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The impact of behavioral and physiological maternal effects on offspring sex ratio in the common snapping turtle, *Chelydra serpentina*

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Abstract Theory suggests that maternal effects are especially important in organisms with environmentally-sensitive sex-determining mechanisms. However, there is no substantive body of empirical evidence to confirm this conjecture. We integrated field and laboratory studies to jointly evaluate the significance of behavioral (nest-site choice) and physiological (yolk hormone allocation) maternal effects on offspring sex ratio in the common snapping turtle (*Chelydra serpentina*), a species with temperature-dependent sex determination (TSD). Of the 16 microhabitat variables measured, only three (south, east, and total overstory vegetation cover) were significantly correlated with nest temperature: cooler nests were located under more vegetation cover. In turn, these microhabitat predictors of nest temperature, and nest temperature itself, may influence nest sex ratio: shadier, cooler nests were more likely to produce a higher proportion of male offspring than less shady, warmer nests. Analysis of eggs from these same nests incubated in a common garden design in the laboratory revealed that

clutch sex ratio was unaffected by levels of yolk estradiol, yolk testosterone, or their interaction. Examination of both behavioral and physiological maternal effects revealed no concordant impact on offspring sex ratio. However, eggs from nests that produced male-biased sex ratios in the field yielded higher proportions of males under constant-temperature conditions in the laboratory. Our study confirms the importance of behavioral maternal effects in nature on offspring sex ratios in species with TSD, while also revealing the potential presence of a predisposition for sex-ratio production underlying TSD in this system.

Keywords Environmental sex determination · Maternal effects · Nest-site choice · Temperature · Yolk hormones

Introduction

The sex of an individual is a critical component of its life history and has numerous implications for its fitness (Karlín and Lessard 1986). Likewise, the mechanism by which sex is determined is important for both life history and fitness components (Bull 1983; Valenzuela et al. 2003). It is commonly accepted that there are two main categories of sex-determining mechanisms (Bull 1983). The first and most familiar is genotypic sex determination, wherein an individual's sex is determined at conception by genetic factors. The second is environmental sex determination (ESD). Environmental sex determination has several forms, one of which is temperature-dependent sex determination (TSD). Under TSD, the sex of offspring is determined predominantly by temperature during embryonic development. However, recent research into the mechanisms underlying TSD suggests that physiological pathways may play a role in determining sex in addition to the recognized effects of temperature (e.g., Bowden et al. 2000). Given rapid local and global ecological changes (climate warming, endocrine disrupting contaminants, etc.), the status of species with ESD is of special concern, as their fitness could be directly im-

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pacted (Gibbons et al. 2000). A deeper exploration of ESD (i.e., underlying mechanisms and microevolutionary potential) is essential for a full understanding of species that exhibit this remarkable life-history trait.

Temperature-dependent sex determination is widespread among turtles, lizards, tuatara, and crocodylians (Janzen and Paukstis 1991; Valenzuela and Lance 2004). For instance, in most turtles females are produced at higher temperatures and males at lower temperatures, with an intermediate (pivotal) temperature at which a 1:1 sex ratio should be produced (Mrosovsky and Pieau 1991). Despite considerable research into the patterns and processes of TSD, there is nonetheless much to learn concerning the basic biology underlying TSD, including why it evolved and is maintained in so many fish and reptile species (Morjan and Janzen 2003; Valenzuela 2004).

Bulmer and Bull (1982) proposed a model for the microevolution of TSD which states that, subsequent to its origin, this sex-determining mechanism could be regulated by heritable variation in two respects. The first is the thermal sensitivity of embryonic sex determination (e.g., the pivotal temperature) and the second is the behavioral choice of thermal qualities of a nest site by the mother. A great deal of work has addressed aspects of the thermal sensitivity of embryonic sex determination (e.g., Bull et al. 1982; Janzen 1992; Ewert et al. 1994), but relatively little attention has been paid to the behavioral choice of nest sites (e.g., Janzen and Morjan 2001). No consideration, theoretically or empirically, has been devoted to covariation between these two traits.

Despite considerable research effort focused on addressing the adaptive value of TSD in reptiles, no clear empirical answer has yet appeared (Shine 1999; Valenzuela 2004). Beyond a growing recognition of their general biological importance (Mousseau and Fox 1998), there has been an increasing focus on the role that maternal effects might play in the persistence of TSD (Roosenburg 1996; Reinhold 1998; Freedberg and Wade 2001; Janzen and Morjan 2001; Valenzuela and Janzen 2001; Morjan and Janzen 2003). Maternal effects can vary in their magnitude and nature. Nest-site choice by a female is a behavioral maternal effect that can have dramatic effects on the sex of offspring, and there is evidence supporting possible heritable variation for choice of nest-site microclimate (Janzen and Morjan 2001). Behavioral investment is not the only maternal factor affecting clutch sex ratio. Recent laboratory evidence implies that endogenous hormone levels in egg yolk alter clutch sex ratios of reptiles with TSD (Janzen et al. 1998; Bowden et al. 2000, 2001). Variation in endogenous yolk hormone levels thus provides another means by which a female could skew the sex ratio of her offspring.

In this study, we evaluated the significance of behavioral (nest-site choice) and physiological (yolk hormone allocation) maternal effects on offspring sex ratio in the common snapping turtle, *Chelydra serpentina*. We first determined if the microclimate properties of a nest could accurately predict nest temperature, and therefore clutch

sex ratio, in the field. Second, we evaluated the relationship between levels of endogenous steroid hormones in egg yolks and clutch sex ratio under common garden conditions in the laboratory. Finally, we explored whether the behavioral and physiological maternal investments varied concordantly (i.e., did females skew the sex ratio of their clutches in the same direction both behaviorally and physiologically?). To accomplish these goals, we examined the sex ratio of clutches from each of which one subset of eggs was incubated in the laboratory and one subset in the field (i.e., in the natal nest).

Methods

Study organism and field site

The common snapping turtle, *Chelydra serpentina*, is a widespread freshwater turtle, occurring in the eastern two-thirds of North America as well as in Central America and northern South America (Ernst et al. 1994). This species exhibits pattern II TSD, with females produced at both low and high temperatures and males produced at intermediate temperatures (Ewert et al. 1994). Therefore, two pivotal temperatures of egg incubation (~21.5°C and ~27.6°C) should yield a 1:1 sex ratio. Only the upper pivotal temperature is likely to be ecologically relevant in our population, though, because the lower pivotal temperature is near the limit for successful embryonic development and is rarely approached during the temperature-sensitive period of sex determination (Janzen 2004).

We conducted the field research in the Upper Mississippi River National Wildlife and Fish Refuge in Carroll and Whiteside Counties, Ill. We monitored two large sections of the refuge adjacent to the Mississippi River for nesting snapping turtles. One section had residential dwellings and the second section was largely undisturbed by humans. Both locations are primarily sand prairie with a riparian zone near the river. A more detailed description of the field site is provided elsewhere (Kolbe and Janzen 2002).

Field methods

We patrolled the field site for nesting activity daily from 1 to 30 June 2001. Nesting events ($n=48$) occurred between 7 and 27 June. Because the turtles primarily oviposit during darkness, we patrolled the sections at dawn, and located 26 intact nests.

We recorded a number of microhabitat variables at nest sites using methods similar to those described in Kolbe and Janzen (2002). When a nest was encountered, we first measured the proportion of overstory cover at ground level at the nest site using a Model-A spherical densiometer facing each of the four cardinal directions [see Janzen (1994a) for a more complete description]. The other nest-site variables measured included: distance of the nest from the river, maximum vegetation height, modal vegetation height, slope, aspect, proportion of bare ground, proportion of litter cover, proportion of grass cover, proportion of forb cover, proportion of cactus cover, and total proportion of ground vegetation cover. Because there was always some disturbance at the nest site, such as vegetation flattened during nesting, we obtained these latter measurements from a 1×1-m quadrat located in a random cardinal direction 0.5 m from the nest site. These locations appeared to differ very little from the actual nest site (St. Juliana, personal observation).

After recording the vegetation measurements, we removed 12 eggs from the top of each nest for laboratory incubation and steroid hormone determination. This sampling procedure is appropriate because all eggs in a clutch are produced simultaneously within an individual turtle (reviewed in Moll 1979), thus order effects in egg size and yolk hormones are negligible (Janzen et al. 1998; Tucker

and Janzen 1998). We placed the eggs immediately into chicken egg cartons containing damp sand and stored the cartons in a shaded Styrofoam cooler for 1–4 days before transporting them to Iowa State University. Only the top eggs were removed from the nest to minimize disturbance to the remaining eggs (24–65 eggs/nest), which were left to incubate in their natal nests.

We protected each nest from predation using a cage (with a 3-cm grid of white, plastic-coated wire) placed over the center of the nest and secured with gardening rebar. The grid allowed plants to persist through its openings, while minimizing disturbance to the nest. Several nests located on a sand road at the residential site did not receive a cage for practical reasons. Temperature loggers (HOBO Temp, Onset Corp.) were placed into 14 nests between 27 and 30 June. Nests were briefly re-opened for insertion of an external thermocouple probe into the middle of the nest and the data loggers were buried approximately 1 m from the nest cavity. Loggers were set to record temperature every 45 min for about 7 weeks.

On 12 August, we excavated all nests that survived predation ($n=15$) and removed loggers and eggs (or hatchlings) for transport to Iowa State University. Most nests contained unhatched eggs, so their incubation was completed in the laboratory. For statistical analyses involving nest temperature, we used data from the first half of July because several loggers were disabled by predators (even though the nest survived) or malfunctioned after 15 July ($n=4$). The first half of July also coincides broadly with the temperature-sensitive period of offspring sex determination for most of the clutches in this study (Kolbe and Janzen 2002) because, all else being equal, embryos in later nests have accelerated development due to warmer ground temperatures at oviposition compared to embryos in earlier nests. All nests in our study completed this sex-determining period prior to excavation on 12 August.

Laboratory methods

Of the 12 eggs from each clutch brought to the laboratory, we stored two eggs at -20°C for subsequent hormone analysis. Previous work with this population has not detected much variation in yolk testosterone among four eggs, two from the first and two from the last laid eggs in each of 20 clutches (Janzen et al. 1998). We placed the other 10 eggs from each clutch in an incubator nominally set at the upper pivotal temperature (i.e., $\sim 27.6^{\circ}\text{C}$) (Janzen 1992; Janzen et al. 1998). We incubated eggs only at the upper pivotal temperature, because the lower pivotal temperature is unlikely to be ecologically important in our population (Janzen 2004). However, due to human error, incubator temperature was near 30°C in late June and early July, causing an average temperature during incubation of $28.37 \pm 0.18^{\circ}\text{C}$ (measured with a HOBO temperature logger in the center of the chamber). We randomly assigned one egg from each clutch to a covered plastic shoebox and then to a random position within that shoebox until all 10 eggs were placed for each clutch. The shoeboxes contained moistened vermiculite at -150 kPa (300 g dry vermiculite plus 337 g deionized water). We maintained approximately constant hydric and thermal conditions during embryonic development by adding back any lost water once weekly and by rotating boxes within the incubator every other day. We treated field-incubated eggs that had not yet hatched in a similar manner, although each clutch was assigned to its own box.

We checked all boxes 2–3 times/day for hatchlings, beginning 12 August. When a turtle hatched, we placed it in a cup with a moist paper towel for 2 days to allow for greater solidification of the carapace and absorption of the yolk sac. We held hatchlings with their appropriate group of lab- or field-incubated siblings at room temperature ($\sim 22^{\circ}\text{C}$) in a plastic bin containing water. We sacrificed all surviving hatchlings from lab-incubated eggs (25 clutches, $n=222$ turtles, 6–10 turtles/clutch) for sex determination. For analysis of sex ratio of field-incubated eggs (15 clutches, $n=611$ turtles), we sacrificed 10 randomly chosen hatchlings from each clutch except one (hatchlings in that clutch were unavailable for sex ratio assessment). The euthanasia procedure consisted of a pericardial injection of 0.5 ml of 1:1 deionized water:Sleepaway. We

noted the sex of each sacrificed turtle by macroscopic examination of the gonads (Janzen 1992). All individuals were preserved in 70% EtOH as voucher specimens. We released all surviving hatchlings from field-incubated eggs at the field site on 16 September.

We used a competitive-binding steroid radioimmunoassay (RIA) to measure levels of testosterone and 17β -estradiol in *C. serpentina* yolk samples from 20 clutches. To prepare yolk samples for the assay, we separated frozen yolks from the other egg components and homogenized each yolk. We collected approximately 50 mg of homogenized yolk from each egg and suspended the sample in 500 μl of dH_2O . We followed the RIA procedure of Wingfield and Farner (1975), see also Bowden et al. (2000, 2001): we added 2,000 cpm tritiated testosterone and 17β -estradiol (New England Nuclear, Boston, Mass.) to each sample to serve as a tracer and then vortexed the sample and allowed it to equilibrate overnight at 4°C . We extracted hormones from the samples using petroleum and diethyl ethers, reconstituted them in 90% ethanol (Schwabl 1993), stored the reconstituted samples at -20°C overnight to allow for sedimentation of neutral lipids, and then centrifuged and decanted the samples. We evaporated the supernatant under nitrogen gas and resuspended each sample in 10% ethyl acetate in isooctane in preparation for column chromatography. The columns consisted of a celite:ethylene glycol:propylene glycol upper phase and celite:water lower phase. We applied the samples directly to the columns and completed hormone separation by eluting each fraction with a unique ratio of ethyl acetate:isooctane (20% for testosterone and 40% for 17β -estradiol). In the end, we dried the fractionated samples under nitrogen gas, resuspended them in phosphate buffer, and measured hormone concentrations by competitive-binding RIA using antibodies specific for each hormone of interest (antibody for testosterone from Wien Laboratories, Succasunna, NJ; antibody for 17β -estradiol from Arnel, New York, N.Y.).

We ran all yolk samples in duplicate in a single assay and compared hormone concentrations to a standard curve that ranged from 1.95 to 500 pg for both testosterone and 17β -estradiol. Recovery values averaged 69.7% for testosterone and 64.2% for 17β -estradiol. The intra-assay variation, calculated as the coefficient of variation for the standards, was 6.68% for testosterone, and 6.62% for 17β -estradiol.

Statistics

Because we evaluated 16 environmental variables to assess nest-site choice, we implemented a principal components analysis in StatView 512+ to obtain uncorrelated factors for further statistical evaluation. We used JMP 4.0.4 (SAS Institute 2001) for all linear regression and correlation analyses as well as for analyses of variance, and Proc Logistic in SAS 8.2 to perform logistic regression for all analyses involving clutch sex ratio (expressed as proportion male). We log-transformed hormone values prior to their statistical evaluation. All figures present untransformed data for clarity.

Results

Behavioral maternal effect

To explore the thermal effects of microhabitat variables on nests, we regressed the factor scores from the first three principal components of these variables (PC1, PC2, and PC3), which explained $>87\%$ of the original variance, on nest temperature for the first half of July. Only PC1, which was mainly comprised of approximately equal loadings of the five overstory cover variables, was significantly correlated with nest temperature ($r=-0.77$, $n=14$, $P=0.0014$; $P>0.37$ in the other two cases). This

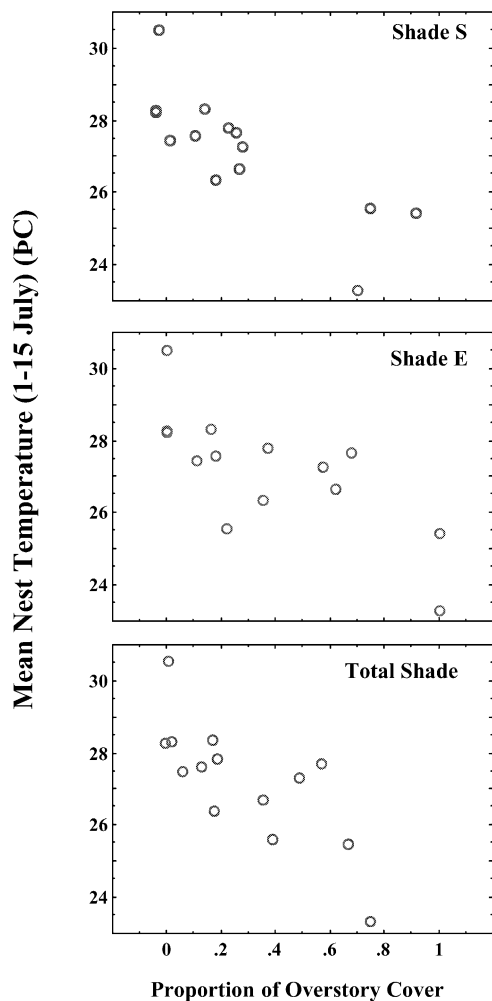


Fig. 1 Relationship between mean nest temperature and proportion of southern (a), eastern (b), and total (c) overstory nest cover. Mean nest temperature was calculated by averaging temperatures recorded every 45 min between 1 and 15 July 2001 for 14 nests. Overstory vegetation cover was determined using a spherical densiometer (see text). All nests were laid between 7 and 27 June and temperature loggers were implanted into the nests between 27 and 30 June. Mean nest temperature was significantly correlated with each of the three measurements of overstory cover ($P=0.0006$, $P=0.0006$, and $P=0.0013$, respectively)

result accords with linear regression analysis of the 14 normally-distributed microhabitat variables measured (i.e., all variables but aspect and proportion of cactus cover). Only three of these variables, all of which were important contributors to PC1, were substantially correlated with nest temperature: proportion of overstory cover on the south side of the nests (“shade S”: $r=-0.80$, $n=14$, $P=0.0006$), proportion of overstory cover on the east side of the nests (“shade E”: $r=-0.79$, $n=14$, $P=0.0006$), and total proportion of overstory cover around nests (“total shade”: $r=-0.77$, $n=14$, $P=0.0013$). No other comparison had a P -value < 0.01 . Overall, cooler nests were located under more overstory cover than warmer nests (Fig. 1).

The overall sex ratio of the hatchlings deriving from field-incubated eggs was strongly female-biased (22.1%

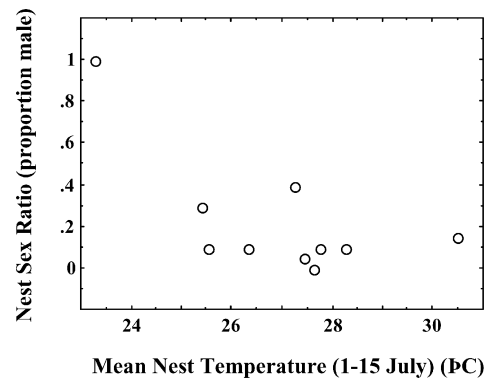


Fig. 2 Relationship between the proportion of males resulting from incubation in the natal nest (nest sex ratio) and mean nest temperature ($n=10$ nests with both hatchlings and functional temperature loggers) ($P=0.1816$). Sex ratios were determined using 10 randomly chosen hatchlings from those recovered from each natal nest. Note that the overall sex ratio of field-incubated hatchlings was strongly female-biased, and that the nest with the lowest mean nest temperature produced only males

male, $n=14$ nests), although nest sex ratio varied from all male in one case to all female in four other instances. Logistic regression analysis of the relationships between nest sex ratio and the four variables that predicted nest temperature were not significant ($n=14$ in each case: $\chi^2=2.20$, $P=0.1381$ for PC1; $\chi^2=2.63$, $P=0.1046$ for “shade S”; $\chi^2=3.20$, $P=0.0735$ for “shade E”; $\chi^2=2.51$, $P=0.1131$ for “total shade”). More male-biased sex ratios resulted from nests with more overstory cover in all cases, concordant with previous field and laboratory research on this population of *Chelydra* (e.g., Kolbe and Janzen 2002; Janzen 2004). Although the pattern was similar and consistent with prior research, nest temperature for the first half of July did not significantly affect nest sex ratio ($\chi^2=1.78$, $P=0.1816$), possibly due to small sample size ($n=10$ nests with both functional loggers and sexable hatchlings). Within the range of temperatures observed in this study, cooler nests were more likely to produce a higher proportion of males than warmer nests, with the coolest nest yielding all males (Fig. 2). Thus, the overstory vegetation cover at a site chosen by a female snapping turtle affects the nest temperature and, in turn, can influence the nest sex ratio.

Physiological maternal effect

Across all eggs sampled, the average estradiol level in the yolk was 2.50 ng/g yolk (range=1.14–4.75 ng/g, $n=20$ clutches) and the average testosterone level in the yolk was 3.02 ng/g yolk (range=0.57–5.19 ng/g, $n=20$ clutches). To determine if yolk hormone levels were repeatable within a clutch, we compared the hormone values for the two eggs from each clutch. There was a significant relationship between hormone values in the two eggs sampled from each clutch for both estradiol ($r=0.64$, $n=20$, $P=0.0023$) and testosterone ($r=0.71$, $n=20$, $P=0.0005$), consistent with

previous endocrine work on this population (Janzen et al. 1998). Average estradiol and testosterone levels within clutches were independent of each other ($r=0.04$, $n=20$, $P=0.8674$).

As in the natural nests, the overall sex ratio of the hatchlings deriving from lab-incubated eggs was strongly female-biased (19.4% male, $n=25$ clutches). Several clutches were male-biased, but 10 clutches produced only female offspring. To evaluate a potential physiological maternal effect underlying this pattern, we adopted a multiple logistic regression approach to assess the impact of hormone levels in eggs. The proportion of male offspring from eggs incubated in the laboratory was not significantly affected by estradiol or testosterone levels, or by their interaction ($n=20$ in each case: $\chi^2=1.32$, $P=0.2513$; $\chi^2=0.03$, $P=0.8639$; $\chi^2=0.68$, $P=0.4084$; respectively). Thus, although yolk hormone levels varied among clutches, these steroids apparently did not influence the sex ratios of offspring from eggs incubated in the laboratory in this study.

Concordance of behavioral and physiological maternal effects

We tested for a concordant relationship between the behavioral and physiological maternal investments prior to embryonic development that might influence offspring sex ratio. None of the three principal components or three selected microhabitat variables was linked with the two measure of yolk hormone levels or their interaction ($F_{3,16}<1.77$, $n=20$ for all six analyses of variance, $P>0.19$). Additionally, there was no relationship between the sex ratio of lab-incubated eggs and any of the three principal components or three focal microhabitat variables from nests in the field ($\chi^2\leq 0.50$, $n=25$ for all six comparisons, $P>0.48$). Interestingly, though, nests that produced males in the laboratory had significantly more shade than those that did not produce any males in the laboratory (PC1: $F_{1,24}=4.39$, $P=0.0473$; "shade S": $F_{1,24}=6.87$, $P=0.0153$; "shade E": $F_{1,24}=4.30$, $P=0.0495$; "total shade": $F_{1,24}=5.91$, $P=0.0232$), and this pattern was not influenced by the timing of oviposition ($F_{1,24}=0.01$, $P=0.9113$). Thus, females who chose shadier (cooler) nest sites, which produced more male offspring than warmer nest sites, also laid eggs with a greater innate propensity to be male. Indeed, although none of the key behavioral predictor variables was correlated with any of the targeted physiological factors, logistic regression revealed a positive concordance between the sex ratios of lab- and field-incubated eggs ($\chi^2=4.52$, $n=14$, $P=0.0335$). Eggs from nests that produced male-biased sex ratios in the field yielded higher proportions of males under constant-temperature conditions in the laboratory as well (Fig. 3).

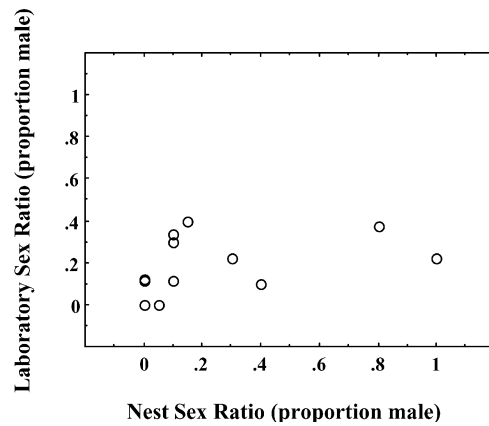


Fig. 3 Intra-nest relationship between the proportion of males resulting from incubation under constant temperatures in the laboratory and the proportion of males resulting from incubation in the field ($n=14$ clutches with hatchlings from both natal nests and eggs incubated in the laboratory) ($P=0.0335$). Laboratory sex ratios were determined using all hatchlings from eggs incubated in the laboratory (~9 hatchlings/clutch). As in the natural nests, strongly female-biased sex ratios resulted from the laboratory incubation of eggs

Discussion

Behavioral maternal effect

Organisms exhibit phenotypic differentiation that is subject to context-dependent (e.g., environmentally-induced) variation in fitness. For instance, certain oviposition microhabitats, in addition to having developmental implications (e.g., determination of offspring sex in species with ESD), may be more subject to predation, parasitism, or flooding (e.g., Wilson 1998; Spencer 2002; Morjan 2003a). Most populations of oviparous organisms are likely to have been subjected to such selective pressures, which may result in preference for certain types of oviposition locations (e.g., Singer et al. 1993; Spencer and Thompson 2003). For example, the population used in this study exhibits an overall preference for nesting in sandy patches, which have higher success rates and incubation temperatures (Kolbe and Janzen 2002). Although a population as a whole may exhibit a particular pattern of non-random nesting, there can still be biologically meaningful variation in nest-site choice among individuals within the population (e.g., Janzen and Morjan 2001; Kolbe and Janzen 2002).

Female choice of oviposition site is a maternal effect that can impact individual fitness in a wide variety of organisms (Roitberg 1998). Unlike in other taxa, the nest microenvironment, which is greatly affected by oviposition location, also influences sex in species exhibiting TSD. For an important trait like nest-site choice to have microevolutionary potential, though, it must be heritable. The potential for heritable nest-site choice in relation to overstory vegetation cover for turtles has been documented under natural conditions (Janzen and Morjan 2001; Spencer and Thompson 2003). However, inherit-

ance of nest-site choice in some species could be due to nest-site philopatry as a cultural means of “heritability”, although the behavior of philopatry itself could be genetically controlled (Freedberg and Wade 2001). For the microevolution of nest-site choice to occur, the environmental cue used by an organism to select a nest site (e.g., overstory vegetation cover) must also be chosen consistently (i.e., exhibit repeatability). Given the necessity of repeatability for an evolutionarily dynamic system, one would also expect the chosen cue to be a reliable indicator of nest temperature for organisms exhibiting TSD.

Of all the microhabitat variables we measured, only shade provided by vegetation cover was significantly correlated with nest temperature (Fig. 1). The quantity of overstory vegetation cover at oviposition can be an accurate indicator of nest temperature later in embryonic development (e.g., Morjan and Janzen 2003), particularly when the shade is provided by mature trees (e.g., Janzen and Morjan 2001; this study). Because of the relationship between vegetation cover and nest temperature, as well as that between nest temperature during the approximate temperature-sensitive period of sex determination and offspring sex ratio (Fig. 2; see also Kolbe and Janzen 2002), the lack of statistically significant correlations between nest sex ratio and vegetation cover and between nest temperature and nest sex ratio in this study is likely to be due to small sample size and/or limited among-nest variation in sex ratio. Indeed, vegetation cover can be a reliable indicator of nest sex ratio in other turtles with TSD (e.g., Vogt and Bull 1984; Janzen 1994a; Roosenburg 1996). Even so, we cannot account for the impact, if any, on nest sex ratio due to sampling 12 eggs from the top of each nest. Because the top of a nest may experience warmer temperatures than the lower portion, these upper eggs might have yielded female-biased offspring sex ratios had we not removed them, possibly reducing variation in nest sex ratios. Nonetheless, offspring sex ratio may be impacted more by the substantial daily fluctuations in nest temperature (as great as 10°C in the middle of a nest; St. Juliana, personal observation) than by small differences in egg position (~2.5 cm) within a nest (but see Wilhoft et al. 1983). Given our results and those of previous studies, overstory vegetation cover thus appears to provide a relatively reliable environmental predictor of subsequent offspring sex ratio for nesting turtles with TSD. This finding allows for the possibility that a female could exert behavioral “control” over the sex ratio of her offspring.

The maternal effect of nest-site choice may or may not (now or in the past) have undergone selection in relation to TSD (Morjan 2003a). Certainly other factors, like predation on females (Spencer and Thompson 2003) or on nests (Kolbe and Janzen 2002), can play a role in the evolution of nest-site choice. However, species with TSD cannot “ignore” the thermal consequences of nest-site choice because a biased sex ratio is evolutionarily unstable (Charnov 1982; Bull 1983; Karlin and Lessard 1986). Consequently, females could benefit from the ability to use an environmental cue to direct their nesting

behavior to also maximize sex-related fitness under TSD. Fitness could be maximized under TSD by producing male or female offspring at temperatures for which they were more fit (Charnov and Bull 1977). Some sources (recently reviewed by Shine 1999) suggest this sex-specific differential fitness could be realized through altered post-hatching growth rate (Rhen and Lang 1995; Roosenburg and Kelley 1996; Janzen and Morjan 2002; but see Morjan and Janzen 2003), antipredator behavior (Janzen 1995), or mating behavior (Gutzke and Crews 1988). The general applicability of these findings remains to be established, however. Another adaptive scenario arises if a female is able to nest in relation to the sex ratio of the population (at least as she perceives it) (sensu Conover and Van Voorhees 1990; Komdeur et al. 1997). For instance, in organisms with TSD there could be an evolved cue related to the sex ratio of the adults in the population, perhaps involving the stimulus of mating encounters. When females in a given year encounter numerous reproductive males, as they might in polygynous mating systems like those that characterize turtles (Pearse and Avise 2001), they may tend to nest in microhabitats likely to produce clutch sex ratios biased toward female offspring. Such an adaptive scenario has yet to be explored in reptiles with TSD.

Nesting behavior may also (or instead) result from the genetic acuity with which an organism can respond to cues from the environment, and the cues in the environment themselves. Females with TSD who excel at selecting favorable, sex-specific nest sites would realize a higher fitness (Roosenburg 1996; Reinhold 1998; Freedberg and Wade 2001). The ability of females to manipulate offspring sex ratio in response to present environmental conditions is difficult to test, especially in the wild. Still, more experimental studies will be necessary to discern whether nesting behavior in regards to TSD has adaptive value (e.g., Bull et al. 1988; Janzen 1995; Bragg et al. 2000).

Physiological maternal effect

While temperature clearly influences sex determination in organisms with TSD, other factors may also affect sex determination in these taxa. Recent work suggests that endogenous steroid hormones in yolk may play a role in influencing clutch sex ratios in turtles with TSD (Janzen et al. 1998; Bowden et al. 2000, 2001). Consequently, elucidating the effects of endogenous yolk hormones on clutch sex ratios may be critical to understanding the mechanistic basis, as well as the microevolution, of TSD. For example, Bulmer and Bull’s (1982) model for the microevolution of TSD highlights the importance of the thermal sensitivity of embryonic sex determination. Among-clutch disparity in yolk hormone concentrations could, in fact, be a major factor determining variation in thermal sensitivity of embryonic sex determination. Thus, if individual choice underlies among-female differences in hormone allocation to egg yolk (e.g., Pilz et al. 2003), a

female could bias the sex ratio of her clutch through this physiological effect instead of, or in addition to, choice of nest thermal quality, the other key factor in models of the microevolution of TSD (Bulmer and Bull 1982). Viewed in this light, hormone allocation to egg yolks could be interpreted as a type of female choice. The extent of female control over this maternal effect remains to be examined empirically.

As in previous studies (e.g., Janzen et al. 1998; Bowden et al. 2000), we detected statistically consistent hormone levels between eggs within clutches. However, our results do not support the concept that endogenous yolk hormones affect clutch sex ratio in snapping turtles, because we did not find a significant relationship between clutch sex ratio in the laboratory and estradiol or testosterone concentrations or their interaction. Yolk hormones therefore may not affect the thermal sensitivity of embryonic sex determination in *C. serpentina*. Two caveats should be noted though. First, eggs were frozen from 1–4 days into development, which might have caused among-clutch variation in the concentrations of hormones detected in the sampled yolks. Moreover, the incubation temperature used in the laboratory portion of our study (~28.4°C) unfortunately diverged significantly from the targeted pivotal temperature of sex determination (~27.6°C). We thus cannot rule out the possibility that the strongly female-biased sex ratios that resulted may also have masked a mechanistic relationship between clutch sex ratio and yolk hormone levels at the pivotal temperature (sensu Janzen et al. 1998 for snapping turtles). Even if true, our results suggest at best a limited role for yolk hormones in influencing clutch sex ratio in snapping turtles, unlike studies on painted turtles where a more consistent link between endogenous yolk hormones and clutch sex ratios has been detected (Bowden et al. 2000, 2001). Nonetheless, the two studies of snapping turtles (the present study and Janzen et al. 1998) were conducted on the same population, so more work with *C. serpentina* (e.g., Elf et al. 2002) and other species with TSD (e.g., Conley et al. 1997) is warranted before we can draw any general conclusions concerning the effects of yolk hormones on TSD.

Concordance of behavioral and physiological maternal effects

We predicted that the behavioral and physiological investments would covary positively. This hypothesis was based on the adaptive concept that a female most able to manipulate both maternal investments would more predictably influence the sex ratio of her clutch. We assessed behavioral and physiological maternal investments by examining, accordingly, nest microhabitat variables (especially overstory vegetation cover) and steroid hormone allocations to egg yolk. These traits could serve as indicators of maternal investment to manipulate offspring sex ratio. In other words, clutch sex ratio is the ultimate result, but the actual allocation strategy used by a female should be a better indicator of her “perceived” investment. We

found no indication that any microhabitat variable was correlated with yolk hormone levels. However, in this study hormone levels in egg yolk were not an accurate indicator of the sex of hatchlings incubated at a nearly constant temperature. Our research does confirm, though, that temperature is a major factor in determining the sex of embryonic *C. serpentina*.

The thermal sensitivity of sex determination in snapping turtles and many other reptiles highlights the potential vulnerability of populations to dramatic changes in climatic temperature (Janzen 1994b). How have such taxa responded to climate change historically? Can they respond successfully to the relatively rapid alterations in climatic temperature occurring currently and, if so, how? We did not detect evidence of a relationship between yolk hormones and offspring sex ratio nor between potential behavioral and physiological maternal effects that could influence offspring sex ratio in this species. However, our results suggest that behavioral choice of nest-site characteristics might be able to evolve in response to selection if heritable variation is present for this behavior (Janzen and Morjan 2001; but see Morjan 2003b).

But another evolutionary option may exist in this system. We found that sex ratios in the laboratory and field were positively correlated (Fig. 3), implying that some intrinsic factor in the eggs might influence the sex ratio of the clutch. Although the correlation may be affected by the overall female-biased sex ratios both in the laboratory and in the field, it could nonetheless reflect an among-female predisposition (genetic or otherwise) for sex-ratio production. For example, it has long been known that clutches of eggs from some female snapping turtles do not yield male offspring under any thermal conditions of incubation, even those that regularly produce 100% male offspring for most other females in the population (Janzen 1992; Ewert et al. 1994). Moreover, we noted that the propensity to produce male offspring in the laboratory was statistically linked to key microhabitat variables related to nest-site choice (Kolbe and Janzen 2002) and nest temperature in the field (Fig. 1). This concordance might indicate a positive association between the behavioral maternal effect of nest-site choice and some unmeasured property of the eggs that influences clutch sex ratio. Thus, the relatively reliable factor of nest-site choice in affecting offspring sex ratio in this system may be augmented by an unidentified physiological or genetic effect as well.

Additional studies that adopt a similar split-family design to incubate eggs in the laboratory and the field could provide a great deal of insight into this system. Of particular interest would be manipulative or cross-fostering experiments (Roff 1998) to separate the genetic, environmental, and genetic X environment control of the nesting behavior and thermal sensitivity of embryonic sex determination. These experiments would elucidate the key factors that actually underlie maternal investments in populations with TSD, providing a better mechanistic understanding of this system and improved ability to predict its microevolutionary trajectory in response to selection.

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