Several factors potentially influence hatchling sex ratios in animals with ESD, including attributes of the

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*Corresponding author. E-mail: doody@aerg.canberra.edu.au mother, embryo, and environment. The mother can influence where and when she lays, and the depth of the nest. Influential attributes of the embryo include the relationship between temperature and developmental rate (Georges, 1989), the value of the pivotal temperature that separates male-producing temperatures from female-producing temperatures (e.g. Mrosovsky, 1988; Mrosovsky & Pieau, 1991), and the period during incubation when sex is influenced by temperature (the thermosensitive period, Bull, 1987; Mrosovsky & Pieau, 1991). Environmental factors include the magnitude of fluctuations in temperature (Georges, 1989; Georges, Limpus & Stoutjesdijk, 1994), seasonal trends in temperature (Vogt & Bull, 1984), and stochastic events such as rainfall, which temporarily depress nest temperatures. On a broader temporal scale, overall climate will be influential (Vogt & Bull, 1984).

Importantly, understanding the influence of the incubation environment on offspring sex ratios is a prerequisite to answering why temperature-dependent sex determination (TSD) has evolved in reptiles. The most popular explanations for the evolution of TSD involve models derived from notions of differential fitness of male and female offspring incubated under particular thermal regimes (reviewed in Shine, 1999). These models link incubation temperature, phenotype, and fitness, and posit that TSD can enhance maternal fitness by enabling the embryo to develop as the sex best-suited to the particular environmental conditions that it experiences during incubation (Shine, 1999). According to these models, incubation temperatures influence differential fitness between the sexes, primarily as a result of nest site choice or timing of nesting (reviewed in Shine, 1999; Harlow & Taylor, 2000). However, evidence for these models is meagre (Harlow & Taylor, 2000; Valenzuela & Janzen, 2001).

Despite empirical evidence for relationships among lay date, nest site choice, embryonic survival, and offspring sex in reptiles (Mrosovsky, 1994; Wilson, 1998; Madsen & Shine, 1999; Weisrock & Janzen, 1999; Harlow & Taylor, 2000), two areas need attention. First, few studies have rigorously examined those relationships in an environmental context. For example, what annual variation in lay date exists, and how does that variation influence embryonic survival? Second and surprisingly, no study has examined these relationships comprehensively, despite the importance of their interpretation in a combined context (Schwartzkopf & Brooks, 1987; Weisrock & Janzen, 1999). For example, disproportionate survival among nest sites could result in a long-term change in sex ratios in a species with TSD (Brown & Macdonald, 1994). These two shortcomings limit our ability to understand how embryonic survival and offspring sex production might contribute to the evolution of the nesting strategy in a population.

We addressed these issues by quantifying the relationships between three maternal traits (nest site choice behaviour, lay date, and nest depth) and two fitness-related attributes of offspring (hatchling sex and embryonic survival) in the riverine turtle *Carettochelys insculpta*. We used *C. insculpta* because this species exhibits TSD, and because prior research documented variation in the thermal properties of nests, laying a foundation for studying determinants and consequences of that variation (Georges, 1992). We used four years of data from natural nests, environmental data, and a field experiment to elucidate environmental factors underpinning nest site choice and timing of nesting, in order to test the hypotheses that nest site choice and timing of nesting influence hatchling sex ratios and embryonic survival, and to determine which processes might shape the maternal nesting strategy in the population.

METHODS

STUDY AREA AND SPECIES

The pig-nosed turtle (*Carettochelys insculpta*) is a large freshwater species inhabiting rivers, river swamps and billabongs in Australia and New Guinea (Georges & Rose, 1993). This species is appropriate for studies of the ecology and evolution of sex determination because it possesses TSD (Georges, 1992), while its closest living relatives, the softshell turtles, apparently do not (e.g. Vogt & Bull, 1982). We studied *C. insculpta* along a 30-km stretch of the Daly River near Oolloo Crossing (14°04′ 40′-S, 131°15′ 00′-E) in the Northern Territory, Australia. The climate is typical of the wet-dry tropics of northern Australia (Taylor & Tulloch, 1985) with mean monthly rainfall of less than 7 mm from May to September, rising to a peak monthly average of nearly 300 mm in February (Stn 014139/014941, Oolloo 1962–85). The study was conducted during the dry season (July–November) of each year during 1996–98. In northern Australia, *C. insculpta* nests from July to October (Georges & Rose, 1993; Georges, Rose & Doody, 2004). On the Daly River *C. insculpta* nests occupy linear home ranges 7 km in length (Doody, Georges & Young, 2002), and nest on isolated sandy beaches and banks varying in size from a few square metres to several hundred hectares (Georges, 1992; Doody, Georges & Young, 2003a). The turtles lay two clutches of eggs every second year (Doody, Sims & Georges, 2001a; Doody, Georges & Young, 2003b). Embryos complete development during the late dry season, enter aestivation, and hatch and emerge with the onset of early wet season rains (Doody *et al*., 2001b).

TIMING OF NESTING AND NEST SITE CHOICE

We searched for nests daily during the nesting seasons of 1996–98. We accessed nesting areas by boat and located nests by noting turtle tracks and probing for the eggs with a spring steel rod. Because we swept the beaches clean after each survey, we were confident that we missed very few nests. For each nest we recorded laying date and measured the following attributes of the nest site: elevation above water, distance to water, aspect (compass direction of the slope), and slope. Elevation was measured using a level and an extended metre stick. We also recorded depth to the top egg and the maximum depth of the nest chamber. To assess whether turtles were choosing these variables non-randomly, we divided 15 nesting beaches into grids with square metre cells by demarcating lines in the sand. We measured the same attributes for the centre of each cell that were measured for each nest (elevation and distance from water, slope, aspect). Attributes of nest sites and available sites were then quantitatively compared for each of the 15 beaches. Finally, for each of the 15 beaches we estimated the maximum elevation at which a nest chamber could be constructed, based on the cohesiveness of the sand. We did this by attempting to construct a nest chamber by hand to 15 cm depth at the highest point on the beach. If we could not make a chamber (the sand fell in on itself due to low moisture content) we moved progressively lower and repeated the procedure until we were able to construct a chamber. We then measured the elevation above water of this 'cohesive sand line' (Doody *et al*., 2003a).

NEST ELEVATION EXPERIMENT

After finding that turtles did not nest at the highest elevations on most beaches (Doody *et al*., 2003a), we hypothesized that in these areas the sand was not cohesive, preventing turtles from constructing a nest chamber (due to dry sand falling in on itself). To test this hypothesis we conducted a field experiment on a nesting beach in 1998. The selected beach was used heavily in the first nesting period, but also had >20 cone-shaped pits which were evidence of non-cohesive sand at higher elevations.

Prior to the second nesting period, we divided the beach into one metre wide bands perpendicular to the river. Alternate bands were then wetted with river water every ten days, with the remaining bands serving as controls. By wetting the bands we created cohesive sand, despite the first few centimetres on the surface drying out in a few days. We estimated the maximum elevation of cohesive sand ('cohesive sand line') using the method described previously. We predicted that subsequent nests deposited in wetted bands would be higher above water than those placed in control bands, because (1) the constraint of non-cohesive sand was removed, and (2) because turtles benefit through nesting at the highest sites by reducing the probability of nest flooding (Doody *et al.*, 2003a). The beach was subsequently surveyed daily for nests as outlined above.

NEST TEMPERATURES

Continuous temperatures were monitored in 102 nests with either Datataker DT500 multichannel dataloggers (Datataker Corp.) or Stowaway single-channel dataloggers (Onset Computer Corp.). Both data loggers were calibrated just before deployment using a thermometer certified by the National Australian Testing Authority. Temperatures were recorded from the core of each nest at 15 min intervals by the Datataker dataloggers and at 1 h intervals using the Stowaway dataloggers. The probes were fitted as soon as possible after discovery of the nest, usually within 1–2 days. The depth of each egg was measured before its removal and eggs were returned to their original positions and orientations after deployment of datalogger probes.

To ensure that any influences on hatchling sex were due to temperature, we examined relationships between nest temperatures and lay date, nest site attributes, and nest depth. We used a model to calculate a single temperature value (constant temperature equivalent (CTE), Georges, 1989; Georges *et al.*, 1994) for each day of incubation in each nest. The CTE is necessary because the mean temperature often fails to predict offspring sex because development is faster at hotter temperatures (Georges, 1989; Georges *et al.*, 1994). Because the model also determines the cumulative contribution to development for each day throughout the developmental period, we were able to demarcate the temporal window during which sex is determined for each temperature trace, based on knowledge of the developmental window or thermosensitive period (TSP). We estimated the thermosensitive period to be the middle third of development, based on experimental data for other turtle species (Bull, 1987; Mrosovsky & Pieau, 1991) and unpublished data for *C. insculpta* (J. Young, A. Georges, S. Doody, P. West, unpubl. data). We then averaged the daily CTEs during the TSP for each trace to yield a single value that best described the thermal character of each nest during the period when sex was determined. We used the temperature trace recorded at the core of each nest, and we used only data from nests from which the lay date was known.

Because dataloggers were typically deployed 1–2 days after the lay date, we used a second model to back-fill temperature traces to their nesting dates using temperature traces from other nests. Strong predictive relationships can be established between temperature traces from different nests. Values in the response trace can be predicted from values in a second predictor trace by (1) bringing the predictor trace into phase with the response trace with a translational or temporal shift, (2) decomposing both traces into a trend line and stationary time series, (3) developing predictive relationships between the two trend lines and the two stationary series using simple linear regression, (4) using these relationships to predict missing values in the response trend and stationary series, and (5) reconstructing the response trace, with missing values added. Validation using known data deleted from response traces indicated that this approach was accurate to within 0.5°C.

We then used multiple regression analysis to reveal the relative importance of nest site attributes (elevation, distance, slope, aspect), lay date, and nest depth on the CTE for each nest. We also tested the accuracy of the sex-determination model by comparing the CTEs to the observed sexes (male, female, mixed) for each nest, but these data are being published elsewhere.

EMBRYONIC MORTALITY

Flood mortality was estimated through (1) observations of nest flooding, and, when a clutch had already been removed for determining hatchling sex, (2) by comparing observations of nest site flooding to hatching dates of the respective nest in the laboratory. For example, if a nest with a elevation of 1 m above water was flooded, then all nests with elevations <1 m (that would have been in the ground had we not removed them) were considered to have been flooded. Although there may be some error in our assessment (e.g. small differences in nest temperatures due to different nest sites could lead to small differences in developmental time/incubation period), we see no reason for our estimate to be biased in any direction.

We monitored nests for predation at least every other day throughout incubation. To ensure that we would obtain hatchling sex from nests, we installed flat wire covers (20 cm × 20 cm hardware wire) at the surface of each nest site to protect it from predatory monitor lizards (*Varanus* spp.). Although we protected nests, if a predator attempted to excavate a nest, as evidenced by diggings around the covers, we scored the nest as destroyed by a predator. In this way we could estimate mortality data without sacrificing sex ratio data. We also noted clutches of eggs that failed to hatch due to intrinsic reasons (e.g. infertility, developmental problems). Because we removed many clutches prior to their natural hatching date, our predation estimates are conservative. However, because most predated nests are taken within 24 h of laying (S. Doody, unpubl. data, Congdon *et al.*, 1983, 1987), our predation estimates should be realistic.

HATCHING AND HATCHLING SEX

When a nest contained eggs that were near-term (development was nearly complete and sex already determined), as estimated by knowledge of lay date and previous incubation data (A. Georges, unpubl. data), we removed the clutch and housed it in a makeshift field laboratory until hatching. This allowed isolation of eggs to facilitate determination of sex in relation to depth in the nest (sex was already determined in these embryos). In 1996 and 1997 we obtained hatching dates in the field laboratory. In 1998 we obtained emergence dates in the field using remote camera systems (Doody & Georges, 2000; Doody *et al.*, 2001b).

Hatchlings were measured, weighed, and sacrificed via intercranial injection of pentobarbital or ethanol. In 1996 all hatchlings were sacrificed, thereby obtaining the sex of every hatchling, while in 1997–98 we only sacrificed a few hatchlings from each nest to determine whether a nest contained all males, all females, or mixed sexes. For example, if the hatchling from a top egg in the nest was found to be male, then all eggs in the nest were deemed to be males, because deeper, cooler eggs become males (Georges, 1992).

Hatchling sex was determined by histological examination of the gonads. The right gonad, kidney, and associated ducts were removed, embedded in wax, sectioned, and dyed with haemotoxylin and eosin. The sex of each gonad was assessed by examination under a light microscope according to criteria established by Miller & Limpus (1981). In rare cases where determining sex was difficult, the second gonad was examined.

ENVIRONMENTAL DATA COLLECTION

Water temperatures were monitored from May to November each year on a beach used by nesting *C. insculpta*. Water temperatures were recorded in the shade at approximately 0.5 m depth with four-wire RTD probes fitted to a datalogger (Datataker DT500). To document seasonal changes in air temperature we used data from a weather station *c.* 20 km from the study area (Douglas Daly Research Farm, Department of Primary Industries and Fisheries). Associated changes in sand temperatures were recorded by taking monthly temperatures 50 cm below the surface at 1 m elevation, on each beach. We chose 50 cm because temperatures at this depth are not confounded by time of day (Monteith & Unsworth, 1990).

To examine any association between timing of nesting and the preceding wet season(s), we used mean monthly river stages as an index of the magnitude of the wet season precipitation. River stage data were obtained for Dorisvale Crossing (60 km upstream of the study area) for the years 1995–98. We used only wet season river stages, because dry season river stages were nearly identical among years during the study. We used timing of nesting data from the three study years, and data collected in 1986 from the same population by A.G.

STATISTICAL ANALYSES

Statistical tests were performed using SAS 6.12 (1996) and SYSTAT 8.0 (1998). In some cases data were not normal, and analyses were conducted on log- or square root-transformed data. Step-wise discriminant function analysis was used to examine the influence of lay date, nest site attributes, and nest depth on embryonic survival and hatchling sex, and multiple regression analysis was used to examine relationships between nest temperatures and: lay date, nest site attributes, and nest depth. Lay date and aspect were coded for analyses, and the latter was coded symmetrically about due north because aspect is a circular parameter, and because we expected that aspect might mediate a heating influence. Bonferroni-corrected probabilities were used in correlation analyses.

RESULTS

TIMING OF NESTING

The bimodal temporal distribution of lay dates reflected the production of two clutches in a season (Fig. 1A). The onset of nesting differed significantly among years (ANOVA; $F_{2,150} = 37.19$, $P < 0.001$). In 1996, nesting began 4 and 5 weeks later than in 1997 and 1998 respectively (Tukey's HSD, $P < 0.001$ for both comparisons; Fig. 1), whilst nesting onset in 1997 and 1998 was similar ($P = 0.20$). The onset of nesting was not associated with mean daily water temperature in the weeks or months prior to nesting. Nesting began latest in the warmest year (1996), and earliest in a year with intermediate temperatures (1998).

The onset of nesting was negatively correlated with mean monthly wet season river levels in the year prior to nesting ($r_1 = -0.95$, $N = 4$, $P = 0.05$), an index of the magnitude of wet season precipitation. Turtles nested earlier following 'big' wet seasons (1997, 1998) than they did following 'small' wet seasons (1986, 1996) (Fig. 2). We also examined the association between the onset of nesting and the mean monthly river levels of the two preceding wet seasons (averaged), because *C. insculpta* in the Daly nest every second year (Doody *et al.*, 2003b), indicating that turtles need two years to produce eggs. The correlation was similar but not significant ($r_1 = -0.82$, $N = 4$, $P = 0.18$). However, the mean lay date for each season's first clutch was significantly negatively correlated to mean monthly wet season river levels in the previous two years ($r_1 = -0.99$, $N = 3$, $P = 0.02$; 1986

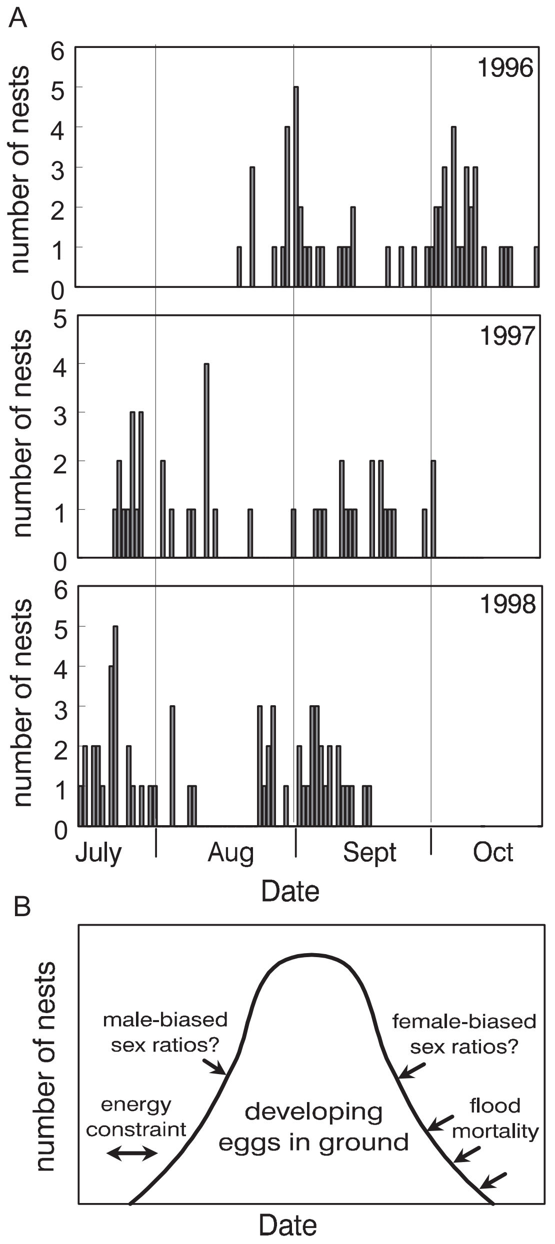

Figure 1. (A) Annual variation in timing of nesting of *C. insculpta* during 1996–98, showing a five week maximum difference in the onset of nesting between years. Data are from daily nest surveys. (B) A hypothetical temporal distribution developing eggs in the population, showing how natural selection and energy constraints likely explain the position of the distribution. The onset of nesting is related to the magnitude of the previous wet season(s), while nests laid too late incur flood mortality.

was not included in the analysis due to inadequate sampling).

NEST SITE CHOICE

Means for nest site attributes and nest depth for the three years combined are listed in Table 1. One hundred and ninety-one nests were found during the three-year study (1996, $N = 65$; 1997, $N = 51$; 1998, $N = 75$). Four beaches with > 10 nests each were used for comparison of available and selected nest sites. On average, nests were closer to water than available sites, but this was significant on only two of 15 beaches ($F_{1,136} = 7.63, P = 0.007$; $F_{1,119} = 10.42, P = 0.002$; $F_{1,38} = 0.92, P = 0.34$; $F_{1,61} = 0.00, P = 0.96$). Similarly, turtles nested at lower elevations than mean available sites on only two of 15 beaches ($F_{1,119} = 9.44$, $P = 0.003$; $F_{1,135} = 0.99$, $P = 0.16$; $F_{1,38} = 0.29$, $P = 0.59$; $F_{1,62} = 0.13$, $P = 0.72$). No pattern existed in slope or aspects of nests relative to mean available sites (all $P > 0.15$), except in one case where significantly gentler slopes were chosen ($F_{1,38} = 8.82, P = 0.005$).

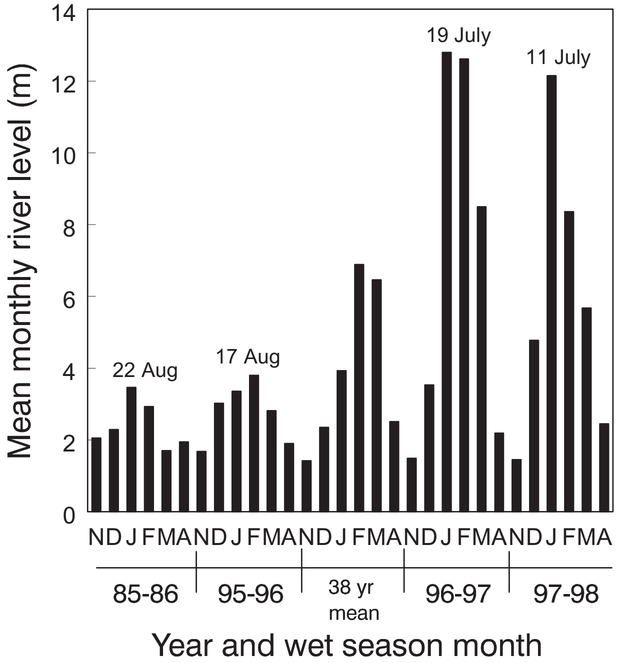

Figure 2. The onset of nesting in *C. insculpta* may be associated with river levels during the previous wet season, an index of the magnitude of the previous wet season's precipitation. Data area mean monthly wet season river levels prior to each year of the study (1996–97, 1997–98), in a previous unpublished study (1986), and a 38 year average (1960–98). Nesting season initiation dates are given above the respective year. Note that 1985–86 and 1995–96 were 'small' wet seasons and that 1996–97 and 1997–98 were 'big' wet seasons. Examining the same association for the previous two wet seasons reveals a similar result. Data are from Dorisvale Crossing and are routinely collected by NT Water Resources.

Although quantitative data revealed no consistent differences in mean elevation between nest sites and available sites, the average variance around mean available sites (3488) was approximately six times that of nest sites (567). This reflected a narrower distribution of nest elevations (mean range = 75.5) compared to available sites (mean range = 194.4). Turtles did not nest at the highest elevations available (Fig. 3), apparently because the sand was not cohesive at these sites on most beaches. Figure 4 shows the distribution of nest site elevations chosen by turtles, and a hypothetical scenario for the maintenance of the distribution. In years with later nesting, low elevation nests will be flooded, while in all years the physical constraint of loose, dry, sand precludes the construction of a nest chamber at higher sites. The resulting distribution of nest sites is leptokurtic relative to the distribution of available sites.

Elevation of nest sites was influenced by year (ANOVA; $F_{2,168} = 16.23, P < 0.001$). In 1998 nests were deposited at higher sites than in 1996 or 1997 (Tukey's HSD, $P < 0.001$ in both cases). Distance of nest sites from water mirrored elevation relationships among years (ANOVA; $F_{2,179} = 8.69, P < 0.001$), with nest sites farther from water in 1998 than in 1996 or 1997 (Tukey's HSD, $P < 0.001$ in both cases). Turtles chose steeper slopes in 1998 than in 1996 (ANOVA; $F_{2,176} = 11.77, P < 0.001$). Slopes were not significantly different between 1997 and the other two years (all $P > 0.05$). Aspect of nest sites differed significantly among years (ANOVA; $F_{2,177} = 5.26$, $P = 0.006$), with

Table 1 Attributes for *C. insculpta* nest sites in 1996–98

Attribute	Mean ± 1 SD (range)	*N*
Elevation above water (m)	0.97 ± 0.437 (0.35–2.88)	178
Distance from water (m)	2.45 ± 1.259 (0.59–9.10)	180
Slope (°)	17.18 ± 9.889 (0–50)	177
Aspect (coded about due N)	13.92 ± 10.025 (0–36)	178
Nest chamber depth (cm)	21.47 ± 1.773 (14.6–26.2)	166

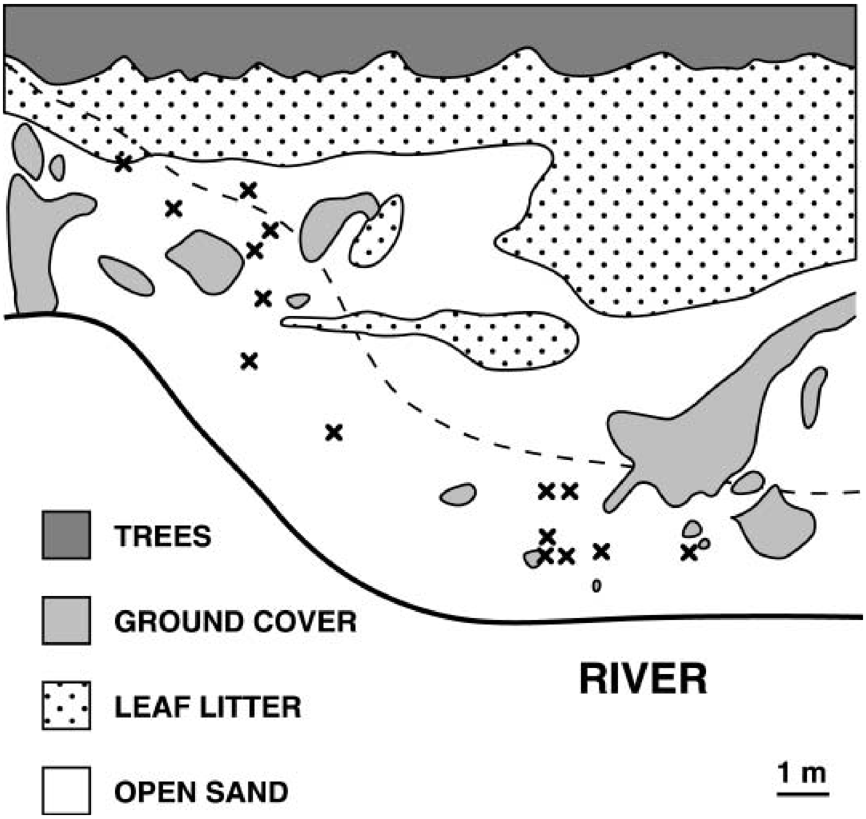

Figure 3. Aerial view of a nesting beach used by *C. insculpta*, showing location of nests (×). Note that the estimated maximum elevation of cohesive sand, denoted by the dashed line, agrees with the maximum elevation of nest sites.

more east-facing nest sites in 1998 than in 1997 ($P = 0.008$).

NEST ELEVATION EXPERIMENT

A field experiment supported our hypothesis that non-cohesive sand was constraining the elevation of available nest sites. Fourteen nests were deposited during the experiment, nine in the treatment (wetted) bands and five in the control (dry) bands (Fig. 5). The position of nests relative to the cohesive sand line (above vs. below) was not independent of band moisture (wet vs. dry; $\chi^2 = 5.83$, d.f. = 1, $P = 0.016$). Elevations of nests in the treatment bands were significantly greater than those in the control bands (ANOVA; $F_{1,13} = 4.75$, $P = 0.001$). Our estimated upper limit of (naturally occurring) cohesive sand agreed closely with the highest nests deposited in the control bands (Fig. 5). Replication of this experiment was not possible due to the small number of nests on most beaches.

NEST TEMPERATURES

Continuous temperatures were successfully recorded in 102 nests for which sex was determined (1996, $N = 30$; 1997, $N = 29$; 1998, $N = 43$). Nest temperatures (CTEs) during the TSP were significantly related to lay date ($\chi^2_1 = 113.51$, $P < 0.0001$); lay date explained 78% of the variation in nest temperatures (linear regression). This pattern was evident for each year (1996, $r^2 = 0.55$, $P < 0.001$; 1997, $r^2 = 0.39$, $P < 0.001$; 1998, $r^2 = 0.65$, $P < 0.001$). Nests laid later in the season were hotter (Fig. 6A) due to the seasonal increase in air temperatures (Fig. 6B). There were no significant relationships between nest temperatures and elevation, distance from water, slope, or nest depth (all $P > 0.15$). Nest temperatures were hotter at more north-facing aspects, but the difference only approached significance ($\chi^2_1 = 3.42$, $P = 0.07$).

Nest temperatures (CTEs) during the TSP differed significantly among years (ANOVA, $F_{2,104} = 11.07$, $P < 0.001$). The mean CTE in 1996 (32.7 ± 1.13°C SD), when nesting was later, was significantly higher than both the mean CTE in 1997 (31.9 ± 1.09°C SD; Tukey's HSD, $P = 0.02$) and 1998 (31.5 ± 1.08°C SD; Tukey's HSD, $P < 0.001$).

HATCHING AND EMERGENCE DATES

Eggs in nests producing all males hatched sooner than eggs in all-female nests in both 1996 and 1997, but in

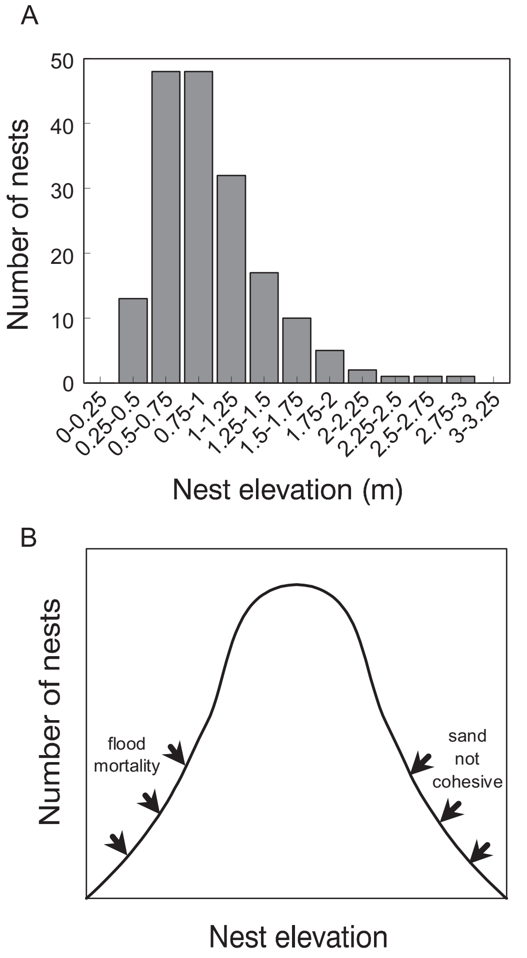

Figure 4. (A) Distribution of nest site elevations chosen by *C. insculpta* in 1996–98. (B) A hypothetical distribution of nest elevations on a beach.

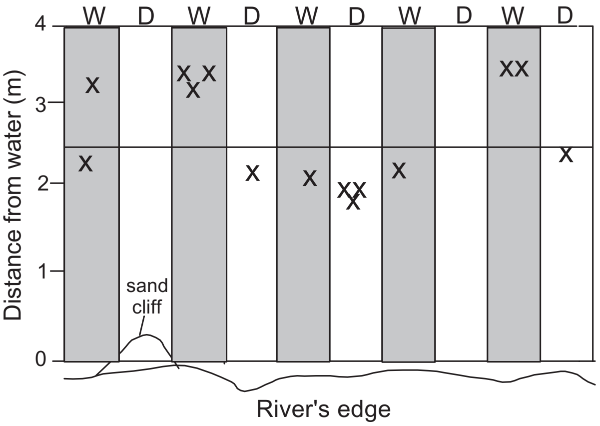

Figure 5. Experimental design (top view) of the 'nest elevation' experiment, showing alternating experimental and control 'bands'. W = wetted, experimental bands (shaded), D = dry, control bands. Experimental bands were manipulated by adding river water every ten days throughout the nesting season. × = nests deposited during the experiment. The horizontal line demarcates the maximum height of cohesive sand. A cliff of sand at the water's edge in the second band from the left may have influenced the probability of turtles nesting in that band.

1996 the difference only approached significance (ANOVA, 1996, $F_{1,28} = 3.49$, $P = 0.07$; 1997, $F_{1,23} = 9.23$, $P = 0.006$). The difference between mean hatching dates was 12 days in both 1996 and 1997. In 1998, the mean emergence date was earlier ($\xi = 19$ days) in all-male nests than in all-female nests (ANOVA, $F_{1,29} = 57.50$, $P < 0.001$) (Doody *et al.*, 2001b).

EMBRYONIC SURVIVAL

Sources of mortality in order of importance were predation, flooding, and intrinsic causes (Fig. 7). Embryonic survival was higher in earlier clutches than in late clutches, but was not influenced by nest site attributes (Table 2). The majority of the variation in embryonic survival was explained by flood mortality. In 1996, 13 nests (3 observed, 10 predicted = 20% of total cohort) were destroyed by a single flood on 22 November. This flood was associated with heavy rainfall during the early wet season, and was 'typical' in timing and magnitude, based on historical river stage data from near the study area (Doody *et al.*, 2001b). Flood survival was lower in nests deposited at low elevations later in the season (together explaining 45% of the variation), while nest depth and other nest site attributes did not influence nest survival (Table 2). No flood mortality occurred in 1997 or 1998; in these years early nesting led to hatching prior to early wet season river rises. A small proportion of nests each year experienced complete embryonic failure for presumably intrinsic reasons (e.g. infertility, developmental problems) (Fig. 7).

Nest predators were the monitor lizards *Varanus panoptes* and *V. mertensi*, based on direct observations and tracks in the sand. Most predation occurred the day after eggs were laid: in 1996, for example, 80% of destroyed nests were taken within 24 h. The small number of destroyed nests precluded statistical tests of the influence of lay date, nest site attributes, and nest depth on the probability of predation.

HATCHLING SEX RATIOS

Hatchling sex was determined in 140 nests (1996, $N = 39$; 1997, $N = 38$; 1998, $N = 63$). Most early (first) clutches produced males, while most later (second) clutches produced females (Contingency analysis; $\chi^2_2 = 83.73$, $P < 0.001$; Fig. 8A). This difference in sex production resulted from the seasonal increase in air temperatures throughout incubation (Fig. 6B).

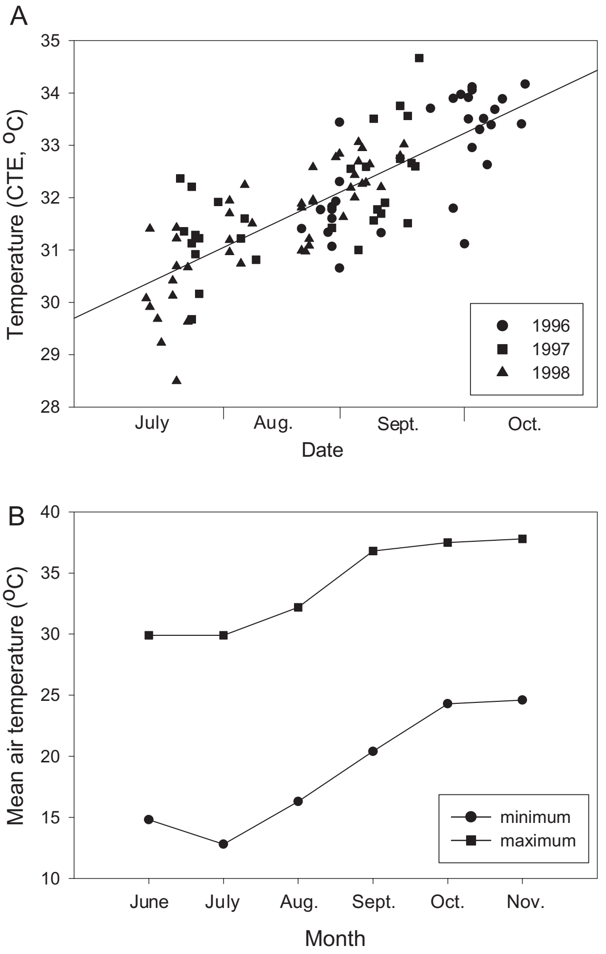

Figure 6. (A) Lay date influences nest temperatures, reflecting (B) a seasonal increase in air temperatures during the nesting season. Temperatures are constant temperature equivalents (CTEs) from the thermosensitive period (TSP). See text for explanation of CTEs and the TSP.

Hatchling sex ratios differed annually, with a preponderance of females in 1996 when nesting was later (140F:62M), compared to sex ratios near unity in 1997 and 1998, when nesting was earlier (Fig. 8B). However, sex ratios in 1996 included ten all-female nests that would have been flooded had they not been removed for sexing prior to the flood. Thus, had we not intervened, hatchling sex ratios would have been near unity in 1996, indicating that when nesting is late, flood mortality offsets the female-biased hatchling sex

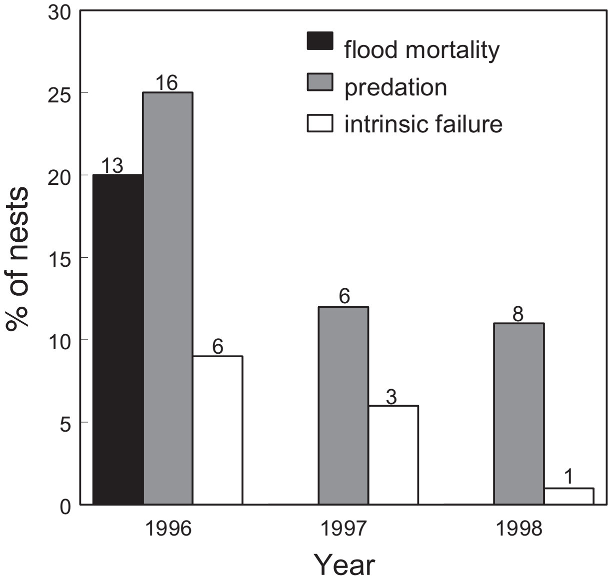

Figure 7. Annual variation in mortality of *C. insculpta* nests during 1996–98. Intrinsic failure refers to clutches that failed to hatch due to inherent causes (e.g. infertility, developmental problems). Sample sizes (number of nests) are presented above bars.

Table 2. Influence of nest site attributes, lay date, and nest chamber depth on embryonic survival, flood survival, and hatchling sex in *C. insculpta*. Results are from Stepwise Discriminant Function Analysis. Flood survival data are from 1996 only

	Total embryonic survival			Flood survival			hatchling sex		
Attribute	partial r^2	$F_{1,113}$	P	partial r^2	$F_{1,33}$	P	partial r^2	$F_{2,95}$	P
Lay date	0.148	20.636	0.000**	0.305	17.987	0.000**	0.464	47.104	0.000**
Elevation	0.017	2.161	0.144	0.140	6.501	0.015*	0.015	0.781	0.461
Distance	0.000	0.005	0.945	0.072	3.018	0.090	0.043	2.428	0.093
Slope	0.001	0.107	0.303	0.015	0.571	0.455	0.066	3.829	0.025*
Aspect	0.003	0.407	0.525	0.016	0.594	0.446	0.014	0.722	0.488
Depth	0.002	0.216	0.643	0.000	0.001	0.980	0.020	1.104	0.335

Distance = from water, depth = nest chamber
*= $P < 0.05$, **$P < 0.0001$

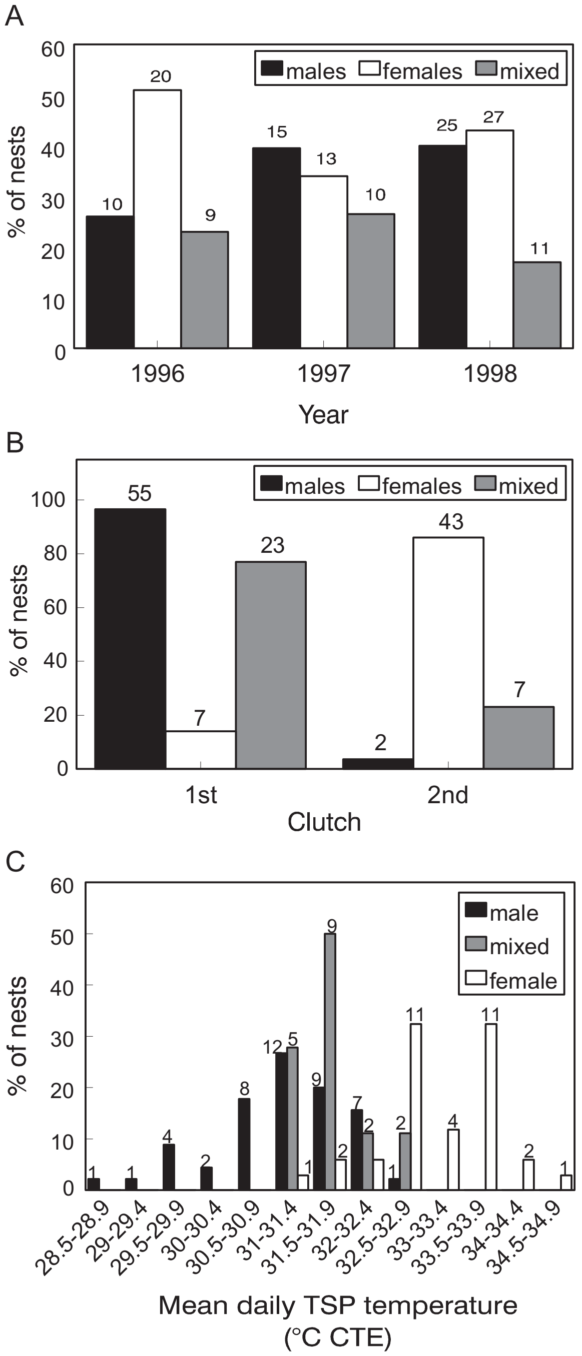

Figure 8. Hatchling sex production in *C. insculpta* in 1996–98, as a function of clutch within a season, year, and nest temperature. (A) Early (first) clutches produced mostly males, while late (second) clutches produced mostly females. (B) Resulting annual variation in hatchling sex ratios as influenced by annual variation in timing of nesting. (C) Hatchling sex production as a function of the mean daily temperature (constant temperature equivalents) during the thermosensitive period (TSP). Samples sizes (number of nests) are presented above bars.

ratio. In a given year 17–26% of nests contained both males and females (mixed nests, Fig. 8B).

The distribution of sexes produced in field nests as a function of mean daily CTE during the thermosensitive period, is given in Figure 8C. Males were produced at cooler temperatures ($\xi = 31.2 \pm 0.92$°C SD; range = 28.5–32.8°C, $N = 45$), mixed sexes resulted from intermediate temperatures ($\xi = 31.8 \pm 0.46$°C SD; range = 31.1–32.7°C, $N = 19$), and females were produced at hotter temperatures ($\xi = 33.1 \pm 0.75$°C SD; range = 31.4–34.7°C, $N = 35$) (Fig. 8).

Hatchling sex was determined primarily by lay date, which explained 47% of the variation in sex production (Table 2; Fig. 6A). The analysis revealed also revealed a weaker but significant effect of slope on hatchling sex (Table 2). Female-producing nests were deposited in gentler slopes than male-producing nests. Nest depth and other nest site attributes did not influence hatchling sex (Table 2).

DISCUSSION

The evolution of environmental sex determination has been evaluated interspecifically by the comparative method (Ewert & Nelson, 1991; Ewert, Jackson & Nelson, 1994), and intraspecifically by identifying mechanisms generating phenotypic variance in nature (reviewed in Shine, 1999). Although this approach has provided valuable insights, we still do not understand why ESD has evolved in reptiles (Shine, 1999; Valenzuela & Janzen, 2001). Differential fitness models for the evolution of TSD pivot around a critical life history stage: the developing embryo (Shine, 1999). Quantifying embryonic survival in nature therefore is essential for careful interpretation of the role of offspring sex ratios in the evolution or maintenance of TSD (Valenzuela & Janzen, 2001). Using this approach, the present study examined the influence of timing of nesting and nest site choice on embryonic survival and hatchling sex in a combined and natural context. We found evidence that two maternal trait distributions that are important determinants of hatchling sex and embryonic survival in *C. insculpta*, are shaped by multiple processes, including natural selection, physical constraints, and phenotypic plasticity.

TIMING OF NESTING

High annual variation in lay date and its consequences in embryonic survival and sex production in *C. insculpta* highlight the importance of considering timing of reproduction in studies of reproductive tactics (Olsson & Shine, 1997). The 5-week difference in the onset of nesting between 1996 and 1998 (Fig. 1A) is high for turtles (e.g. in *Chelydra serpentina*, 22 days, Congdon *et al.*, 1987; 14 days, Obbard &

Brooks, 1987; ten days; Iverson *et al*., 1997; in *Emydoidea blandingi*, six days, Standing, Herman & Morrison, 1999; 23 days, Congdon *et al*., 1983; in *Chrysemys picta*, < 15 days, Congdon & Gatten, 1989; in *Terrapene ornata*, 12 days, Doroff & Keith, 1990), and possibly for reptiles in general. We might expect that turtles would nest earlier in years with warmer air or water temperatures (Congdon *et al*., 1983, 1987; Obbard & Brooks, 1987; Iverson & Smith, 1993; but see Christens & Bider, 1987), due to enhanced rates of egg maturation (Whittow & Balazs, 1982; Congdon *et al*., 1987; Adolph & Porter, 1993). However, timing of nesting of *C. insculpta* was not associated with water or air temperatures in the months prior to nesting.

Instead, we found a correlation between timing of nesting and river levels during the previous one to two wet season(s) (Fig. 2). Direct evidence of food uptake influencing lay date is lacking for reptiles, but correlational evidence exists for lizards and snakes (Olsson & Shine, 1997; Weatherhead *et al*., 1999). In some birds, lay date advances with natural or experimental increases in food availability (see reviews in Drent & Daan, 1980; Rowe, Ludwig & Schluter, 1994). Timing of nesting in *C. insculpta* may be related to energy acquisition during the previous wet season(s), if the wet season is a critical period of energy accumulation (Christian *et al*., 1995, 1999a; Madsen & Shine, 1996; Christian, Bedford & Shultz, 1999b). In support of this (1) biennial reproduction in the population indicates that females are energy-limited (Doody *et al*., 2003b), and (2) dry season food is constant but energy-poor (Heaphy, 1990; Welsh, 1999), suggesting that annual differences may be related to wet season food intake (Doody *et al*., 2003b). How might the magnitude of wet season precipitation dictate energy accumulation in *C. insculpta*? The wet season diet of the population is unknown, but continuous flooding of the river channel would generally displace turtles (Doody *et al*., 2002) away from their principal dry season food (aquatic vegetation, Heaphy, 1990; Welsh, 1999). A test of the importance of the wet season to *C. insculpta* reproduction would require knowledge of the wet season diet, and a study of seasonal variation in field energetics.

NEST SITE CHOICE

Several studies have demonstrated that turtles actively choose a nest site by selecting nest site attributes non-randomly (Schwartzkopf & Brooks, 1987; Wilson, 1998; Valenzuela & Janzen, 2001; Kolbe & Janzen, 2002). What variables were *C. insculpta* assessing when choosing a nest site? We experimentally removed a constraint on nest site choice, demonstrating that (1) loose dry sand limits elevation of the nest site, and that (2) turtles will nest at higher elevations if given the opportunity (Fig. 6). Nesting higher can be critical, as demonstrated by flood mortality in lower elevation nests, and the lowest elevations are generally avoided (Figs 3, 4). The resulting distribution of nest site elevations appears to reflect opposing mechanisms: non-cohesive sand at higher elevations and natural selection through flood mortality at lower elevations (Fig. 4B). This spatial difference between nest sites and 'available sites' can be clearly seen in our map of a beach utilized by nesting *C. insculpta* (Fig. 3). Similarly, distance from water of nests sites differed from available sites; however, distance from water is likely an artefact of elevation above water, as turtles would often nest close to water when the beach was steep (see also Doody, 1995). Coarse, dry sand has been implicated in the difficulty of constructing a nest chamber in some turtle populations (reviewed in Mortimer, 1990; Bjorndal & Bolten, 1992). The difficulty in constructing a nest chamber by turtles in our study reflects a vertical moisture gradient through the sand, and extremely dry conditions during the nesting season at our study site (e.g. mean monthly rainfall for August = 1 mm).

Annual variation in most nest site attributes, especially elevation, was unexpected. In theory, turtles should select nest sites with similar elevations among years if natural selection is favouring the avoidance of the lowest elevation sites. However, the magnitude of the upper limit on nest elevation (cohesive sand availability) could vary annually depending on weather patterns (Bjorndal & Bolten, 1992) or among-beach variation in factors influencing cohesiveness of the sand (e.g. substrate type and characteristics, Mortimer, 1990). Few individual beaches are available to turtles in consecutive years due to their ephemeral nature (Doody *et al*., 2003a, 2003b). In contrast, weather patterns during the dry season in this region of northern Australia show very little annual variation. Therefore, the annual variation in nest site attributes that we observed is likely an artefact of beach (i.e. site-specific differences in beach size, aspect, shape, or silt content of sand). This idea is supported by considerable among-beach variation in aspect and beach temperatures measured in a companion study (Doody *et al*., 2003a).

EMBRYONIC SURVIVAL AND ITS DETERMINANTS

Embryonic mortality in our study had two major sources: predation and flooding, and the latter was strongly influenced by timing of nesting and nest site choice. Our finding of lower survival in nests laid later and at lower elevations (Table 2) may not be unusual, given low annual variation in the timing of early wet season flooding (river rises in mid- to late November), as evidenced by historical river stage data (Doody *et al*., 2001b). Although *C. insculpta* typically aestivates in the egg to coincide emergence with the onset of early wet season rains (Webb, Choquenot & Whitehead, 1986; Doody *et al*., 2001b), our mortality estimates rest upon the assumption that embryos must be fully developed to hatch and survive flooding.

Flooding is a common source of embryonic mortality in riverine turtle populations (Roze, 1964; Plummer, 1976; Doody, 1995). Although populations can presumably respond through selection for the behaviour of nesting at higher elevations, substantial mortality occurs when (1) all available nest sites are flooded in extreme years (e.g. Plummer, 1976; Doody, 1995), or when (2) incubation extends into a wet season (present study, Alho & Padua, 1982). Elevation of nest sites has been shown to influence embryonic mortality in other turtle species (e.g. Whitmore & Dutton, 1985), and one species nests at high tide, apparently as an adaptation to avoid nest flooding (Burger & Montevecchi, 1975).

DETERMINANTS OF NEST TEMPERATURES AND HATCHLING SEX

The pattern of sex determination in natural nests was cooler temperatures producing all males, intermediate temperatures producing mixed sexes, and hotter temperatures producing all females (Fig. 8C). These data confirmed a type 1a pattern of TSD (Ewert & Nelson, 1991) suggested by laboratory and fieldwork (Webb *et al*., 1986; Georges, 1992). The range of temperatures producing mixed sexes (pivotal temperature) within a nest was about 1.5°C (31.1–32.7°C, CTE). In comparison to other species this pivotal temperature range is narrow but falls within range of pivotal temperatures determined for other species (Vogt & Flores-Villela, 1992; Mrosovsky & Yntema, 1980; Viets *et al*., 1993; Lang & Andrews, 1994).

A strong seasonal component to hatchling sex ratios within years (Fig. 8A) was due to a seasonal increase in air temperatures during nesting and incubation (Fig. 6B). Seasonal trends in hatchling sex ratios are known in several temperate reptiles (Vogt & Bull, 1984; Mrosovsky, Hopkins-Murphy & Richardson, 1984b; Harlow & Taylor, 2000; but see Spotila *et al*., 1987). We agree with Shine (1999) that a seasonal pattern in sex production is likely to be common in tropical reptiles with TSD (i.e. in species with extended nesting seasons). However, such data are currently scarce for tropical TSD species (but see Mrosovsky, Dutton & Whitmore, 1984a; Webb & Smith, 1984; Lang, Andrews & Whitaker, 1989; Godfrey, Barreta & Mrosovsky, 1996).

In the present study nest site choice exerted a weaker but significant influence on hatchling sex ratios (Table 2). Female-producing nests were constructed in gentler slopes than male-producing nests. However, the difference between means was small and seems biologically insignificant because slope did not influence nest temperatures. Surprisingly few studies have investigated the influence of nest site choice on hatchling sex ratios in reptiles. The major spatial determinant of sex ratios in studies to date was the extent of shading vegetation, although this was not quantified for individual nest sites (Bull & Vogt, 1979; Morreale *et al*., 1982; Vogt & Bull, 1984; Spotila *et al*., 1987; Roosenberg, 1996). Hatchling sex ratios can also be influenced by nest site attributes such as aspect and solar exposure (Janzen, 1994). Other studies on TSD species have found little correlation between nest site attributes and sex ratios (e.g. Schwartzkopf & Brooks, 1987).

MATERNAL NESTING STRATEGY: POSSIBLE CAUSES

Our study supports the recent suggestion that spatial and temporal variation in the reptilian embryonic environment may generate substantial variation in life history traits (Shine & Harlow, 1996; Madsen & Shine, 1999). We obtained circumstantial evidence for the evolution or maintenance of two attributes of the maternal nesting strategy in *C. insculpta*. Nest site choice behaviour and timing of nesting in *C. insculpta* appear to reflect multiple processes, including natural selection, physical constraints, and phenotypic plasticity (Figs 1A, 4B).

If the onset of nesting was related to the magnitude of the previous wet season(s) (Fig. 2; see also Doody *et al*., 2003b for evidence of other reproductive correlates of the wet season), then temporal placement of the reproductive season may reflect a left tail dictated by phenotypic plasticity (energy accumulation constraint), and a right tail shaped by natural selection via flood mortality of nests (Fig. 1B). Under this scenario, the onset of nesting would vary among years, while the right tail would likely represent a 'harder line', based on the average timing of flood mortality among years (Doody *et al*., 2001b). We are unaware of any previous quantitative studies on turtles demonstrating that timing of nesting or lay date influences embryonic mortality through nest flooding.

The idea that embryonic mortality can shape nest site choice has some empirical support in reptiles (Madsen & Shine, 1999; Valenzuela & Janzen, 2001; Kolbe & Janzen, 2002; Spencer, 2002), and a genetic underpinning to behaviour associated with nest site choice is likely (Janzen & Morjan, 2001). The distribution of nest elevations appeared to reflect opposing mechanisms: flood mortality in the lowest elevation sites (left tail) and non-cohesive sand imposing an upper limit on high elevation sites (right tail) (Fig. 4B). Studies of nesting strategies in turtles have rarely identified the ultimate causes of variation in nest elevation or distance from water, although the placement of nests in aquatic species presumably reflects opposing factors at some scale (Mrosovsky, 1983; Bjorndal & Bolten, 1992). A notable exception was a study experimentally demonstrating that nest site choice in *Emydura macquarii* appeared to reflect a trade-off between adult and offspring survival (Spencer, 2002). Interestingly, nest site choice exerted no clear influence on hatchling sex in our study. Rather, a strong temporal component (lay date) was implicated in sex production, leading us to conclude that behaviour associated with nest site choice in *C. insculpta* is not engendered by offspring sex ratios. Our data also reinforce the importance of investigating and explaining environmental variation underpinning trait distributions, because natural selection has presumably already shaped the temporal placement of the nesting season. For example, in the present study we were fortunate to have four years of data following periods of rainfall patterns at both extremes (Fig. 2), allowing us to infer how the reproductive strategy of *C. insculpta* is shaped by the wet-dry tropics. While lay date exerts a strong influence on hatchling sex ratios, nesting early or late will not necessarily result in skewed hatchling sex ratios. Because the two 'big' wet seasons during our study were the largest on record for the catchment, and because big wet seasons apparently lead to earlier nesting (Fig. 2), we may have documented the earliest nesting possible. Yet, sex ratios following these two years were near unity, rather than being male-biased. Similarly, our data indicate that any female-bias in hatchling sex ratios engendered by late nesting would be offset by differential mortality of female nests due to flooding.

FIT OF DIFFERENTIAL FITNESS MODELS OF TSD TO *C. INSCULPTA*

'Differential fitness' models for the evolution of TSD in reptiles propose that TSD can enhance maternal fitness by enabling the embryo to develop as the sex best-suited to those incubation conditions (reviewed in Shine, 1999). Each model posits links among incubation temperature, phenotype, and fitness, but each differs in the mechanisms generating phenotypic variance (Shine, 1999). The model that best fits the *C. insculpta* system is the 'time-matching' model, whereby phenotypic variation is driven by sex ratio adjustment relative to date of hatching (Conover & Kynard, 1981; Conover, 1984; Shine, 1999). In *C. insculpta*, males were produced early and females late, despite this difference being reduced by (1) shorter incubation in female-producing nests than in male-producing nests, and (2) embryonic aestivation in the egg (Doody *et al*., 2001b). About two weeks separated the mean hatching or emergence dates of males and females. There may be a fitness advantage in producing males early in *C. insculpta*, as hypothesized for the lizard *Amphibolurus muricatus* (Harlow & Taylor, 2000). In the fish *Menidia menidia*, a size-induced increase in fitness is higher in females than in males, favouring the early production of females (Conover & Kynard, 1981; Conover, 1984). However, in both the fish and the lizard, maturation occurs in one season (Conover & Kynard, 1981; Harlow & Taylor, 2000), and thus, incubation temperatures may be linked to temperatures during growth (Conover, 1984). Although maturation time in *C. insculpta* is unknown, long-lived animals such as turtles take a minimum of several years to mature (Gibbons, 1987; Iverson, 1991; Shine & Iverson, 1995). This means that, for *C. insculpta* (1) incubation temperatures cannot predict temperatures during growth and maturation (2) a 2–3 week growth advantage is less likely to translate into adult fitness, and (3) establishing links among incubation temperature, phenotype, and fitness, relative to sex, will likely be difficult. We should also note that covariation between hatching date and nest temperatures is likely to be common in reptile species laying multiple clutches (Shine, 1999; Harlow & Taylor, 2000), and so differences in hatching dates between sexes is not necessarily evidence for the 'time-matching' model.

We also note that Reinhold's (1998) natal homing hypothesis for the evolution of TSD, proposed for sea turtles, is not upheld in *C. insculpta* (see also Kolbe & Janzen, 2002). The majority of nesting beaches in our study system are ephemeral among years due to wet season flooding, precluding philopatry to nest site or beach. Further, a prediction of the natal homing hypothesis, that male-producing nest sites are of lower quality than female-producing nest sites (Reinhold, 1998), was not upheld in our study.

The evolution of TSD introduces an additional problem, achieving balanced sex ratios, into the ecology of a species. Although our study does not conclusively demonstrate a role for hatchling sex ratios in the maternal nesting strategy of *C. insculpta*, previous work indicates that understanding reproductive strategies is crucial to understanding how and why TSD might evolve (Conover, 1984; Roosenberg, 1996). Our knowledge of the reproductive strategy has not yet revealed why TSD occurs in *C. insculpta*, but our research has provided a framework for pursuing that question by reducing the number of competing explanations for an increasingly enigmatic phenomenon (Shine, 1999; Harlow & Taylor, 2000).

ACKNOWLEDGEMENTS

We are grateful to the following for assistance in the field: R. Alderman, M. Pauza, A. Pepper, S. Thomson, M. Welsh, and P. West. We thank R. Sims for reviewing an earlier draft. The Douglas Daly Research Farm of the Department of Primary Industries and Fisheries provided laboratory space and accommodation. This research was sponsored by an Australian Research Council Large Grant awarded to A. Georges.

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