Original Article

Temporal variation in maternal nest choice and its consequences for lizard embryos

Jenna E. Pruett, Amélie Fargevieille, and Daniel A. Warner

Department of Biological Sciences, Auburn University, 101 Rouse Life Sciences, Auburn, AL 36849, USA

Microhabitat choice of nest sites is an important maternal effect that influences the survival and development of embryos in oviparous species. Embryos of many species display a high degree of plasticity in response to developmental environments, which places maternal nesting behavior under strong selective pressure, particularly in temporally changing environments. Nesting behavior varies widely across taxa that exhibit diverse reproductive strategies. The brown anole (*Anolis sagrei*), for example, lays one egg every 7–10 days across an extended reproductive season from April to October. This aspect of their reproduction provides an opportunity to examine temporal shifts in nesting behavior and its consequences on egg survival and offspring development under seasonally changing climatic conditions. We conducted a two-part study to quantify temporal variation in maternal nesting behavior and its effect on development of *A. sagrei* embryos. First, we measured nest micro-environments over the nesting season. Second, we "planted" eggs across the landscape at our field site to examine the influence of nest conditions on egg survival and hatchling phenotypes. We also incubated eggs inside chambers in the field to decouple effects of nest moisture from those of other environmental variables (e.g., temperature). Females chose nest sites with higher moisture and lower temperatures relative to what was generally available across the landscape during the nesting season. In addition, eggs exposed to relatively cool temperatures had higher hatching success, and high nest moisture increased egg survival and body condition of hatchlings. Overall, we provide evidence in the field that maternal nesting behavior facilitates offspring survival.

Key words: **Anolis sagrei, developmental plasticity, maternal effects, microclimate**

INTRODUCTION

Maternal effects play a critical role in generating phenotypic variation and determining the fitness of offspring (Moore et al. 2019). Because early life stages are often very sensitive to developmental environments, mothers have the potential to impact survival and phenotypes of their progeny via variation in their own physiology or behavior beyond the effects of heritable genetic material (Mousseau and Fox 1998). Although maternal effects can be energetically costly to females (e.g., increased provisioning to embryos, long-term parental care), they can enhance fitness by improving offspring survival and perpetuating the mother's genetic material into future generations (Marshall and Uller 2007). Parental care is a prime example of how behavior can impact the fitness of offspring. In many birds, for example, parental attendance of eggs or food provisioning to offspring can influence a suite of fitness-relevant traits of offspring, such as growth and immunocompetence (Saino et al. 1997; Tinne et al. 2005). In oviparous organisms, maternal nesting behavior can be viewed as another form of parental care because it determines the environmental conditions offspring will experience during development (Mainwaring et al. 2017).

Nesting is common across vertebrate and invertebrate taxa (Hansell 2005), and much of our understanding of nesting behavior and its consequences comes from studies of avian systems (Refsnider 2016). Birds provide extensive post-laying parental care and invest considerable energy towards maintaining consistent nest temperatures through brooding and caring for their young post-hatching (Deeming and Reynolds 2015). In species that exhibit extended post-laying parental care, mothers have continued opportunities to modify the developmental conditions experienced by their offspring. In many species, however, females lay eggs and leave them unattended for the rest of development (Shine 2005), which limits opportunities for parents to modify the nest environment during the development of their offspring. Therefore, the timing of nesting, environmental conditions chosen by females, and design of nests are particularly important at the time of oviposition (Deeming and Ferguson 1991; Telemeco et al. 2009).

Evidence that females make active choices of oviposition sites is needed to classify nesting behavior as a true "maternal effect" (Wolf and Wade 2009). This evidence is observed in many

Address correspondence to Jenna E. Pruett. E-mail: jep0057@auburn.edu.

reptiles whereby females actively select specific microclimates for nests (Brown and Shine 2004; Mitchell et al. 2013a; Li et al. 2017). Furthermore, nesting is a plastic behavior that is dependent on prevailing conditions. For example, the onset of nesting is influenced by winter temperatures in painted turtles (Schwanz and Janzen 2008), and nest-site choice can vary to match local climates (Refsnider et al. 2013) or change depending on predation risk (Spencer 2002; but see Refsnider et al. 2015). Aspects of nest construction by three-lined skinks, such as nest depth, correlate with changes in ambient temperatures (Telemeco et al. 2009). Several lab-based studies reveal that females choose nesting conditions that are favorable for offspring fitness, suggesting that this behavior is adaptive (Warner and Andrews 2002; Pike et al. 2010; Reedy et al. 2013). Importantly, most species inhabit temporally heterogeneous environments, and consequently, available nesting conditions shift through the reproductive season (Warner and Shine 2008). This heterogeneity could influence nesting behavior and its consequences for offspring development and survival. Few studies have examined the importance of temporally shifting environments because many organisms have a relatively short window for reproduction due to seasonal constraints.

Anolis lizards provide a unique and understudied system for examining adaptive nesting behavior. Anoles produce one egg at a time, approximately once a week (Smith et al. 1973) over a reproductive season that can extend 6 or more months. This means that energy allocation toward reproduction is temporally extended in anoles compared to species that produce large, but infrequent clutches. Moreover, this reproductive pattern could enable females to spread their eggs across multiple sites that experience different conditions. This aspect of *Anolis* life history leads to seasonal variation in nesting conditions across the long reproductive season, thereby providing females an opportunity to adjust their nest choices to changes in ambient conditions.

Although *Anolis* lizards have been models for testing concepts in behavior, ecology, and evolution (Losos 2009), and are emerging as a model for developmental biology (Sanger et al. 2007) and plasticity (Warner 2014), nearly nothing is known about their nesting behavior in nature. Anecdotal reports of anole nesting suggest that these lizards lay eggs in a variety of habitats (Delaney et al. 2013) and might nest communally (Rand 1967; Swierk et al. 2019), and lab studies show that females prefer moist substrate with leaf cover for nesting (Reedy et al. 2013; Socci et al. 2005). Empirical work on anole nesting in the field is, however, limited (Tiatragul et al. 2019). Relatively more is known about the effects of developmental conditions on anole embryos (Goodman and Heah 2010; Warner et al. 2012; Pearson and Warner 2016, 2018), but much of this work has also been done in lab settings and does not fully capture the environmental fluctuations embryos would experience in the field. Nevertheless, these studies have provided valuable insight into this crucial life stage of a very prolific model system, but we need a better understanding of nesting behavior in the field to advance research on their reproduction and development. Furthermore, the unique nature of anole reproductive biology enables us to answer new questions that will advance the understanding of maternal investment in nesting.

We sought to address these knowledge gaps using a two-part study on the brown anole, *Anolis sagrei*. The first objective was to quantify the nest microhabitats chosen by females in the field and to characterize general qualitative patterns of anole nesting behavior. We did this by searching for anole eggs using randomized and targeted approaches in the field. Furthermore, because females lay approximately one egg per week from April to October, we repeated our approach three times to examine seasonal changes in nesting behavior. We predicted that females would choose relatively cool and moist nesting sites because these microhabitats would prevent desiccation and limit exposure to extreme thermal fluctuations that might be lethal to embryos (Hall and Warner 2018). The second objective was to examine the effects of microclimate conditions on embryo development in the field. To do this, we planted anole eggs across our field site at two times during the nesting season; our approach exposed eggs to a broad range of temperature and moisture conditions and enabled us to assess the effects on hatching success and offspring phenotype. We predicted that cooler sites with more moisture would be beneficial for both hatching success and fitness-relevant hatchling phenotypes, which would suggest that nesting behavior is adaptive (according to our first prediction). Lastly, given that ambient conditions change through the season, we expect that patterns of maternal nesting behavior and egg hatching success will also shift seasonally.

METHODS

Study species and field site

The Cuban brown anole (*A. sagrei*) is native to Cuba and the Bahamas, and invasive across the southeastern United States. This species produces about one egg per week from April to early October, and eggs hatch continuously throughout the season (Mitchell et al. 2018). Embryos exhibit considerable plasticity in response to incubation conditions over the season (Pearson and Warner 2018).

This study was conducted on an island in the Intracoastal Waterway located within the Guana Tolomato Matanzas National Estuarine Research Reserve near Palm Coast, Florida (Figure 1a). Based on continuous surveys since 2011, the numbers of adult lizards on this island has varied from about 73 to 486 individuals (unpublished data). The island also has a diversity of habitat types, including a central area of dense tree canopy (cedar trees and palm trees) surrounded by open areas. The north, south, and west ends of the island are densely covered in needlerush (*Juncus roemerianus*). To facilitate habitat measurements and egg searches for our study, we installed a permanent grid system across the island using PVC stakes that were spaced 5 m from each other, which created a 9 × 11 grid of 5 × 5 m squares across the island (Figure 1b).

Quantifying maternal nesting behavior

To quantify maternal nesting behavior, we searched for *A. sagrei* eggs at our field site (Figure 1) during early, middle, and late periods of the nesting season in 2018 (June 25–27, July 21–24, and October 3–5, respectively). We randomly located plots to search for eggs to ensure that our sampling was not biased towards any specific microhabitat. During each trip, a random number generator was used to select 20 stakes in the grid and to determine a distance (1–500 cm) and direction (0–359°) from each stake. At each of the 20 randomly selected locations, a flag was placed at the designated distance and direction from the stake, and a 1 × 1 m quadrat was placed around it. The quadrat was searched thoroughly for eggs by turning up 1–2 cm of soil with a spoon and searching under any rocks or debris within the quadrat. We also conducted targeted searches for eggs in locations that appeared to be suitable incubation habitat based on information from laboratory studies (Reedy et al.
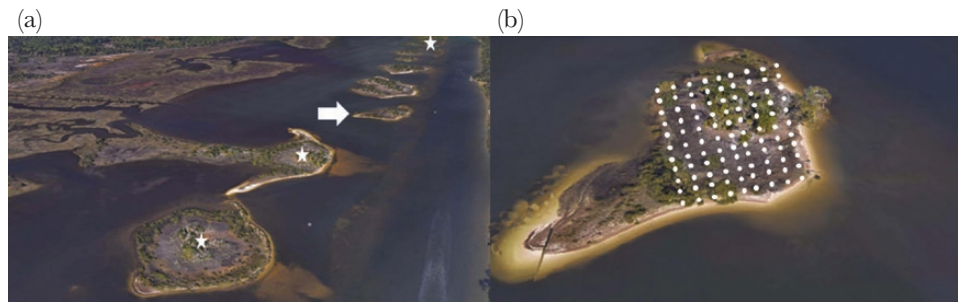
Figure 1
Aerial photographs of the field site. The island marked with a white arrow (a) was used to quantify nesting microhabitat and for the egg placement experiment, and the islands with white stars (in addition to the mainland) are locations used to collect the breeding colony. The permanent grid system is represented by white dots (b).

2013) and anecdotal field observations (Delaney et al. 2013). Targeted searches were conducted independently of the grid system and were opportunistic in nature, with each potential nest site being searched as we came upon a suitable location based on our previous anecdotal observations of nests; the key factors that led us to choose targeted sites were presence of leaf litter, nearby trees, and cover objects (e.g., rocks, fallen palm fronds). When an egg was located, a 1 × 1 m quadrat was placed around it, and the quadrat was searched thoroughly for eggs as described above for the random searches. A search was only counted as a targeted search when an egg was found, but we searched more areas that did not contain eggs.

When an egg was found using either search method, a soil sample (~2 tablespoons) was collected at the location of the egg, hemispherical photographs were taken with a digital camera (Nikon Coolpix L30) with a 180° fish-eye lens (Zykkor), and Thermocron iButtons (programmed to take hourly temperature readings) were placed next to the egg. When multiple eggs were found near each other (within 25 cm), a single soil sample, photograph, and iButton were used to quantify microhabitat of that site. We conducted 20 random searches and 20 targeted (until we found 20 sites with eggs present) searches in June, July, and October to capture potential temporal variation in nesting behavior. However, in October only 7 maternal nest sites were found, likely because this was the end of the nesting season. Therefore, only 7 successful targeted searches were conducted for this final time period.

Microhabitat measurements were taken at every other stake (n = 50 stakes) across the grid (henceforth, "available nest site") to compare to maternally selected sites. iButtons were buried under ~2 cm of soil at the base of the stake, and soil samples (~2 tablespoons) were collected. These samples were taken concurrently with samples from maternally selected sites. Temperature was recorded hourly for 15 days for each period after loggers were placed in the ground. Soil moisture was determined by weighing samples before and after drying (to a consistent mass) and calculating the percent water content (Warner and Andrews 2002).

An analysis of variance (R Version 3.4.2) was used to quantify temporal differences in microhabitat parameters between maternally selected and available nest sites. Temperature, canopy openness, and soil moisture were the dependent variables in three separate models; month (June, July, and October), nest type (Maternal or Available), and their interaction were the independent variables. A Least Squares Means test was used for pairwise comparisons among months and nest type. Analyses of variance were

also used to quantify differences in microhabitat variables (temperature, soil moisture, and canopy openness) between eggs found individually versus those in aggregations, using the microhabitat variable as the dependent variable and aggregation status as the independent variable.

Animal husbandry and egg collection

In addition to egg searches, we planted eggs across the island landscape to assess the effects of a broad range of microclimate variables on egg survival and embryo development. To obtain eggs for this study, we housed a captive breeding colony of *A. sagrei* at Auburn University. Adult lizards for the breeding colony (321 females and 46 males) were collected from 1 to 3 April 2018 from nearby islands (Figure 1a) and the mainland of Palm Coast, Florida. Adults were housed outdoors at Auburn University in screen cages (0.61 m long × 0.61 m wide × 1.22 m high) (~9 females and one male per cage) that contained an upright bamboo perch (1.37 m) wrapped in artificial leaves for hiding and climbing. Each cage contained a plastic nesting pot (19 cm long × 12 cm wide × 9 cm high) filled with potting soil and placed at the base of the bamboo perch. Lizards were fed crickets (dusted with calcium and vitamins) twice per week and misted with water daily if there was no rain.

Nest pots were checked for eggs twice weekly. Eggs found were weighed and placed in Petri dishes (radius: 26 mm; height: 15 mm) filled halfway with moist vermiculite (−150 kPa) and sealed with parafilm. Eggs were incubated (Peltier-cooled Incubator IPP55 Plus, Memmert GmbH, Schwabach, Germany) under fluctuating incubation regimes that mimicked conditions measured from the early season (Supplementary Figure S1). This cool, early-season temperature regime was chosen because it slows development and thus minimized the amount of development that occurred prior to being placed in the field for incubation. All eggs were between 1 and 14 days (Stage 4–10; Sanger et al. 2007) from oviposition at the time of their placement, and, on average, only 18.6% of the incubation period occurred in the lab prior to egg placement in the field (Supplementary Figure S2).

Development of embryos under field conditions

On 22 June and 22 August 2018, eggs were transported to the field in compartmentalized tackle boxes with moist vermiculite (−300 kPa), and were weighed before being placed on the island. To control for possible effects of transportation, 40 additional eggs traveled to the field site and then immediately back to Auburn for

incubation; hatching success was 92.5%, suggesting minimal, if any, impact of transportation. Four eggs were placed at every other stake on the island grid system starting with the south-westernmost stake (four eggs at 50 stakes = 200 eggs). Eggs at each stake were randomly assigned one of the cardinal directions and buried 25 cm from the stake under ~2 cm of soil. A small piece of hardwire cloth (3 × 3 cm square of 0.5 cm mesh size) was placed over each egg and buried just under the soil surface to narrow the search area for relocation when eggs were recovered for assessment of survival. This cloth was not in physical contact with the egg and had no measurable impact on the nest environment. Microclimate data (e.g., temperature, percent soil moisture, and canopy cover) were collected from each egg placement location; similar to methods outlined above, ibuttons were buried 2 cm at the base of each stake, and soil samples and canopy photos were taken at the time of egg placement for measurements of substrate moisture and canopy openness, respectively.

Because of the importance of substrate moisture for development (Packard and Packard 1988) and due to its correlation with other microhabitat variables (i.e., open/warm sites tend to be drier than shaded cooler sites), we performed an additional experiment to isolate the effects of moisture on egg hatching success from other variables. To do this, an additional 30 eggs were placed in moisture-controlled chambers and buried at randomly selected stakes. These chambers were Petri dishes filled halfway with moist vermiculite (−150 kPa) and sealed with parafilm and duct tape, which controlled moisture level and allowed comparisons with eggs that were subjected to natural moisture conditions. This comparison enabled us to determine the relative importance of moisture versus other microhabitat variables (temperature, substrate) towards egg survival and offspring phenotypes.

Eggs incubated on the island for 18 days (percentage of incubation prior to collection: min: 51.3%; mean: 68.9%; max: 85.7%), at which point they were collected, weighed, and returned to the laboratory where they were placed in fresh Petri dishes with moist vermiculite (−150 kPa) and placed in a programmable incubator set at a natural fluctuating incubation regime (Supplementary Figure S1) until hatching. Collecting eggs prior to hatching was necessary to ensure that hatching success was reliably scored and offspring phenotypes measured. Because several eggs hatched within 24 hours of collection, this confirmed that the timing of collection was appropriate. Moreover, the 18-day incubation period in the field subjected eggs to predation and extreme thermal and hydric conditions, which led to some eggs not being recovered on each trip (though signs of predation were never observed as hardwire cloth always remained in position). Rather than counting those eggs as dead, they were removed from the analyses to take the most conservative approach. Hatchlings were measured (mass, snout-vent length [SVL], and tail length) before being euthanized according to our animal care protocol. We calculated individual body condition as residuals from a regression of hatchling mass against SVL. This protocol was performed in June and August to capture temporal changes in the effect of nest conditions on embryo development and survival.

A general linear model was used to determine differences in microclimate parameters between June and August. A generalized linear mixed model with a binomial distribution (R package lme4; Bates et al. 2015) was used to determine the effect of average daily mean and maximum temperatures, canopy cover, month (June or August), treatment (Petri dish or no Petri dish),

and a month by treatment interaction on hatching success with egg mass as a covariate and stake as a random factor. A similar model was used to analyze survival using only eggs contained within Petri dishes. This model provided an analysis of survival for eggs under constant moisture conditions known to be suitable for development. To analyze hatching success of eggs placed in the ground in August (low survival in June prevented further analysis), a generalized linear mixed model was used with average daily mean and maximum temperature, canopy cover, and percent soil moisture as fixed effects, egg mass as a covariate, and a random effect of stake.

Mass gained during incubation for eggs with no Petri dish was analyzed using a linear mixed model with initial egg mass, average daily mean temperature, canopy openness, and soil moisture as fixed effects and stake as a random effect. Incubation duration was analyzed by using the number of days before hatching after eggs were removed from the field as the dependent variable and estimated egg stage (Supplementary Figure S2), average daily mean temperature, canopy cover, percent soil moisture, and initial egg mass as independent variables. Hatchling body condition was analyzed using a linear mixed model with mass as the dependent variable, and with SVL, soil moisture, canopy cover, and average daily mean temperature as independent variables, and stake as a random variable. Similar models were used to analyze these traits for eggs in Petri dishes, but soil moisture was not included in these models. A principal component analysis (PCA) was conducted (loadings in Supplementary Table S1) to collapse the variation in microhabitat variables into uncorrelated axes, but produced the same results as the raw values, so we elected to use raw values for ease of interpretation.

RESULTS

Nest microhabitat

Females consistently used relatively cool and moist sites to lay their eggs. Maternally selected sites were significantly cooler ($\beta = -1.43 \pm 0.19$, $F_{1,190} = 53.80$, $P < 0.0001$; Figure 2) with higher soil moisture ($\beta = 5.36 \pm 1.21$, $F_{1,199} = 43.47$, $P < 0.0001$; Figure 2), and lower canopy openness ($\beta = -6.12 \pm 2.66$, $F_{1,193} = 8.79$, $P = 0.0222$) than what was available across the island. This pattern remained consistent between time periods for temperature ($F_{2,190} = 2.81$, $P = 0.063$; Figure 2). Additionally, temperature for both maternally selected and available sites declined as the season progressed with June being the warmest and October being the coolest (Figure 2, Supplementary Table S2). Although soil moisture of maternally selected sites in June and July differed from that available across the island, this pattern was not evident in October ($F_{2,199} = 9.10$, $P < 0.0001$; Figure 2). Canopy openness was consistent in June and October, but a significant interaction was driven by lower canopy openness for maternally selected sites in July ($F_{2,193} = 6.20$, $P = 0.002$). Of the 80 eggs that we found, 3.75% were found using the random search method, and 96.25% were found using the targeted method. Most eggs (68.75%) were found under cover objects (e.g., rocks, palm fronds) or in aggregations (21 sites that were searched contained two or more eggs; the largest aggregation was five eggs). Nests with aggregations versus those with a single egg did not differ in temperature ($\beta = 0.16 \pm 0.32$, $P = 0.609$), moisture ($\beta = -0.26 \pm 3.30$, $P = 0.938$) or canopy openness ($\beta = 4.35 \pm 4.52$, $P = 0.341$).
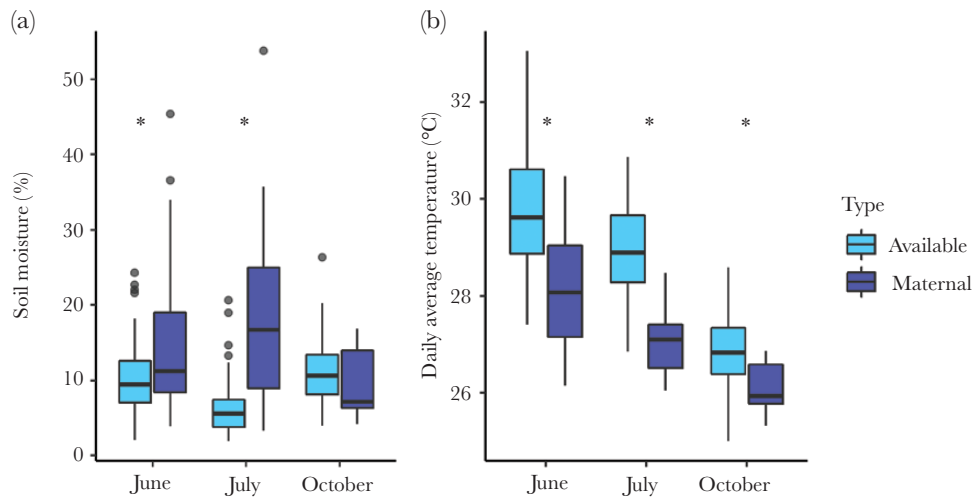
Figure 2
Soil moisture (a) and average daily mean nest temperature (b) for maternally selected nest sites and available sites for each month of egg searches (see text for statistics). The bold line represents the 50th percentile lower and upper bounds represent the 1st and 3rd quartiles, respectively. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 × interquartile range and the points represent outliers in the dataset.
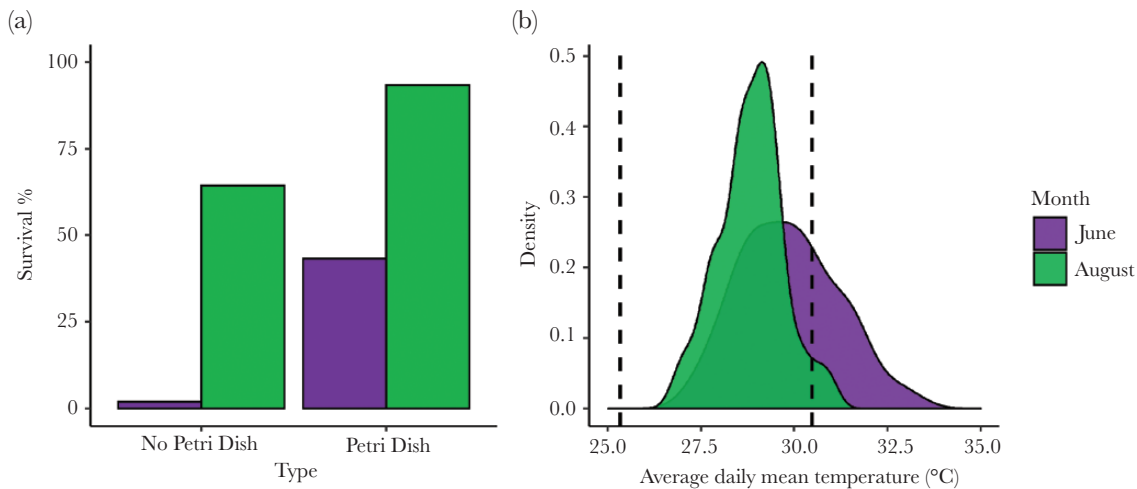

Figure 3
Survival percentages for eggs with and without a Petri dish in June and August (a), and a density distribution of average daily mean temperatures for egg placement locations in June and August (b). The vertical dashed lines in panel b represent the range of the average daily mean temperatures selected by females across the season.

Effect of nest microclimate on development

Microclimate conditions and egg survival differed between the two egg placement trials (Figure 3). Eggs placed in June experienced warmer mean ($\beta = 1.02 \pm 0.23$, $P < 0.001$) and maximum temperatures ($\beta = 4.24 \pm 0.91$, $P < 0.001$) than those in August with no significant difference in soil moisture ($\beta = 1.08 \pm 0.96$, $P = 0.263$). Of the 200 eggs placed in the June trial, 138 were recovered, and only four hatched (2.89% survival). In the August trial, 182 eggs were recovered and 118 eggs hatched (64.83% survival). Furthermore, hatching success of the eggs placed in the field within sealed Petri dishes showed similar temporal trends with 43.3% and 93.3% survival for June and August, respectively (Figure 3). Eggs incubated in Petri dishes were 10.68 ± 2.23 times more likely to survive than eggs incubated with no Petri dish ($P = 0.003$), and eggs from both treatments had lower survival in June than in August ($F_{1,376} = 26.57$, $P < 0.001$, Supplementary Table S3).

Because of low egg survival in June (2% survival for eggs not in Petri dishes) further analyses that examined relationships between egg survival and microhabitat variables were restricted to the August trial. Survival of eggs incubated in August decreased with increasing canopy openness ($F_{1,173} = 12.19$; $P = 0.001$; Supplementary Table S3) and increasing temperatures (mean temperature: $F_{1,173} = 3.52$, $P = 0.007$; max temperature: $F_{1,173} = 3.03$, $P = 0.014$; Figure 4; Supplementary Table S3). Mass gained by eggs during incubation in the field increased significantly with average daily mean incubation temperature ($\beta = 0.03 \pm 0.005$, $P < 0.001$; Figure 5), but had no relationship with soil moisture or canopy openness. Incubation duration decreased with increasing average daily mean temperature ($\beta = -0.82 \pm 0.25$, $P = 0.002$), stage at placement ($\beta = -1.01 \pm 0.10$, $P < 0.001$), and decreased 1.9 days with each 0.1 g increase in egg mass ($P = 0.020$). Hatchling body condition increased with soil moisture ($\beta = 0.001 \pm 0.0004$; $F_{1,35} = 5.76$, $P = 0.022$; Figure 5b).

For eggs incubated within Petri dishes, egg survival probability was higher in August than in June ($F_{1,51}$ = 5.69; P = 0.004) and decreased with increasing average daily maximum temperatures ($F_{1,35}$ = 2.75; P = 0.045). Body condition of hatchlings from eggs incubated in Petri dishes was not affected by temperature or canopy cover (P > 0.335). Incubation duration of eggs in Petri dishes was not affected by stage at placement (β = 0.785 ± 0.530; $F_{1,8}$ = 3.39, P = 0.103), average daily mean temperature (β = −0.416 ± 0.857; $F_{1,8}$ = 0.77, P = 0.407), canopy cover (β = −0.014 ± 0.045; $F_{1,26}$ = 0.022, P = 0.884), or egg mass (β = −62.108 ± 28.96; $F_{1,8}$ = 4.60, P = 0.064).
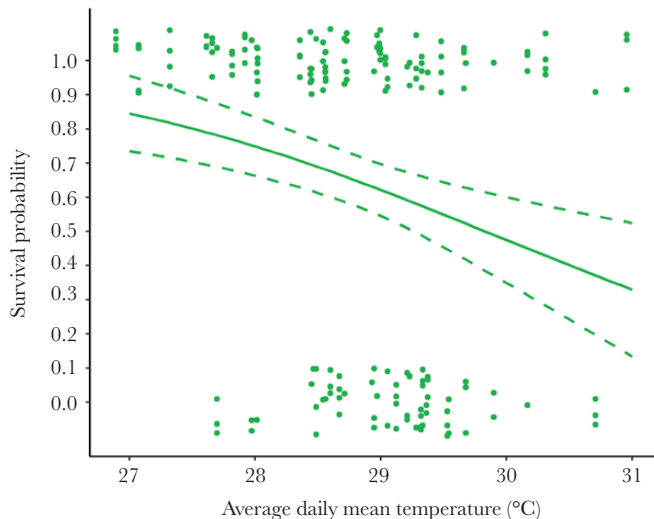

Figure 4
Probability of egg hatching success in relation to average daily mean temperature of the egg placement location. The survival probability curves were generated using generalized linear models of survival plotted against temperature. The bold line represents survival probability and the dashed lines represent the upper and lower (95%) confidence limits. The raw survival data (1 = alive, 0 = dead) is plotted and jittered.
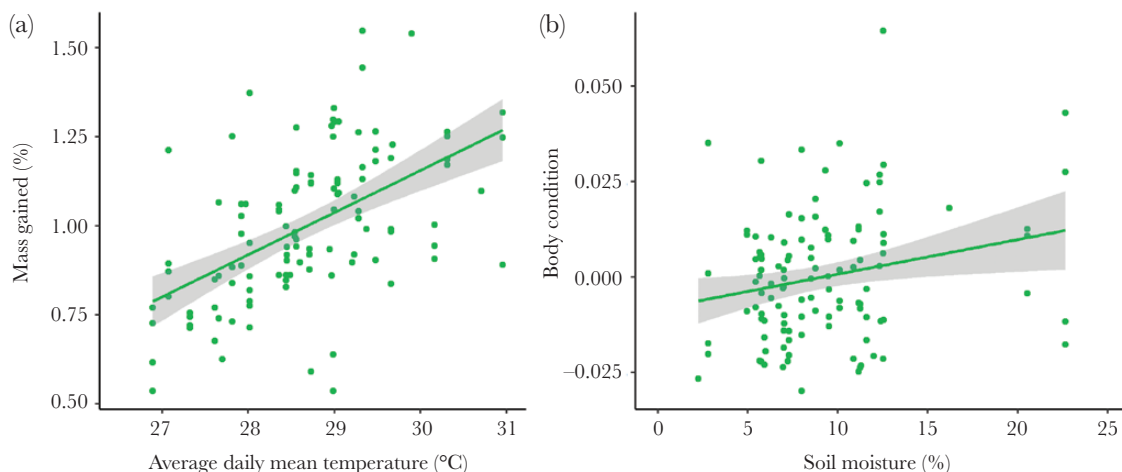
DISCUSSION

The consequences of maternal nesting behavior on offspring are well documented via correlative and experimental studies on a wide range of organisms (Morse 1990; Lloyd and Martin 2004; Mitchell et al. 2013b; Pruett et al. 2019). Importantly, temporally changing micro-environments impose challenges to reproductive females when selecting nest sites, since the consequences of a particular nest site may be unpredictable. In this study, we quantified maternal nesting behavior in the brown anole by assessing micro-habitat of nest sites across three periods during the reproductive season. We also performed an experiment to determine the effects of nest microhabitat on egg survival under field conditions. To our knowledge, we gathered the most comprehensive data set on anole nesting in the field and provide critical insights into this poorly understood aspect of *Anolis* biology. We show that nest sites are relatively cool compared to available microhabitat across the landscape, and that nest sites used by mothers facilitate embryo development and increase egg hatching success. These patterns of microhabitat choice remained consistent across the reproductive season as ambient temperature changed, but eggs suffered substantially greater mortality early in the season.

Maternal nesting behavior

In line with our predictions, females chose sites that were relatively cool and moist compared to what was broadly available on the island across the season. In addition, maternally selected sites generally followed the seasonal ground temperature trends observed in the available sites, which steadily decreased across the season. This seasonal decrease in ground temperature contrasts past work at our field site that shows increases in temperature as the season progresses (Pearson and Warner 2018). However, average air temperature in June and July in 2018 did not differ substantially from each other (27.8 vs. 28.6°C), and average October air temperature was considerably lower (25.2°C) during our study (Supplementary Figure S3). This seasonal decline in air temperature in 2018 parallels the decline in ground temperature that we measured. The decline in ground temperature from June to July can also occur due

Figure 5
(a) The effect of average daily mean incubation temperature on percent egg mass gain during incubation in the field. Each dot represents an individual egg from the August placement experiment. The gray cloud represents the standard error. (b) A linear regression of hatchling body condition (residuals of snout-vent length plotted against body mass) against soil moisture for August hatchlings. Each dot represents an individual hatchling. The gray cloud represents the standard error.

to increased rainfall over the season (a common weather pattern in Florida), which has been shown to dampen nest temperatures in other reptiles (Warner and Shine 2008; Lolavar and Wyneken 2015). Importantly, even with the seasonal decline in temperature, females were still able to find nest sites that were significantly cooler than other sites available across the island, even during the cooler part of the season. Indeed, nearly all nests found were in shaded areas and undercover objects (rocks, leaf litter), which remain cooler than the average ground temperature across the island landscape.

The similar seasonal trends of maternal and available nest temperatures suggest that females choose similar sites across the season rather than changing their behavior, and the thermal characteristics of those sites change with ambient conditions. Importantly, average daily mean temperatures of nest sites used by females are within the range of temperatures that are favorable for development (Warner et al. 2012). Females also nested in sites with relatively high levels of substrate moisture, which has been previously observed in lab studies (Socci et al. 2005; Reedy et al. 2013). However, the consequences of substrate moisture in the field are difficult to interpret for several reasons. First, our index of nest moisture was a snapshot at the time when eggs were found. Given that substrate moisture changes temporally (Robbins and Warner 2010) we can neither directly determine the moisture conditions female select for nesting, nor examine how nest hydration changes during incubation. Second, although percent water content of substrate moisture provides some index of moisture availability to eggs, this can vary depending on substrate type. Indeed, quantifying substrate water potential would provide a better indication of water availability for eggs; this might explain why substrate moisture from the field (mix of sand, shell, soil) was consistently lower than what females prefer in the lab (potting soil; Reedy et al. 2013). Moreover, soil type might be another factor used by females when choosing nest microhabitats (Tiatragul et al. 2019). Despite these caveats, we still detected differences in substrate moisture between nest sites and available sites in the expected direction. Although the difference was consistent among all sampling periods, only October exhibited no statistically significant difference; this may be due to a relatively small sample size at this period, or the cooler temperatures could have reduced variation in moisture between maternally selected and available nesting locations.

Most eggs were found under cover objects (e.g., rocks, palm fronds, seashells, dense leaf litter) and close to the soil surface, suggesting that females prefer areas that conceal their nest. This strategy, along with egg aggregations, has been observed in other species (Doody 2009; Telemeco et al. 2009) and could reduce the amount of time and energy spent during nesting activities (thereby reducing female exposure to predators; Warner and Shine 2008; Huang and Pike 2012). These nesting sites could also provide similar benefits to developing embryos than those of relatively deep nests (e.g., protection from predators, direct sunlight, or moisture loss). Whether this nesting behavior is favored by selection as a result of reducing threats to nesting females or due to benefits for embryo development is unknown, but both may synergistically explain female preference for cover objects when nesting.

Eggs were often found in aggregations which have been observed previously in other anoles (Robinson et al. 2014; Godfrey et al. 2018; Tiatragul et al. 2019; Swierk et al. 2019). These egg aggregations may be explained by either 1) nest site philopatry where individual females repeatedly choose the same site to nest or 2) communal egg-laying where multiple females choose the

same site to lay their eggs. Our study does not directly address egg aggregation behavior, but the variation in egg size suggests that both these explanations are likely. For example, some aggregations contained eggs of similar size; given that eggs substantially increase over time, the low variation in egg size suggests that eggs were of similar ages (Supplementary Table S4), and hence, laid by multiple females. However, we also observed aggregations with large variation in egg size (Supplementary Table S4), suggesting that one female could have repeatedly returned to that site to lay eggs over multiple weeks. Further investigation is needed to determine if egg aggregation is due to nest philopatry, communal nesting or both, as well as the fitness consequences of these aggregations (Doody 2009; Mateo and Cuadrado 2012). Finally, while the random search method forced us to look for eggs across the landscape, and hence reduced bias in our search area, this method did not locate many nests. In contrast, the success of our targeted searches suggests that eggs were largely where we predicted them to be.

Effect of nest microclimate on development

The results of our egg placement experiment generally support our predictions that nest microhabitats used by females are beneficial for offspring. Moreover, incubation of a subset of eggs within moisture-controlled conditions (i.e., within sealed Petri dishes) enabled us to decouple the effects of moisture with other environmental factors, such as temperature. First, we show that eggs that experienced controlled moisture conditions consistently had higher survival than those buried directly in the soil, suggesting that sustained moisture to eggs conveys a survival advantage (Reedy et al. 2013). Thus, females that select moist nest sites could improve the success of her offspring. Secondly, eggs within and outside Petri dishes had higher survival in August than in June, which suggests that temperature may have also influenced variation in egg hatching success. For instance, the distribution of August nest temperatures was more similar to the temperature range selected by mothers across the season (c.f., Figures 2b and 3b) than the June temperature distribution—this pattern coupled with higher egg survival in August provides evidence that nesting females select thermal conditions that facilitate hatching success. Indeed, maximum temperatures at many of the stakes during the June experiment were considerably higher than the lethal limit for brown anole embryos (Hall and Warner 2019). Moreover, substrate conditions appeared very dry when eggs were collected during the June trial, which also could have contributed to the low egg survival at this time. Although we did not detect differences in substrate moisture between June and August trials, these measurements were taken at the time eggs were placed in the field, and subsequent nest moisture was not monitored.

Although relatively high mean and maximum temperatures negatively affected egg hatching success, maximum temperatures likely drove most of the variation in embryo survival (Angilletta et al. 2013; Hall and Warner 2018). Thus, females may improve offspring survival by choosing sites that are less likely to reach high maximum temperatures. Because these two metrics are tightly correlated, avoiding sites with high maximum temperatures would reduce overall mean nest temperature and increase incubation duration (Noble et al. 2018). Consistent with this prediction, we show that incubation duration in the field increases with decreasing temperatures. Such an effect has potential fitness consequences, as prolonged incubation time might increase the risk of exposure to predators or adverse weather conditions (Doody 2011).

Consequently, this presents a major challenge for nesting females because they must select thermal conditions that optimally balance those that favor hatching success (cooler temperature) versus those that shorten the length of incubation (warmer temperature). This is particularly critical late in the nesting season when temperatures are comparatively low; not only are embryos developing slowly, but late-produced eggs hatch late in the season, which puts hatchlings at a further survival disadvantage (Pearson and Warner 2018). Thus, the shift in ambient temperature across the nesting season can generate significant temporal changes in how selection may operate on maternal nesting behavior. Temporal shifts from small to large egg size over the season, as reported previously (Mitchell et al. 2018), could enable offspring to cope with late-season nest temperatures, as larger eggs had shorter incubation durations. However, stochasticity in ambient conditions across time makes this a complex system that may sometimes constrain female nest choice.

The phenotypic consequences of the nest environment on offspring (Noble et al. 2018) adds another level of complexity to understanding how selection operates on maternal nesting behavior. Higher temperatures also increased the percentage of mass eggs gained during incubation (via water uptake), which could have been due to increased metabolic rate at higher incubation temperatures (Warner et al. 2012; Hall and Warner 2020). This effect on mass gain of eggs, however, did not have phenotypic influences on hatchlings, and thus, may not be biologically meaningful. Consistent with lab experiments (Warner et al. 2012); however, substrate moisture positively influenced hatchling body condition but the effect size was extremely small. Thus, this small range of body conditions may not generate much variation in offspring survival (Pearson and Warner 2018). Given these minimal phenotypic effects of nest conditions in the field, most variation in fitness due to nesting behavior is likely driven by environmentally induced variation in survival during embryonic development, rather than at post-hatching stages.

CONCLUSIONS

Maternal nest choice has an important influence on development and early life of offspring in oviparous species. While nesting behavior and its consequences are relatively well studied in some organisms (e.g., turtles; Kolbe and Janzen 2002; Janzen and Morjan 2002), the reproductive biology of anoles (single egg clutches produced over a long season) provides a unique opportunity for examining temporal variation in this important maternal effect. Our large data set on anole nests in the field shows that females consistently select cool and moist sites for nesting across the season and that these microhabitat choices enhance egg survival. In addition, eggs are almost exclusively laid under cover objects and commonly found in aggregations. These aspects of nest-site choice may further facilitate egg survival by retaining moisture and reducing exposure to temperature extremes, which are two factors that we demonstrated to positively impact hatching success in the field. Thus, this study adds to a growing literature demonstrating that maternal nesting behavior enhances fitness in wild populations, and provides evidence that this important maternal effect is under selection in temporally changing environments.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at *Behavioral Ecology* online.

REFERENCES

Andrews RM 1982. Spatial variation in egg mortality of the lizard *Anolis limifrons*. Herpetologica. 38(1):165–171.

Angilletta MJ Jr, Zelic MH, Adrian GJ, Hurliman AM, Smith CD. 2013. Heat tolerance during embryonic development has not diverged among populations of a widespread species (*Sceloporus undulatus*). Conserv Physiol. 1:cot018.

Bates D, Maechler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. J Stat Softw. 67(1):1–48.

Brown GP, Shine R. 2004. Maternal nest-site choice and offspring fitness in a tropical snake (*Tropidonophis mairii*, Colubride). Ecology. 85(6):1627–1634.

Deeming DC, Ferguson MWJ. 1991. Incubation and embryonic development in reptiles and birds. In: Tullett SG, editor. Avian incubation. London: Butterworths-Heinemann, pp. 3–37.

Deeming DC, Reynolds SJ. 2015. Nests, eggs, & incubation: new ideas about avian reproduction. Oxford, UK: Oxford University Press.

Delaney DM, Reedy AM, Mitchell TS, Durso AM, Durso KP, Morrison AJ, Warner DA. 2013. *Anolis sagrei. Nest-site choice*. Herpetol Rev. 44(2):314.

Doody JS. 2011. Environmentally cued hatching in reptiles. Integr Comp Biol. 51:49–61.

Doody JS, Freedberg S, Keogh JS. 2009. Communal egg-laying in reptiles and amphibians: evolutionary patterns and hypotheses. Q Rev Biol. 84:229–252.

Godfrey ST, Duberstein JA, Mota J, Moore W. 2018. *Anolis carolinensis* (green anole) nest sites and communal nesting. Herp. Rev. 49(1):115.

Goodman RM, Heah TP. 2010. Temperature-induced plasticity at cellular and organismal levels in the lizard Anolis carolinensis. Integr Zool. 5:208–217.

Hall JM, Warner DA. 2018. Thermal spikes from the urban heat island increase mortality and alter physiology of lizard embryos. J Exp Biol. 221:jeb181552.

Hall JM, Warner DA. 2019. Thermal tolerance in the urban heat island: thermal sensitivity varies ontogenically and differs between embryos of two sympatric ectotherms. J Exp Biol. 222:jeb210708.

Hall JM, Warner DA. 2020. Thermal sensitivity of lizard embryos indicates a mismatch between oxygen supply and demand at near-lethal temperatures. J Exp Zool. doi:10.1002/jez.2359.

Hansell MH. 2005. Animal architecture. Oxford: Oxford University Press

Huang WS, Pike DA. 2012. Effects of intraguild predators on nest-site selection by prey. Oecologia. 168:35–42.

Janzen FJ, Morjan CL. 2002. Egg size, incubation temperature, and posthatching growth in painted turtles (*Chrysemys picta*). J Herpetol. 36(2):308–311.

Kolbe JJ, Janzen FJ. 2002. Impact of nest-site selection on nest success and next temperature in natural and disturbed habitats. Ecology. 83:772–781.

Li SR, Hao X, Wang Y, Sun BJ, Bi JH, Zhang YP, Janzen FJ, Du WG. 2017. Female lizards choose warm, moist nests that improve embryonic survivorship and offspring fitness. Funct Ecol. 32(2):416–423.

Lloyd JD, Martin TE. 2004. Nest-site preference and maternal effects on offspring growth. Behav Ecol. 15(5):816–823.

Lolavar A, Wyneken J. 2015. Effect of rainfall on loggerhead turtle nest temperatures, sand temperatures and hatchling sex. Endanger Species Res. 28:235–247.

Losos JB. 2009. Lizards in an evolutionary tree: ecology and adaptive radiation of anoles. Berkeley, CA: University of California Press.

Mainwaring MC, Barber I, Deeming DC, Pike DA, Roznik EA, Hartley IR. 2017. Climate change and nesting behaviour in vertebrates: a review of the ecological threats and potential for adaptive responses. Biol Rev Camb Philos Soc. 92:1991–2002.

Marshall DJ, Uller T. 2007. When is a maternal effect adaptive? Oikos 116(12):1957–1963.

Mateo JA, Cuadrado M. 2012. Communal nesting and parental care in Oudri's fan-footed gecko (*Ptyodactylus oudrii*): field and experimental evidence of an adaptive behavior. J. Herpetol. 46(2):209–212.

Mitchell TS, Hall JM, Warner DA. 2018. Female investment in offspring size and number shifts seasonally in a lizard with single-egg clutches. Evol Ecol. 32(2–3):231–245.

Mitchell TS, Maciel JA, Janzen FJ. 2013a. Does sex-ratio selection influence nest-site choice in a reptile with temperature dependent sex determination? Proc R Soc B. 280(1772):20132460.

Mitchell TS, Warner DA, Janzen FJ. 2013b. Phenotypic and fitness consequences of maternal nest-site choice across multiple early life stages. Ecology. 94:336–345.

Moore MP, Whiteman HH, Martin RA. 2019. A mother's legacy: the strength of maternal effects in animal populations. Ecol Lett. 22:1620–1628.

Morse DH. 1990. Leaf choices of nest-building crab spiders (*Misumena vatia*). Behav Ecol Sociobiol. 27(4):265–267.

Mousseau TA, Fox CW. 1998. Maternal effects as adaptations. New York: Oxford UP.

Noble DWA, Stenhouse V, Schwanz LE. 2018. Developmental temperatures and phenotypic plasticity in reptiles: a systematic review and meta-analysis. Biol Rev Camb Philos Soc. 93:72–97.

Packard GC, Packard MJ. 1988. The physiological ecology of reptilian eggs and embryos. In: Gans C, Huey RB, editors. Biology of the reptilia, Vol. 16. New York: Alan R. Liss, p. 523–605.

Pearson PR, Warner DA. 2016. Habitat- and season-specific temperatures affect phenotypic development of hatchling lizards. Biol Lett. 12(10):20160646.

Pearson PR, Warner DA. 2018. Early hatching enhances survival despite beneficial phenotypic effects of late season developmental environments. Proc R Soc B 285(1874):20180256.

Pike DA, Webb JK, Shine R. 2010. Nesting in a thermally challenging environment: nest-site selection in a rock-dwelling gecko, *Oedura lesueurii* (Reptilia: Gekkonidae). Biol. J. Linn. Soc. 99(2):250–259.

Pruett JE, Addis EA, Warner DA. 2019. The influence of maternal nesting behavior on offspring survival: evidence from correlational and cross-fostering studies. Animal Behav. 153:15–24.

Pruett JE, Fargevieille A, Warner DA. 2020. Data from: temporal variation in maternal nest choice and its consequences for lizard embryos. Behav Ecol. http://doi:10.5061/dryad.jq2bvq86c.

Rand AS. 1967. Communal egg laying in anoline lizards. Herpetologica. 23(3):227–230.

Reedy AM, Zaragoza D, Warner DA. 2013. Maternally chosen nest sites positively affect multiple components of offspring fitness in a lizard. Behav Ecol. 24(1):39–46.

Refsnider JM. 2016. Nest-site choice and nest construction in non-avian reptiles: evolutionary significance and ecological implications. Avian Biol wwRefsnider JM, Bodensteiner BL, Reneker JL, Janzen FJ. 2013.

Experimental field studies of species' responses to climate change: challenges and future directions. Anim Conserv. 16(5):498–499.

Refsnider JM, Reedy AM, Warner DA, Janzen FJ. 2015. Do trade-offs between predation pressures on females versus nests drive nest-site choice in painted turtles? Biol J Linn Soc. 116(4):847–855.

Robbins TR, Warner DA. 2010. Fluctuations in the incubation moisture environment affect growth but not survival of hatchling lizards. Biol. J. Linn. Soc. 100:89–102.

Saino N, Calza S, Moller AP. 1997. Immunocompetence of nestling barn swallows in relation to brood size and parental effort. J. Anim. Ecol. 66:827–836.

Sanger TJ, Losos JB, Gibson-Brown JJ. 2007. A developmental staging series for the lizard genus anolis: a new system for the integration of evolution, development, and ecology. J. Morphol. 269:254–274.

Schwanz LE, Janzen FJ. 2008. Climate change and temperature-dependent sex determination: can individual plasticity in nesting phenology prevent extreme sex ratios? Physiol Biochem Zool. 81:826–834.

Shine R. 2005. Life-history evolution in reptiles. Annu Rev Ecol Evol Syst. 36(1):23–46.

Smith JM, Sinelink G, Fawcett JD, Jones RE. 1973. A survey of the chronology of ovulation in anoline lizard genera. Trans Kans Acad Sci. 75(2):107–120.

Socci AM, Schlaepfer MA, Gavin TA. 2005. The importance of soil moisture and leaf cover in a female lizard's (*Norops polylepis*) evaluation of potential oviposition sites. Herpetologica 61(3):233–240.

Spencer RJ. 2002. Experimentally testing nest site selection: fitness trade-offs and predation risk in turtles. Ecology. 83(8):2136–2144.

Swierk L, Carriere A, Delfin M, Fondren A, Lopera D, Putnam BJ. 2019. *Anolis aquaticus (=Norops aquaticus)* (Water Anole), egg size and oviposition site. Herpetol. Rev. 50(2):360.

Telemeco RS, Elphick MJ, Shine R. 2009. Nesting lizards (*Bassiana duperreyi*) compensate partly, but not completely, for climate change. Ecology. 90:17–22.

Tiatragul S, Hall JM, Pavlik NG, Warner DA. 2019. Lizard nest environments differ between suburban and forest habitats. Biol J Linn Soc. 126(3):392–403.

Tinne S, Rianne P, Marcel E. 2005. Experimental removal of the male parent negatively affects growth and immunocompetence in nestling great tits. Oecologia. 145:165–173.

Warner DA. 2014. Fitness consequences of maternal and embryonic responses to environmental variation: using reptiles as models for studies of developmental plasticity. Integr Comp Biol. 54:757–773.

Warner DA, Andrews RM. 2002. Nest-site selection in relation to temperature and moisture by the lizard *Sceloporus undulatus*. Herpetologica 58(4) 399–407.

Warner DA, Moody MA, Telemeco RS, Kolbe JJ. 2012. Egg environments strongly impact embryonic development, but have minimal consequences for hatchling phenotypes in an invasive lizard. Biol J Linn Soc. 105:25–41.

Warner DA, Shine R. 2008. Maternal nest-site choice in a lizard with temperature-dependent sex determination. Anim Behav. 75(3):861–870.

Wolf JB, Wade MJ. 2009. What are maternal effects (and what are they not)? Philos Trans R Soc Lond B Biol Sci. 364:1107–1115.