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Abstract

Temperature-dependent sex determination, where egg incubation temperature irreversibly determines offspring sex, is a common sex-determining mechanism in reptiles. Weather is the primary determinant of temperature in reptile nests, yet the effects of weather are mediated through the nest microhabitat selected by the mother (e.g., overstory canopy cover). One potentially important aspect of the nest microhabitat is the physical substrate used for nesting. However, the influence of substrate type on nest temperature and offspring sex determination has never been experimentally assessed in the field. We incubated eggs of Painted Turtles (*Chrysemys picta*) in three substrate types similar to those commonly selected for nesting within our study population. Within a single study site, we constructed pits, which we refilled with loam, sand, or gravel. Then, we created artificial nests in each substrate type, and randomly assigned eggs to a substrate treatment. Substrate type influenced nest temperature and soil moisture, and affected incubation duration, but no other phenotype measured beyond offspring sex ratios. The cooler loam yielded the most male-biased outcome. This finding illustrates the potential importance of nesting substrate as a component of nest-site choice and as a factor in modeling future nest temperature scenarios.

Keywords

Chrysemys picta, Hydric conditions, Nest-site choice, Painted Turtles, Sex determination, Soil temperature

Disciplines

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**Substrate Influences Turtle Nest Temperature, Incubation Period, and Offspring Sex Ratio
in the Field**

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ABSTRACT: Temperature-dependent sex determination, where egg incubation temperature irreversibly determines offspring sex, is a common sex-determining mechanism in reptiles. Weather is the primary determinant of temperature in reptile nests, yet the effects of weather are mediated through the nest microhabitat selected by the mother (e.g., overstory canopy cover). One potentially important aspect of the nest microhabitat is the physical substrate used for nesting. However, the influence of substrate type on nest temperature and offspring sex determination has never been experimentally assessed in the field. We incubated eggs of Painted Turtles (*Chrysemys picta*) in three substrate types similar to those commonly selected for nesting within our study population. Within a single study site, we constructed pits, which we refilled with loam, sand, or gravel. Then, we created artificial nests in each substrate type, and randomly assigned eggs to a substrate treatment. Substrate type influenced nest temperature and soil moisture, and affected incubation duration, but no other phenotype measured beyond offspring sex ratios. The cooler loam yielded the most male-biased outcome. This finding illustrates the potential importance of nesting substrate as a component of nest-site choice and as a factor in modeling future nest temperature scenarios.

Key words: *Chrysemys picta*; Hydric conditions; Nest-site choice; Painted Turtles; Sex determination; Soil temperature

REPTILE embryos are highly sensitive to environmental conditions experienced during development (Deeming 2004; Noble et al. 2018). Many reptiles have evolved mechanisms to exert considerable control over the embryonic environment (viviparity, egg brooding, etc.) (Shine 2005; Shine and Thompson 2006). For oviparous reptiles that lack parental care, however,

embryonic development is primarily influenced by the ambient environment (Warner 2014). Environmental factors, such as nest temperature or hydric conditions, can substantially influence diverse phenotypes, including morphology, size, physiology, and even sex (Booth 2006; Noble et al. 2018).

Weather is the primary determinant of nest temperature and hydric conditions in reptile nests (Shine and Elphick 2001; Schwanz et al. 2010), yet the effects of weather are mediated through the nest microhabitat selected by the mother (Janzen 1994; Mitchell et al. 2013a). For example, the relationship between canopy cover above a nest and the nest thermal regime is well documented (e.g., Weisrock and Janzen 1999). Similarly, precipitation affects nest hydric conditions, which can have thermal ramifications as well (Bodensteiner et al. 2015). Some aspects of the microhabitat have received considerably less attention, yet are also important in influencing nest conditions. In particular, nesting substrates might differ in a number of ways that affect developing eggs. Indeed, incubation substrate alters egg environments (Ackerman et al. 1985; Packard et al. 1987), including hydric (Ratterman and Ackerman 1989; Mortimer 1990; Cagle et al. 1993; Packard and Packard 2000) and thermal (Milton et al. 1997; Hays et al. 2001; Tornabene et al. 2018) conditions. Consequently, research investigating the influence of nest substrate type on environmentally mediated offspring traits would provide important information for understanding nest-site choice and modeling the effects of environmental change on developing reptiles.

Painted Turtles (*Chrysemys picta*) provide an excellent system for investigating the influence of nest substrate on offspring phenotypes for several reasons. First, Painted Turtles dig subterranean nests and, given their broad geographic range, these nests occur in a variety of substrate types (Ernst and Lovich 2009). Within our field site, Painted Turtles nest in natural and

human-modified areas with varying substrate types (Kolbe and Janzen 2002; Schwanz et al. 2009). Painted Turtles have temperature-dependent sex determination: cool incubation temperatures produce males, intermediate temperatures produce mixed sex ratios, and warm nests produce all females. Additionally, Painted Turtles have flexible-shelled eggs that are permeable to water, such that hydric conditions of the nest influence offspring phenotypes.

In this field experiment, we incubated *C. picta* eggs in three substrate types similar to those commonly selected for nesting within our study population. We created artificial nests in each substrate, assigned eggs to a substrate treatment, and tracked fates of eggs. We then quantified the influence of substrate type on nest temperature and moisture and on hatchling phenotypes, including offspring sex. In this way, we tested the hypothesis that substrate type affects important traits of neonatal reptiles under field conditions.

MATERIALS AND METHODS

On 15 May 2014, we established experimental pits filled with three different substrates at the Iowa State University Horticulture Research Station (HRS). We excavated six $76 \times 76 \times 45$ cm (W \times L \times D) pits on level ground in a 3×2 array, each spaced 50 cm from one another. The area was fully exposed and all blocks were within a 330×200 cm rectangle. Because of this spatial proximity, all nests received comparable solar radiation and ambient precipitation (Janzen and Morjan 2001). We randomly assigned each pit in a row (hereafter block) to be backfilled with one of the three substrates, such that each substrate type was represented once in each of the two blocks. Because of logistical constraints we did not acquire substrate from the field site, so we used readily available alternatives that approximated substrates from the field site. We filled each pit with either locally mined limestone road gravel (hereafter gravel), commercial sandbox

sand (hereafter sand), or loamy soil (hereafter loam). The loam substrate occurred naturally at the HRS. Hence, for this treatment, we used soil removed from a pit to refill that pit. We tamped substrates until level with the surrounding ground and weeded the surfaces weekly to eliminate plant growth.

We obtained eggs from a population of *C. picta* at the Thomson Causeway Recreation Area (TCRA) in Thomson, IL. These turtles commonly nest in moist loam, sand, or road gravel (Schwanz et al. 2009). We collected 12 clutches (mean clutch size = 11 eggs) from fresh nests between 28 and 30 May 2014 at the TCRA. We weighed eggs (± 0.01 g), marked them with a permanent marker, and placed them in moistened loam in plastic containers, which were stored in coolers during transport to the HRS on 30 May.

On 31 May 2014, we created two artificial cylindrical nests to a depth of 8.0 cm within each substrate pit. We divided each clutch into three artificial nests as evenly as possible—one in each of the different substrates within a block—such that every nest was comprised of eggs from three mothers (Fig. 1). We replicated this design four times with other clutches and artificial nests. We arranged 10–12 eggs in a single layer in the bottom of the nest and placed a Thermocron iButton data logger (Maxim Integrated; San Jose, CA) programmed to record hourly temperatures in the center of the cavity among the eggs. We waterproofed each iButton with layers of latex and parafilm. We covered nests with 1 cm mesh hardware cloth, secured with tent stakes, as predator proofing. Using a Hydrosense instrument (CD620, Campbell Scientific Australia), we measured percentage volumetric water content (VWC%) twice weekly, taking four measurements per nest (10 cm away in each cardinal direction) at every sampling event (Bodensteiner et al. 2015).

On 9 August 2014, we excavated the nests, re-weighed the eggs, and half buried them in moistened vermiculite (-150 kPa) to complete incubation at a constant 28°C . We monitored eggs daily for pipping and hatching, weighing and measuring the carapace length (± 0.01 mm) of turtles within 24 hours of hatching. We calculated incubation duration as the number of days from oviposition until pipping. We also calculated a daily rate of egg mass gain by subtracting the egg mass at oviposition from the egg mass upon excavation from the substrate experiment, divided by days since oviposition. An egg gains mass via absorption of water from the surrounding substrate under moist conditions (i.e., where soil water potential > -750 kPa; Ackerman 1991). Because sexing hatchlings in *C. picta* is terminal and our collection permits required us to minimize mortality, we targeted sexing one hatchling from each nest/mother combination via macroscopic examination of the gonads. We haphazardly selected additional hatchlings for sexing, resulting in some clutches being sampled more thoroughly than others ($n = 47$ total sexed hatchlings). Most *C. picta* nests produce hatchlings all of one sex (Janzen 1994), so our limited sampling for sex ratio nonetheless should provide reliable inference. All other hatchlings were released at the TCRA in May 2015.

We collected soil samples from each substrate type, and estimated the relationship between water potential and water content following the pressure plate method described by Klute (1986). We used ceramic pressure plates and a 1600F1, 220V mercury-free manifold compressor from Soil Moisture Equipment Corporation (Santa Barbara, CA).

To assess the effect of substrate type on nest temperature, we plotted mean hourly temperatures for each substrate type. We also extracted mean daily mean, mean daily minimum, mean daily maximum, and mean daily range for each nest from the iButton hourly thermal profiles. We are missing data from one loam nest because of an iButton failure. We calculated

constant temperature equivalents (CTE; Georges et al. 1994) based on the mean daily mean and mean daily range with a model parameterized for Painted Turtles (Telemeco et al. 2013). To determine if VWC% differed among substrate types, we performed a mixed-model ANOVA with substrate type as the fixed factor and nest as a random effect.

To explore whether substrate type influenced incubation period or rate of egg mass change, we performed mixed-model ANOVAs. Substrate type was a fixed factor, and maternal ID nested within block and nest nested within treatment were random effects. To explore whether substrate type influenced carapace length or hatchling mass, we performed identical mixed-models to those described above, except we included initial egg mass as a covariate. Because we were unable to sex all hatchlings, we had insufficient sample sizes to fit logistic regression models that included substrate type, maternal ID, and nest as factors. Therefore, we performed Fisher's Exact tests to assess whether substrate type affected sex ratio. We used SAS (v9.4, SAS Institute, Cary, NC) for analyses.

RESULTS

Gravel and sand nests were thermally similar, whereas loam nests had cooler maximum temperatures and, consequently, lower means, CTE, and smaller daily thermal ranges (Table 1; Fig. 2). However, minimum nest temperatures did not differ among substrate types. Substrate type influenced VWC% ($F_{2,708} = 266.42, P < 0.001$): gravel (mean \pm 1 SE = $6.8 \pm 1.1\%$) and sand ($7.3 \pm 1.1\%$) generally had less water than loam ($38.5 \pm 1.1\%$) in the field. Water retention curves from laboratory analysis revealed that loam had a stronger water holding capacity than sand or gravel, which had similar curves (Fig. 3).

The overall mean incubation duration was 76.4 d (range = 74–85 d). Eggs were in the experimental nests for 70 d, or 91.7% of the incubation period. The egg that took 85 d to hatch was in its experimental nest for 82.3% of development. Incubation duration was influenced by substrate type: embryos developing in eggs in gravel nests took the longest to hatch, with those in loam having an intermediate duration and those in sand hatching the soonest. Nonetheless, most other phenotypes—rate of egg mass change, carapace length, and hatchling mass—were not substantially influenced by substrate type (Table 2).

Of 47 hatchlings sexed in the experiment, 43 were female and 4 were male. All 18 turtles in gravel and all 16 in sand were females, with the 4 males originating from the loam nests. Moreover, these four males came from two loam nests constructed in separate blocks, and derived from four different clutches. The four male individuals were identified for sexing through a systematic, randomized sampling protocol and no further individuals were haphazardly selected from those male-producing nests. Thus, all individuals haphazardly selected for sexing came from other nests, and all turned out to be female. The sex ratios of loam nests differed statistically from those of gravel ($P = 0.02$) and sand ($P = 0.03$) nests, whereas sex ratios of sand and gravel nests were identical ($P = 1.00$).

DISCUSSION

Egg-laying reptiles can influence the microenvironment of their nests by several mechanisms. Nest-site choice with regards to canopy cover above a nest, nest depth, and phenology have garnered considerable research attention (e.g., Janzen 1994; Refsnider et al. 2013; Telemeco et al. 2013; Mitchell et al. 2017), whereas factors such as the physical substrate in which nests are constructed have received much less focus (but see Milton et al. 1997;

Refsnider et al. 2010; Tornabene et al. 2018). Here we demonstrate, through a common-garden incubation experiment in the field, that commonly used nesting substrates influence nest temperatures, soil moisture, incubation period, and sex determination in Painted Turtles.

The measured environments of sand and gravel nests in this field experiment were indistinguishable from each other, but different from loam nests. Gravel and sand nests had similar thermal properties, whereas loamy nests exhibited cooler CTEs, maximum and mean daily temperatures, and smaller thermal ranges. Loam nests also differed by having higher water content than gravel and sand nests, which might explain the cooler temperatures recorded. Higher soil moisture increases heat conductivity which draws solar heat deeper into the ground, and enhances the potential for surface evaporation and convection. Thus, temperatures might be cooler towards the surface in wetter substrates (Ackerman and Lott 2004; Bodensteiner et al. 2015).

It is unsurprising that we observed higher water content in loam nests in the field, as our lab assay demonstrated a higher ability for loam to retain water (Fig. 3). Despite having both water retention curves and field measurements of VWC%, it is difficult to estimate water potential within nests. The steep slope at low water content for gravel and sand (Fig. 3) challenges the estimation of the actual water potential within these nests, because slight errors in VWC% input would yield different outcomes. Nevertheless, by using VWC percentages measured in the field and water retention curves generated from the lab, we can coarsely estimate that loam nests have higher field potential (approximately 0 to -10 kPa) than sand and gravel nests (approximately -100 to -500 kPa). These values should be interpreted with caution. Although we did not conduct further analyses beyond soil retention curves, loam nests might

differ in other properties that influence temperature, including thermal conduction, which can depend on albedo, particle size, etc. (Ackerman and Lott 2004).

The environmental differences observed between substrates influenced sex determination. We were unable to sex all *C. picta* hatchlings produced in our field experiment. Even so, the only males we detected derived from the cooler loam nests; we did not identify a single male hatchling from any nest constructed in sand or gravel. As the thermosensitive period for Painted Turtles is the middle third of incubation (Telemeco et al. 2013), sex determination occurred in the field for all eggs in our experiment. Therefore, we demonstrate that variation in abiotic properties of nesting substrates—most likely related to temperature (Fig. 2)—have the capacity to influence the sex of developing turtles in the field. This finding is relevant for modeling thermal properties of reptile nests (Kearney et al. 2014; Carter et al. 2015) and for improved understanding of the evolution of nest-site choice, which typically focuses on overstory vegetation cover (e.g., Janzen 1994).

Because loam nests were cooler and wetter than sand and gravel nests, we might have expected additional phenotypic differences between the hatchlings that incubated in different substrates (Deeming 2004). Surprisingly, substrate type had minimal influence on some measured phenotypes. Rate of egg mass change, hatchling length, and hatchling mass were similar among substrate types, which contrasts with prior controlled laboratory studies (e.g., Packard 1991). For example, at a series of constant temperatures, hatchling Snapping Turtles (*Chelydra serpentina*) from eggs incubated in sand generally weighed more than those deriving from eggs incubated in vermiculite (Packard et al. 1987). Because of complex interactions between nest temperature and substrate water potential (Ackerman 1991), generalities from laboratory studies might not translate well into the field. Nonetheless, because embryo water

potential is around -750 kPa, eggs in our study should have gained mass in all nests regardless of substrate type given our crude measurements of VWC% and water retention curves, which is what we documented.

Incubation duration varied among the substrate types, although in an unexpected way. Despite being environmentally similar, embryos from gravel-incubated eggs took ~ 2 more days to hatch than those from the sand-incubated eggs. The cooler, wetter loam nests produced embryos with intermediate incubation periods. This finding was unexpected because prior field research in this system has shown eggs incubating in warmer nests hatch sooner (Mitchell et al. 2013b, 2015). In the laboratory, wet substrates did not influence incubation duration at cool temperatures, but increased incubation duration at warmer temperatures (Gutzke et al. 1987). We expected the cooler loam nests to have the longest incubation durations, but they did not. We have no clear explanation for why our results did not accord with the prior research from laboratory and field based experiments. Other factors unmeasured in our experiment, such as oxygen levels, might have varied among substrate types and influenced developmental rates and other phenotypes of eggs (Ackerman 1981; Cordero et al. 2017). Nevertheless, the failure of prior work to predict the patterns observed in this experiment highlights a gap in our knowledge. We should incorporate more complexity and ecological relevance into laboratory studies (e.g., experiments with thermal and hydric fluctuations; Tucker et al. 2001), and conduct additional manipulative field experiments to move the field forward.

Do reptiles discriminate among substrate types when selecting a nest? Different substrate types might appear, smell, or feel differently to predators, which could influence predation rates (Oddie et al. 2015; Voves et al. 2016), and thus, exert direct fitness consequences of nest location for offspring. Perhaps more indirectly for offspring fitness, ease of nest construction, which also

affects subsequent hatchling emergence, might vary between substrate types and thus influence nest-site choice (Mortimer 1990). Some lizards dig test nests and even spend time within the nest cavity prior to laying eggs (Harlow and Taylor 2000). Such behaviors could allow ovigerous reptiles to discriminate between alternative substrate types by directly assessing cavity temperature or moisture (Plummer and Snell 1988; Reedy et al. 2012; Li et al. 2018). Regardless, because substrate type affects thermal and hydric conditions of a nest in the field, the influence of these three factors on nest-site choice would be difficult to dissociate. Given the possibility that reptiles might use substrate characteristics as a cue for nesting, however, experiments explicitly testing nest-site choice with regards to substrate type are warranted.

Our research has explored an understudied factor of nesting reptiles—substrate type—and demonstrated that thermal differences in substrate types located in close proximity in the field are large enough to influence sex ratios of developing offspring. This finding highlights the importance of nesting substrate as a factor to be investigated in studies of nest-site choice and in modeling nest temperatures.

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TABLE 1.—Mean values (± 1 SE) for daily mean, mean daily maximum, mean daily minimum, and mean daily range calculated from hourly temperatures recorded from 31 May–9 August 2014 in experimental *Chrysemys picta* nests in gravel ($n = 4$), loam ($n = 3$), and sand ($n = 4$) substrates at the Iowa State University Horticulture Research Station (Story Co., IA). Constant temperature equivalents (CTE) were calculated for each nest based on the mean daily mean and mean daily range.

Temperature parameter ($^{\circ}\text{C}$)	Gravel	Loam	Sand
Mean	24.4 ± 0.2	23.9 ± 0.2	24.6 ± 0.2
Maximum	31.3 ± 0.4	29.2 ± 0.4	31.5 ± 0.4
Minimum	18.8 ± 0.2	19.3 ± 0.2	19.0 ± 0.2
Range	12.5 ± 0.4	9.9 ± 0.5	12.5 ± 0.4
CTE	27.5 ± 0.3	26.0 ± 0.3	27.7 ± 0.3

TABLE 2.—Least-squares means (± 1 SE) obtained from statistical analyses of *Chrysemys picta* embryo and hatchling phenotypes from eggs incubated in experimental nests in gravel ($n = 4$), loam ($n = 3$), and sand ($n = 4$) substrates at the Iowa State University Horticulture Research Station (Story Co., IA).

Phenotype	Gravel	Loam	Sand	Statistic
Incubation period (d)	77.9 ± 0.67	76.2 ± 0.66	75.2 ± 0.67	$F_{2,9} = 4.26, P = 0.05$
Rate of egg mass change (mg/day)	31 ± 2	29 ± 2	36 ± 2	$F_{2,9} = 2.84, P = 0.11$
Carapace length (mm)	26.56 ± 0.18	26.16 ± 0.18	26.20 ± 0.18	$F_{2,9} = 2.12, P = 0.17$
Hatchling mass (g)	5.18 ± 0.08	5.10 ± 0.08	5.08 ± 0.08	$F_{2,9} = 0.69, P = 0.52$

FIGURE CAPTIONS

FIG. 1.—Schematic of the experimental design. Eggs from three field-collected clutches of *Chrysemys picta* eggs were randomly and (approximately) evenly divided into artificial nests in three different substrate types, within the same block. This process was repeated four times.

FIG. 2.—Mean hourly temperatures (± 1 SE) recorded in experimental *Chrysemys picta* nests in gravel ($n = 4$), loam ($n = 3$), and sand ($n = 4$) substrates at the Iowa State University Horticulture Research Station (Story Co., IA) during the egg incubation period (31 May–9 August 2014). Temperature readings for hour 0 occurred between 0000–0100 h.

FIG. 3.—Retention curves calculated in the laboratory showing the relationship between water potential and water content for gravel, loam, and sand substrates. Each point represents the average of three sample replicates. Water potentials of 0 kPa represent fully saturated substrates, whereas the lower (more negative) water potentials represent the amount of pressure necessary to remove remaining water that is bound to the substrate (Alberts et al. 1997).