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FEMALE NESTING SITE CHOICE IN BROWN ANOLE (*ANOLIS SAGREI*) LIZARDS:
IMPLICATIONS FOR EMBRYONIC AND POST-HATCHING DEVELOPMENT
ABIGAIL J. DENNIS

A DEPARTMENT HONORS THESIS SUBMITTED TO THE
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Abstract

For oviparous ectotherms, embryonic environment can strongly impact post-hatching phenotype. Therefore, it is advantageous for females to choose nesting sites with conditions conducive to offspring development. In lizards that bury their eggs, the substrate temperature of the embryonic environment can have especially significant consequences for development. Abnormalities in craniofacial development and immobile limbs have been linked to high incubation temperatures outside the normal range of developmental temperatures. However, other factors, such as substrate moisture and depth, can impact offspring fitness as well.

To determine the extent to which temperature influences female nesting site choice in relation to substrate moisture and depth, we housed 35 wild-caught female brown anole lizards (*Anolis sagrei*) in small groups in the laboratory. In each cage, females had the choice of oviposition in one of two “nesting boxes” containing moist substrate: the Heated Box, which was placed over a heating pad controlled by a thermostat, and the Ambient Box, which was maintained in ambient conditions. In both boxes, we measured substrate moisture and depth for each nesting site and calculated the temperature at each location where an egg was laid.

I predicted that the lizards would avoid dangerously warm nesting sites near the base of the Heated Box and that the depth of nesting sites would thus be more variable in the Ambient Box. My findings indicate that females did not exhibit a preference between the Heated and Ambient Box, and on average, nesting sites in the Heated Box were warmer than those in the Ambient Box. The substrate moisture and depth of nesting sites did not differ between the Heated and Ambient Box; however, nesting site depths were more variable in the Heated Box in comparison to the Ambient Box. In both boxes, all oviposition sites were warmer than 26°C and with the exception of one 33.2°C nesting site, all nesting sites were cooler than 31.5°C, indicating

that females avoided nesting site temperature conditions likely to result in low fitness offspring. Temperature may be an important cue for female nesting sites in many taxa. Yet, the progression of climate change may increasingly pose challenges to oviparous ectotherm species in their current ranges.

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Introduction

High Embryonic Environmental Temperature and Phenotype

Across vertebrate taxa, the temperature of the embryonic environment can have far-reaching effects on an organism's phenotype. In development, the thermosensitive periods (TSPs) are defined as those times during development in which animals are most likely to be influenced by temperature. Since most TSPs occur during the embryonic stages of development (reviewed in McCue, 2004), embryos can be especially vulnerable to their environments (Du & Shine, 2015). For instance, in reptiles, embryonic tissue differentiation can be influenced by temperature (Andrews, 2004) and tissue differentiation occurs during the first 30-40% of embryonic development. Therefore, exposure to high temperatures early in development is likely to impact the formation of distinct tissues and organs (Andrews, 2004). During embryonic development, in the case of the European common lizard (*Zootoca vivipara*), temperature impacts neurulation, organogenesis, as well as early growth (i.e., stages 30-34; Dufaure & Hubert, 1961), but has minimal effects on late growth (i.e., stages 35-40; Dufaure & Hubert, 1961; Andrews, 2004).

The impacts of temperature on tissue differentiation in developing embryos is clear when observing the resulting phenotype. In pregnant human (*Homo sapiens*) females, a 39°C fever lasting two days can result in congenital limb disruptions (Martínez-Frías et al., 2001). Across taxa, incubation at temperatures above 38°C can have impacts on embryo phenotype as widespread as abnormalities in hormone expression (zebrafish, *Danio rerio*: Jin et al., 2017), nerve arrangement (chick, *Gallus gallus domesticus*: Primmitt, Stern & Keynes, 1988), and the cytoskeleton (cows, *Bos taurus*: Rivera et al., 2004). Further, excessively high and low temperatures can lead to embryo lethality across taxa, including in organisms such as cyprinid fish species (Herzig & Winkler, 1986).

Thermoregulation in Endotherms and Ectotherms: Life History and Evolutionary Implications

The temperature of the environment may impact ectotherms and endotherms differently, especially in the case of embryos. An endothermic animal can obtain the majority of its body heat from its metabolic processes, while the majority of an ectotherm's body heat originates from its environment (McCue, 2004; Tzschentke & Rumpf, 2011). The metabolic rate of endotherms is five to ten times higher than that of ectotherms, and this high metabolic rate allows endotherms to regulate body temperature despite changing environments (Tzschentke & Rumpf, 2011). In both birds and mammals, embryos transition from ectothermy to endothermy during development (Tzschentke & Rumpf, 2011). In chicken embryos, this transition occurs in the last third of incubation (Nichelmann et al., 1998).

Ectotherms and endotherms can exhibit oviparity or viviparity (Shine, 2005). In the reproductive pattern of viviparity, females retain developing eggs inside their reproductive tracts or body cavities before giving birth to an offspring capable of free existence. Contrastingly, in the reproductive pattern of oviparity, females oviposit or deposit eggs that will develop and hatch in the external environment (Blackburn, 1999).

In many oviparous ectotherms, such as green anole lizards (*Anolis carolinensis*), the mother abandons the egg after burying it (Propper et al., 1991), leaving the egg and the developing embryo inside with no thermoregulatory assistance from parental care.

Oviparous ectotherm embryos abandoned after oviposition are more vulnerable to unpredictable fluctuations in the environment than viviparous organisms are because they are more exposed to the environment (Du & Shine, 2015). In the case of oviparous ectotherm embryos, these organisms cannot maintain body heat through metabolic processes (McCue, 2004; Tzschentke & Rumpf, 2011), so thermoregulation can be difficult. In general, the eggs of

oviparous ectotherms are immobile (Telemeco, 2014; Hall & Warner, 2018), and the embryos are confined to those eggs and to the conditions of the nesting environment (Telemeco, 2014).

Among oviparous ectotherm species, differences exist in embryonic ability to thermoregulate. Egg size can greatly impact thermoregulatory ability in embryos. In large eggs, such as turtle eggs, embryos can move within the egg to exploit subtle thermal gradients, indicating that the environment may be partially under embryonic control in those instances (Du et al., 2013; Ye et al., 2019). However, embryos in smaller eggs are less likely to be able to exert the same control over their environment (Du et al., 2013). While large eggs take a long time to heat or cool, smaller eggs, such as most lizard eggs, heat and cool quickly (Du et al., 2013). Oviparous ectotherm embryos have difficulty behaviorally thermoregulating (Tiatragul et al., 2017; Telemeco et al., 2016), especially those in small eggs, likely due to their undeveloped physiology and small size (Du et al., 2013).

Optimal Range of Temperatures in Oviparous Ectotherms

Most oviparous ectotherms have a range of acceptable temperatures under which high fitness embryos can develop, but this range varies across species. For example, when brown anole lizard (*Anolis sagrei*) embryos were incubated at 26°C, 28°C and 30°C, embryos were able to survive and hatch under all of those conditions (Warner et al., 2012). In the southern alligator lizard (*Elgaria multicaranta*), embryos could survive and hatch between 25-30°C, but the optimal incubation range for development was 27-28°C (Telemeco, 2014). In cyprinid fish such as *Abramis brama* that were exposed to lab incubation temperatures ranging from 9.0-16.2°C, hatching success was highest between 10.5 and 16.2 °C. This species has an especially wide range of tolerance, and this range correlates with the observed spawning temperatures (Herzig & Winkler,

1986). In painted turtles (*Chrysemys picta*), the maximum temperature for embryonic development was 34°C (Telemeco et al., 2013).

In some species, temperatures near the upper limits of the optimal range can be beneficial for development. Tiatragul et al. (2017) found that in the Puerto-Rican crested anole (*Anolis cristatellus*) and in brown anole lizards, temperatures at the higher end of the optimal incubation temperature range (27-30°C) can accelerate embryonic development and may even enhance offspring fitness. Differential developmental rate of zebrafish (*Danio rerio*) incubated at 25°C and 33°C (Kimmel et al., 1995) may be a result of the effect of temperature on the speed of biochemical reactions involved in somitogenesis (Schröter et al., 2008). A segmented body plan is characteristic of vertebrates, and somitogenesis is the process of initiating segmentation during development (Maroto, Bone & Dale, 2012). The length of somitogenesis decreases with increasing temperatures (Schröter et al., 2008). Generally, in oviparous ectotherms, longer incubation times can be dangerous because there is more opportunity for the environment to disrupt the embryo (Du & Shine, 2015). Further, the seasonal timing of hatching can have fitness consequences. Pearson & Warner (2016) found that brown anole lizard eggs hatching too late in the breeding season were at a disadvantage; they grew more slowly and had a lower survival rate.

The speed of developmental rate can have fitness consequences other than those relating to the timing of development. Warner et al. (2012) found that at incubation temperatures of 30°C and under, egg mass seemed to have more of an effect on offspring body mass than the temperature conditions did during development in brown anole lizards. Increasing the incubation temperature from 27 to 30°C could speed up egg incubation, but was unlikely to influence the morphology of the hatchling (Warner et al., 2012). Huang & Pike (2011) also found that at incubation temperatures below 32°C, egg hatching success rates were high in the long-tailed sun skink

(*Eutropis longicaudata*). However, when the temperatures of these nesting sites increased by only 1.5°C, hatching success rate became much lower (Huang & Pike, 2011).

While choosing warm nesting sites can be advantageous for development, this can be a risky strategy. If females choose nesting sites that are a few degrees too warm, there can be serious fitness consequences. Embryo survival can also be impacted by excessively high temperatures in the embryonic environment in the case of oviparous ectotherms, such as certain lizard species. Sanger et al. (2018) found that at incubation temperatures of 36°C, brown anole lizard embryo survival dropped below 50%, and at 39°C, survival dropped below 5%. Hall & Warner (2018) found that when incubated in the presence of thermal spikes, Puerto Rican crested anole lizard embryo survival decreased most significantly in the presence of a 43°C temperature spike, and embryo survival was less affected in the presence of a 39°C temperature spike. Hall and Warner (2018) attributed the embryo lethality to hypoxia resulting from the thermal spike, as high temperatures increase metabolic rate, which requires the consumption of higher levels of oxygen.

In both Cyren's rock lizard (*Iberolacerta cyreni*; Monasterio et al., 2011) and the pine snake (*Pituophis melanoleucus*; Burger, Zappalorti & Gochfeld, 1987), high experimental egg incubation temperatures can lead to low hatching success. High incubation temperatures also impact hatchling phenotype in oviparous ectotherms. At increased temperatures, Cyren's rock lizard neonates exhibited immobility of the forelimbs, and the inability to coordinate head movements, hindering feeding (Monasterio et al., 2011). High incubation temperature was also associated with decreased body condition and smaller juveniles in both Cyren's rock lizard and the Algerian psammodromus (*Psammodromus algrius*), another lacertid lizard (Monasterio et al., 2011). In zebrafish, embryos that developed faster at higher temperatures were also smaller in size (Atkinson, 1994). Mulder (1995) found that abnormalities in pit vipers (*Crotalinae spp.*), such as

kyphoscoliosis, could be linked to high incubation and gestation temperatures in black-tailed pythons (*Python molurus*) and asp vipers (*Vipera aspis*). Studies in the oriental garden lizard (*Calotes versicolor*) have correlated high incubation temperatures with short heads and hind limbs in smaller hatchlings, as well as short eye and tympanum diameters in heat stressed individuals (Ji, Qui & Diong, 2002). In painted turtle embryos, incubation at high temperatures for greater than 60 hours resulted in hatchlings with carapace deformities (Telemeco et al., 2013).

Later ontological effects can also be associated with high incubation temperatures. In reptiles, thermal extremes experienced during development have been shown to lead to skeletal abnormalities that persist into adulthood. The increased abnormality and asymmetry resulting from warm developmental conditions can impact adult performance (Mitchell, Janzen & Warner, 2018). In zebrafish, embryos that developed faster due to high incubation temperatures also developed into smaller adults (Atkinson, 1994).

Molecular Effects of High Temperatures on Development in Oviparous Ectotherms

The temperature-dependent expression of *Hox* genes is an example of how high incubation temperatures can impact gene expression during oviparous ectotherm development. As in all animals, reptile body plans are strongly defined by *Hox* gene expression, and disruption of these genes by exposure to high temperatures can lead to congenital abnormalities (reviewed in Martín-del-Campo, Sifuentes-Romero & Garcia-Gasca, 2019). Abnormalities induced by high incubation temperatures could also be a result of changes to *Hox* gene expression.

The heat shock response, which occurs as a result of exposure to high temperatures, can also impact development in ectotherms (Kingsolver & Woods, 2016). Heat shock response leads to expression of heat shock proteins. Brief exposures to sublethal temperatures can promote heat

hardening, leading to adaptive acclimation and an increased chance of survival when exposed to heat shock again (Kingsolver & Woods, 2016). However, long-term overexpression of heat shock proteins can also alter expression patterns of genes involved in metabolism, ion transport, antioxidant production and negatively affects rates of metabolism, growth and development (Kingsolver & Woods, 2016). Hulbert et al. (2017) found that heat shock can lead to cardiac arrest in brown anole lizard embryos.

Other Factors that Impact Embryo Development in Oviparous Ectotherms

In addition to temperature, moisture of the embryonic environment can impact offspring fitness in oviparous ectotherms. According to Sanger et al. (2008), in environments too high in moisture, water pressure encroaching on the egg can damage anole lizard embryos. Conversely, environments that are too dry can desiccate and kill the embryo (Sanger et al., 2008). Reedy, Zaragoza & Warner (2012) found that female brown anole lizards tend to prefer moist nesting sites (75% moisture content), and these nesting sites result in offspring with higher snout-vent length (SVL) and body mass in comparison to those that developed in drier conditions. In the painted turtle, changes in moisture during embryonic development yielded congenital abnormalities in the offspring, including jaw abnormalities and asymmetrical carapaces (Lynn & Ulrich, 1950).

The depth of substrate at which an embryo develops can also impact fitness in oviparous ectotherms. Doody et al. (2015) found that female lizards may lay eggs at a greater depth to avoid egg predators, and Angilletta, Sears & Pringle (2009) also observed deep nesting sites, possibly to avoid dry or warm substrate conditions near the surface. Numerous other factors have been shown to affect embryo development in oviparous ectotherms as well, including but not limited to maternal stress in brown anole lizards (Sanger et al., 2018), and exposure to toxins such as

cadmium in the painted turtle (Brasfield et al., 2004). Further, in the painted turtle, periods of brief hypoxia have been shown to diminish the embryo's rate of growth, leading to hatchlings that are smaller in size (Cordero et al., 2017).

Female Nesting Site Choice as a Selective Pressure in Oviparous Ectotherms

With all these possible effects of temperature on embryonic development, it is clear that embryonic environment has the potential to impact offspring fitness. Since eggs cannot purposefully move, and the embryos in them have limited means with which to behaviorally thermoregulate, whatever conditions a female chooses for her nesting site will be the conditions under which her offspring develops. Kamel & Mrosovsky (2004) suggest that nest placement behavior has been under strong selection, and the behavior may have a genetic basis, showing evolutionary potential. While their study focused on leatherback sea turtles (*Dermochelys coriacea*), nesting sites in most oviparous ectotherms can similarly impact offspring survival and fitness. Females who do not choose good nesting sites will not have high fitness offspring (Reedy, Zaragoza & Warner, 2012). Therefore, one would expect that females will search for nesting sites that will enhance offspring fitness (Huang & Pike, 2011).

Brown Anole Lizards as a Model

One species in which the effects of nesting site temperature on development are particularly well-characterized is the brown anole lizard. Brown anoles can develop normally at a range of temperatures (26°-33°C), with fitness and developmental rate is highest at 30°C (Warner et al., 2012; Tiatragul et al., 2017). Embryo survival decreases after 33°C (Sanger et al., 2018), and heat

shock can lead to cardiac arrest in brown anole embryos (Hulbert et al., 2017). These studies all indicate a clear link between temperature and fitness.

Not only are the effects of nesting site temperature on development in brown anoles well known, but also the ecology and reproductive biology of the species. Brown anole lizards are native to Cuba and the Bahamas, and their invasive range extends from the Gulf Coast of Texas to Georgia (Fetters & McGlothlin, 2017). Brown anoles are part of the “trunk-ground” class of Caribbean ecomorphs (Calsbeek & Marnocha, 2006), as they inhabit low vegetation, such as barren scrub or the understory of forest (Schoener & Schoener, 1980). Brown anoles prefer perching sites on the ground, or slightly elevated sites on low branches or tree trunks (Calsbeek & Marnocha, 2006). Brown anoles are small-to-medium in size, and are also highly dimorphic in body size. Regardless of sex, animals in this species that are similar in size exhibit an overdispersed spatio-social system. Activity in brown anoles is fairly evenly distributed across the day, and they consume a diet of primarily large insects, with a few fruits as well (Schoener & Schoener, 1980).

The reproductive biology of brown anoles is also well-characterized. Lee et al. (1989) identified the brown anole breeding season as April to July. Invasive populations of brown anoles have also been shown to have short interlaying intervals and eggs that produce hatchlings quickly. Populations in Georgia, Florida and Louisiana were shown to have interlaying intervals around three to five days and egg incubation periods around 29-31 days (Fetters & McGlothlin, 2017). These eggs are generally 0.5-1.5 cm in length, if viable (Sanger et al., 2008). Brown anole lizards are robust in the lab, and will oviposit in a laboratory setting (Sanger et al., 2008). For all these reasons, brown anole lizards are an ideal model species for studies investigating the role of temperature in female nesting site choice.

Experimental Design and Hypotheses

In this thesis, I tested the hypothesis that females would choose nesting site conditions advantageous for the development of their offspring, and they would avoid nesting site conditions that would be detrimental for development. To test this, we caught and housed 35 wild-caught brown anole females in small groups, with each cage containing a single male, 2-3 females, and two nesting boxes. One nesting box, the Heated Box, was filled with moist peat moss and placed on top of a heat source, so that the substrate at the base was much warmer than the substrate at the surface of the box. The other nesting box, the Ambient Box, contained moist peat moss that reflected the cooler, ambient conditions of the room. As incubation temperature can have severe fitness consequences, we measured each nesting site temperature in the Heated and Ambient Boxes. While we did not manipulate substrate moisture or depth, we also measured nesting site moisture and depth in the Heated and Ambient Boxes, since these factors can have fitness consequences as well.

I predicted that the females would avoid nesting site temperatures above 33°C, as well as desiccated nesting sites (if any such sites existed). Incubation temperatures during development above 33°C have been linked to lower fitness offspring, and desiccated nesting sites kill the developing organism. I also predicted that eggs would be buried deeper in the Ambient Box compared to the Heated Box and that the nesting site depths would also be more variable in the Ambient Box. Dangerously high temperatures would be present near the base of the Heated Box, and that could restrict the range of depths where females would find advantageous nesting sites. However, since temperature is uniform with depth in the Ambient Box, I expected that female nest site choice would not be constrained in the same way.

Methods

Animal Collection and Husbandry

We captured 50 brown anole (*Anolis sagrei*) lizards (36 females, 14 males) by dental floss loop from the South Texas Botanical Gardens & Nature Center in Corpus Christi, Texas, in early June, 2019 (within the summer breeding season for this species; Lee et al., 1989). Within one day of capture, the lizards were transported in individual canvas bags to San Antonio, Texas to the Trinity University Vivarium, where we massed the lizards to the nearest 0.1 g using a Pesola spring scale, and measured snout-vent length (SVL) to the nearest mm using a plastic ruler. We marked each lizard with a nontoxic marker on its abdomen for the purpose of individual identification. Lizards were initially housed in 14 groups, each consisting of one male and two or three females, in large plastic Kritter Keepers cages (36.8 cm × 22.2 cm × 24.8 cm; Lee's Aquarium & Pet Products; San Marcos, California, USA). The lizards were allowed to acclimate in these cages for approximately one week.

Within one week of capture, all lizards were moved to mesh cages in preparation for data collection. In each mesh cage (White 12" × 12" × 12" Popup Cage with Vinyl Window; Raising Butterflies; Salt Lake City, Utah, USA), we placed three natural perches (small tree branches). We arranged the mesh cages on a metal rack so that they were directly adjacent to each other, and separated each cage with plywood. The plywood provided a visual barrier to prevent aggression between the males in different cages. We also placed two cylindrical cardboard nesting boxes (described in detail below), in which females could oviposit, in each cage. We loosely filled each nesting box with moist peat moss (The Gold Canadian Sphagnum Peat Moss; Ferti-lome; Bonham, Texas, USA), 2 cm from the surface. In each cage, one box, the Ambient Box, reflected the ambient conditions of the room, and the other box, the Heated Box, was placed on top of a heating mat

(IntelliTemp Reptile Heat Mat 13.8" × 7.9", 20 W or 9.8" × 5.9", 10 W; Big Apple Pet Supply, Inc.; Delray, Florida, USA). Large (20 W) heating mats were shared between two cages, such that the Heated Box in one cage was placed on one side of the heating mat, and the Heated Box in the adjacent cage was placed on the other side of the heating mat (Fig. 1). Only one Heated Box was placed on each 10 W heating mat, because these heating mats were too small to be shared by two cages. All heating mats were controlled by a thermostat (BAH-1000DC Reptile Thermostat with DC Chip Technology; Big Apple Pet Supply, Inc.; Delray, Florida, USA). We originally set each thermostat to 35.6°C at the start of the experiment and incrementally increased them to 40.6°C, in an effort to increase the range of temperatures in the Heated Boxes. After the second day of data collection, the thermostat remained set at 40.6°C. The first day of data collection, the thermostat was set to 36.1°C, and the second day of data collection, the thermostat was set to 37.8°C. Therefore, depending on the nesting day, the thermostat setting may have slightly differed (36.1°C, 37.8°C or 40.6°C). We placed a towel and plywood under the mesh cage and heating mat to provide insulation for the heating mat.

The lizards were housed following the standard lizard care guidelines described in Sanger et al. (2008). The lizard room in the Trinity University Vivarium was maintained at 26-29°C, with 60-72% humidity, on a 13.5:10.5 h light:dark cycle. In addition to the fluorescent room lights, each cage had two full-spectrum cage lights (T8 ReptiSun 5.0 UVB Fluorescent Reptile Lamp, 24-in; Zoo Med; San Luis Obispo, California, USA) directly over the cage. At night, the cage lights turned off 30 min before the room lights to simulate dusk, and in the morning the room lights turned on 30 min before the cage lights to simulate dawn. We fed each lizard 2-3 live crickets or mealworms coated with calcium powder (Repti Calcium with D3 Reptile Supplement; Zoo Med;

San Luis Obispo, California, USA) three times a week, and we misted the cages with water every morning to provide drinking water for the lizards.

Figure 1. Two nesting boxes filled with moist peat moss were placed in each mesh cage. The nesting box placed over the heating mat was the Heated Box; the nesting box not placed over the heating mat was the Ambient Box.



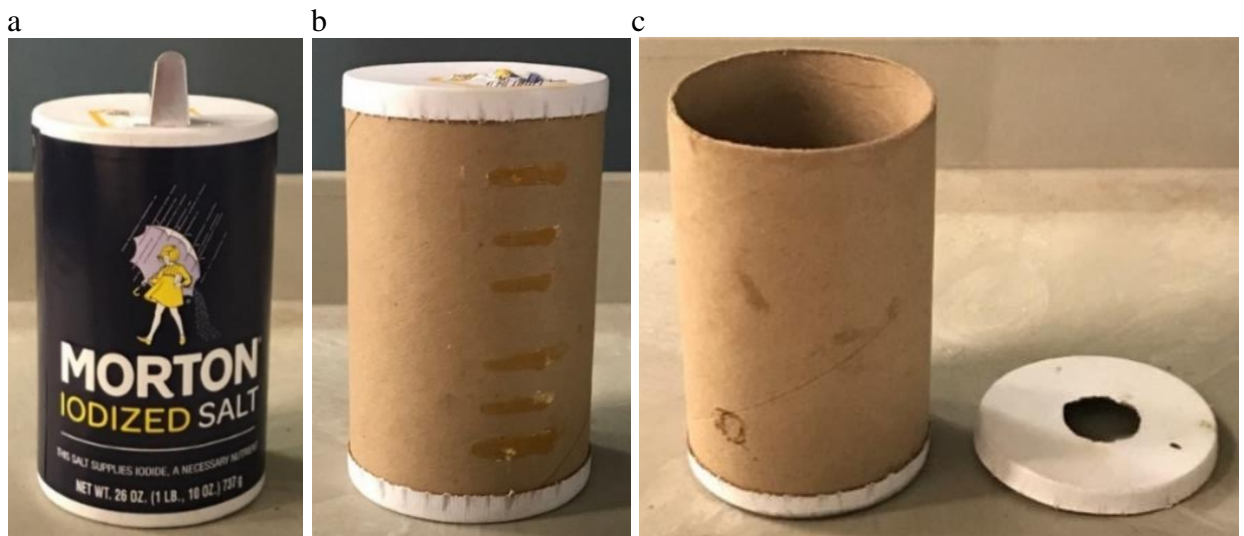
Nesting Box Construction

Since this study sought to examine female nesting site choice across a range of substrate temperatures, we constructed nesting boxes that distributed heat, allowing for multiple thermal microenvironments to exist within a nesting box. We tested a range of commercially available cylindrical containers to determine the size and type of material that produced the largest range of substrate temperature. We filled these containers with moist peat moss, placed them on top of heating mats, and then we measured the temperature at the surface and base of each container using a thermocouple (Type T, Copper/Constantan; REOTEMP instruments; San Diego, California, USA) attached to an automated temperature logger (HH603A; Omega Engineering; Norwalk, Connecticut, USA).

Through this trial-and-error process, we found that we could achieve the highest temperature at the base of the container, and the largest temperature gradient across the box, using Morton Salt cardboard canisters (Morton® Iodized Salt - 26oz and Morton® All Purpose Sea Salt - 26oz; Morton Salt; Chicago, Illinois, USA). We also observed that the lizards perched on and climbed into the nesting box with ease, and we knew of no toxic residue on the salt canisters.

The Morton Salt canister consists of a 14 cm fiber tube wrapped in a paper label, with two fiber, circular endcaps, about 8 cm in diameter. One of the endcaps contains a metal spout; the other one does not (Fig. 2a). After emptying each Morton Salt canister, we removed the metal spout (Fig. 2b). We used the base endcaps on the canisters as lids, and cut a 3 cm hole through which lizards could enter the top of the nesting box (Fig. 2c). We lined the inside of each nesting box with wax paper attached with silicone caulk (GE Silicone 1* All Purpose Silicone Sealant; GE Sealants & Adhesives; Huntersville, North Carolina, USA) to prevent water damage to the containers. On the last two days of data collection, we removed the lining for logistical reasons.

Figure 2. a) Morton Salt canister, prior to nesting box construction. b) To construct nesting boxes from the Morton Salt canister, we removed the label and the metal spout. This exposed the two endcaps enclosing the fiber tube (Morton Salt Product Data Sheet). c) We retained the endcap once containing the spout to serve as the nesting box base, and removed the other endcap so it could serve as a lid. We cut a hole in the lid so that female lizards could enter and exit, and substrate moisture would be retained.



Nesting Site Data Collection

To quantify nesting site substrate temperature, moisture, and depth conditions for each oviposited egg, we conducted egg checks in each nesting box 3 times a week for 4 weeks. Before each egg check, we measured the surface and base temperatures of the substrate in all nesting boxes potentially containing nesting sites. These measurements were later used to calculate estimated nesting site temperatures. We then sifted through the substrate with a scoopula to search for eggs (Fig. 3). Upon finding an egg, we measured the depth of the nesting site from the surface of the container, and collected a small sample of substrate (0.1-1.5 g) from that location to measure the proportion of the wet substrate mass that was water (hereafter, substrate moisture). To this end, we wrapped the substrate sample in wax paper, measured its wet mass, and dried it at 45°C in an oven overnight. The following morning, we measured the dry mass of the sample. We calculated one minus the dry mass divided by the wet mass to determine the proportion of water in the substrate sample.

After 4 weeks of data collection, we concluded the study in mid-July. After again measuring each lizard's SVL and mass, each lizard was euthanized using the two-step MS-222 method (Conroy et al., 2009), and then rapidly decapitated.

Figure 3. Oviposition site located in one of the nesting boxes.



Temperature and Moisture Substrate Gradient Data Collection

To calculate nesting site temperature and to determine the relationship between substrate temperature and moisture, I first measured how temperature and moisture distributed within the Heated and Ambient Boxes in a cage. I set up 21 mesh cages in the vivarium, identical to those used during the experiment, lacking only the lizards. To recreate all conditions that nesting boxes were exposed to over the course of the 30 d of data collection, I set the thermostat in 11 cages to 40.6°C, the thermostat in 5 cages to 36.1°C, and the thermostat in the last 5 cages to 37.8°C. In 5 of the 11 cages set to 40.6°C, I removed the lining inside, but kept the lining in the other six cages. For each cage, I collected data after 1 d. I used a thermocouple to measure the substrate temperature at intervals of approximately 1 cm in a series of 5-6 Heated and Ambient Boxes, depending on the simulated condition. After completing temperature measurements, I took 5 substrate samples at increasing depths of approximately 2 cm intervals in both the Heated and Ambient Boxes and then calculated the proportion of water in the substrate. For substrate moisture gradients, I omitted one cage in the 40.6°C setting, lining present group, so all moisture gradients were $n = 5$.

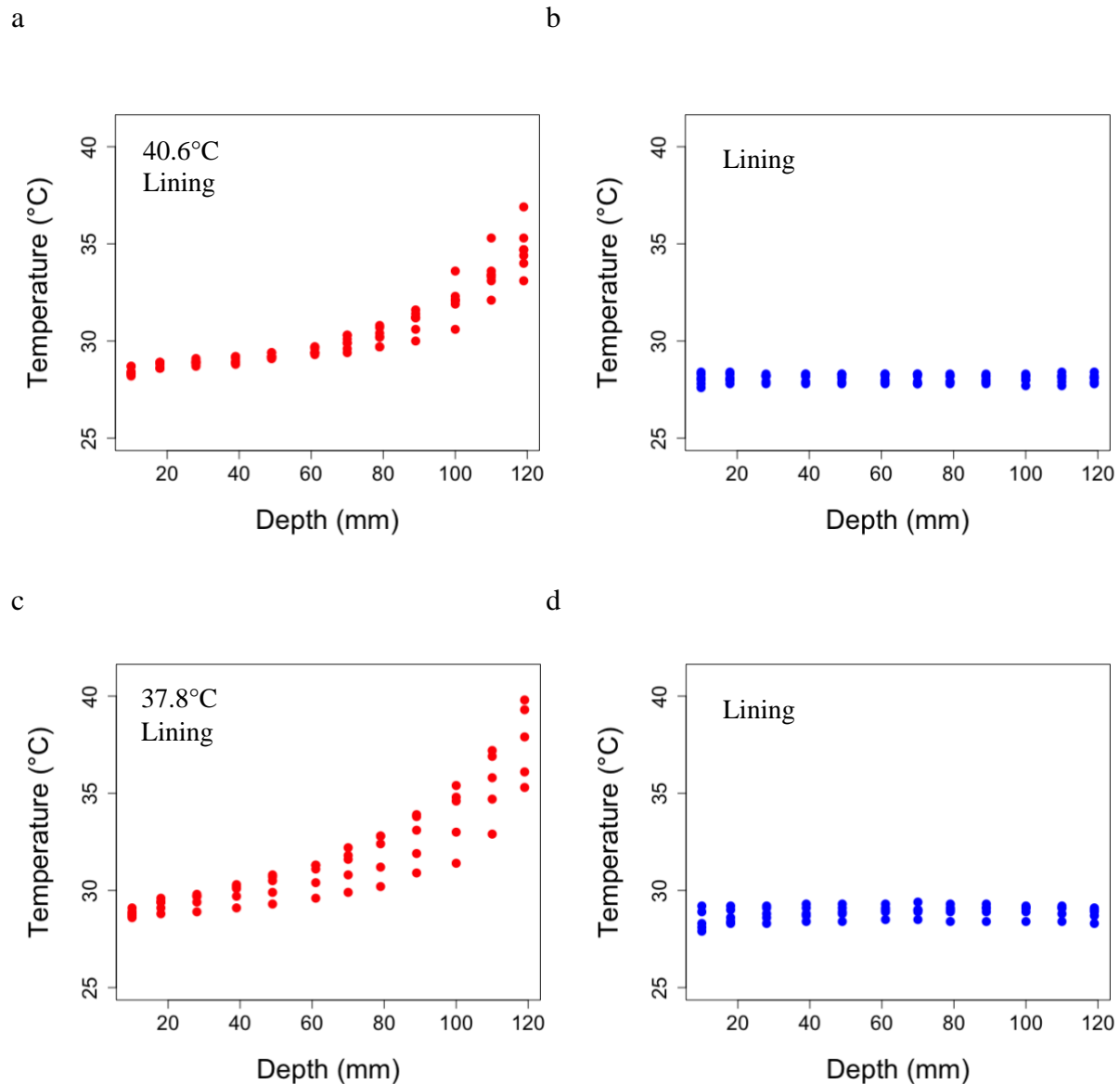
All statistical analyses were performed using RStudio (Version 1.2.5033; RStudio Team). To establish substrate temperature gradients, I plotted the substrate temperature against substrate depth for both the Ambient and Heated Box gradient measures. I conducted an ANCOVA, examining the relationship between temperature and depth, with moisture as a covariate. I found that in the gradient with thermostat settings at 40.6°C and the lining present (the most common condition), temperature did not change with depth in the Ambient gradient ($F_{1,22} = 0.056$, $p = 0.81$), but it did in the Heated gradient ($F_{1,22} = 89.88$, $p = 3.16 \times 10^{-9}$). Moisture did not change with depth in either the Ambient ($F_{1,22} = 0.0041$, $p = 0.95$) or Heated ($F_{1,22} = 1.19$, $p = 0.29$) gradient. For the Ambient Box, temperature did not generally vary with depth (Fig. 4), so I

calculated Ambient nesting site temperatures by averaging the surface and base temperatures of each Ambient Box containing a nesting site. Temperatures were warmer near the base of the Heated Box (Fig. 4), adjacent to the heating pad, than they were near the surface, farther from the heating pad.

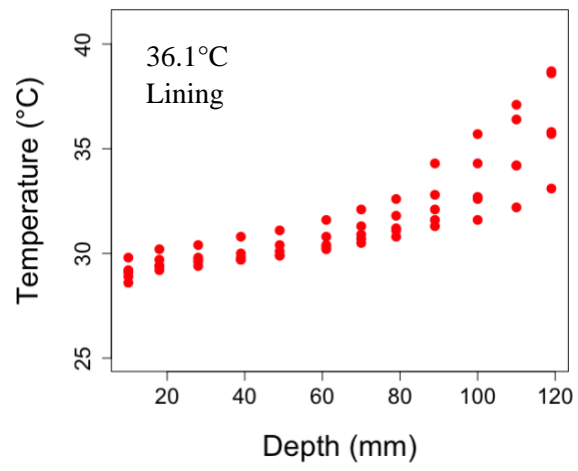
In the Heated Box, since substrate temperature varied as a function of depth (Fig. 4), I calculated nesting site temperatures using a series of linear splines (Fig. 5). The gradient model provided the average slope between consecutive points of temperature plotted against depth. I then tailored that information to each Heated Box containing a nesting site by using the surface and base temperatures. Starting with the surface temperature and 1 cm depth, I calculated the temperature at 2 cm depth based on the slope calculated using the gradients. Once I calculated the temperature at 2 cm depth, I calculated the temperature at 3 cm depth, using the slope from the gradient models, and so on. With this information, I used nesting site depth information from each egg to estimate the nesting site temperature. In the four models used to simulate all conditions over the course of data collection (Fig. 5), temperature increased with depth overall, and between the chosen intervals for the linear splines, but the slope of the increase differed between the models at each depth interval. Therefore, I used different slope values to calculate nesting site temperatures, depending on the nesting site depth and the gradient model used.

To establish the substrate moisture gradients, I plotted substrate moisture against depth (mm) in the Heated (Fig. 6a) and Ambient (Fig. 6b) Boxes. To quantify the relationship between substrate moisture and temperature in the gradients, I plotted moisture (proportion of water in substrate) against substrate temperature (Fig. 7). I also ran an ANOVA examining the relationship between substrate moisture and substrate depth. Moisture did not vary with depth in the Heated ($F_{1,23} = 0.027$, $p = 0.87$) or Ambient gradients ($F_{1,23} = 0.002$, $p = 0.96$).

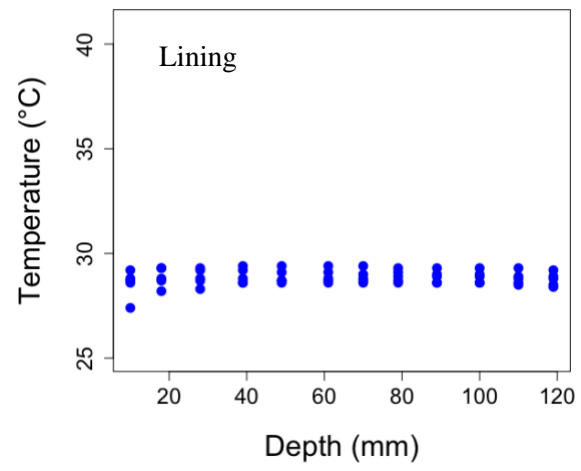
Figure 4. Heated (a, c, e, g) and Ambient (b, d, f, h) temperature gradients, used to calculate nesting site temperatures. Thermostat setting is in the top left corner of panels a, c, e and g. No lining (g and h) indicates that the wax paper lining of the thermostat has been removed. Different thermostat settings and the presence or absence of the lining represent the range of all conditions present across data collection: thermostat set at 40.6°C (n = 6), 37.8°C (n = 5), 36.1°C (n = 5) and 40.6°C without the wax paper lining (n = 5). In all cases, temperature did not vary with depth in the Ambient gradient, but temperature increased with depth in the Heated gradient.



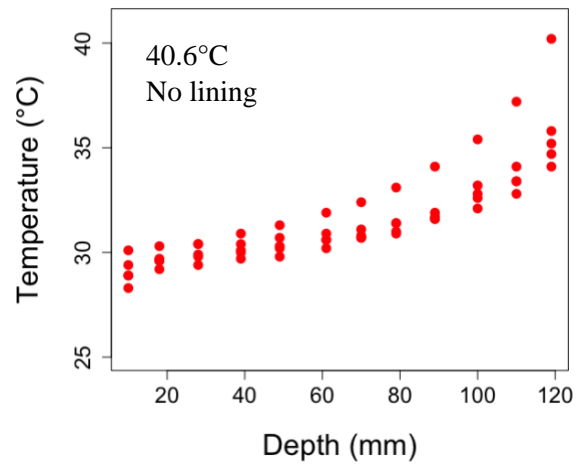
e



f



g



h

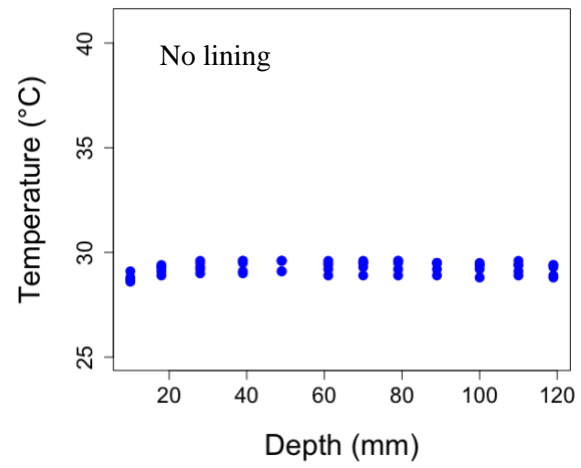


Figure 5. Linear splines of temperature ($^{\circ}\text{C}$) versus depth from top of substrate (mm) in the Heated Box gradients used to calculate nesting site temperature. Thermostat settings and the presence or absence of the wax paper lining is indicated in the top left corner of each panel. The range of all conditions present across data collection: thermostat set at 40.6°C ($n = 6$), 37.8°C ($n = 5$), 36.1°C ($n = 5$) and 40.6°C without the wax paper lining ($n = 5$) are represented.

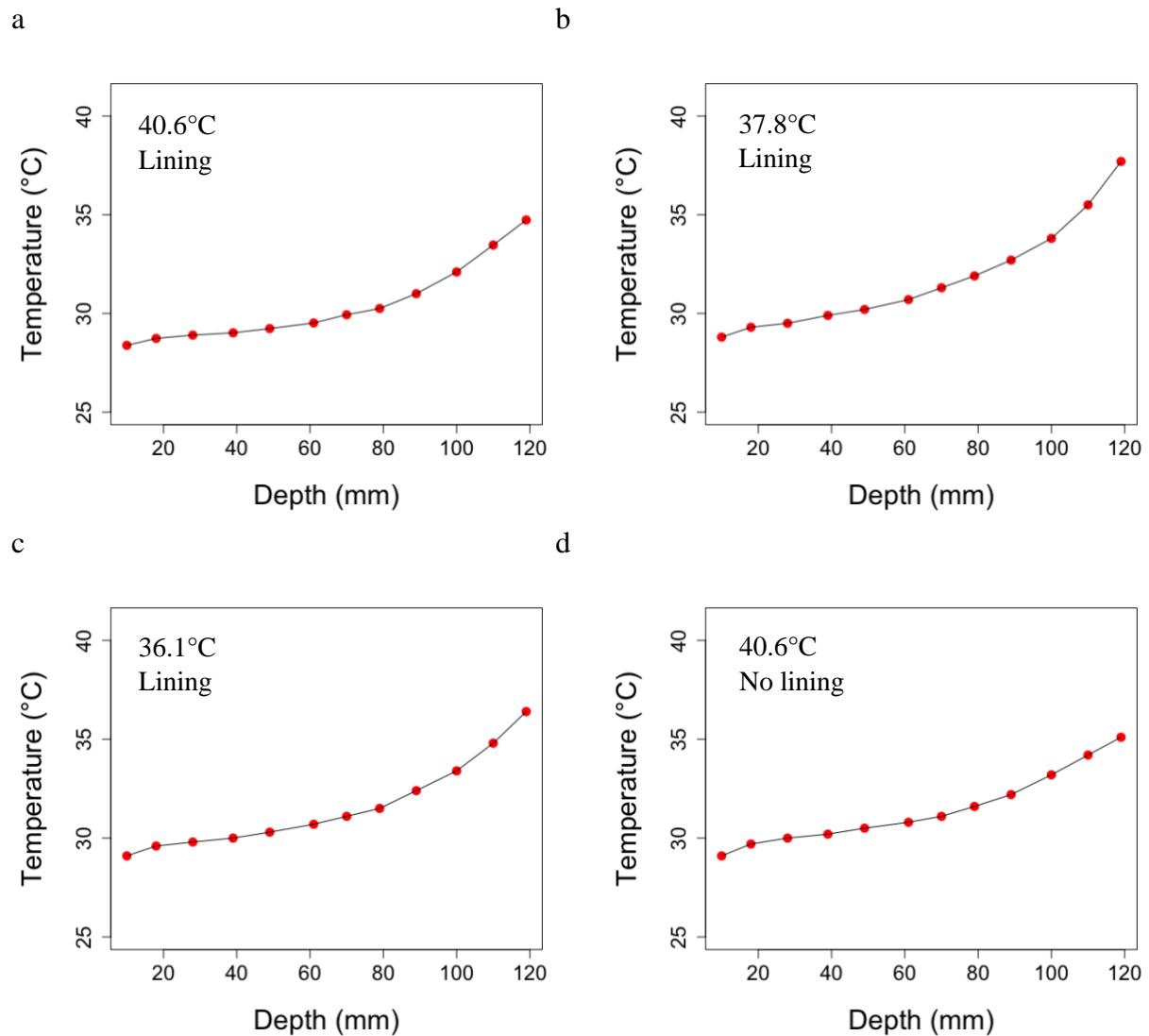
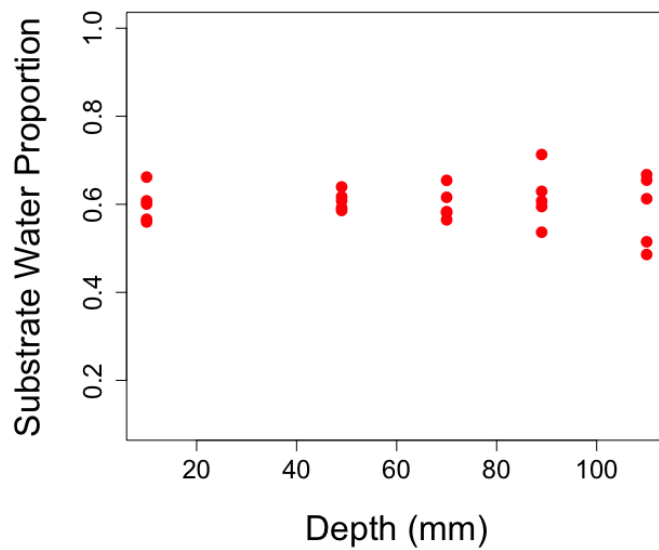


Figure 6. Substrate moisture (proportion of water in substrate) plotted against depth from top of substrate (mm) in the a) Heated and b) Ambient substrate moisture gradients. In both the Heated and Ambient gradients, substrate moisture did not vary with depth.

a



b

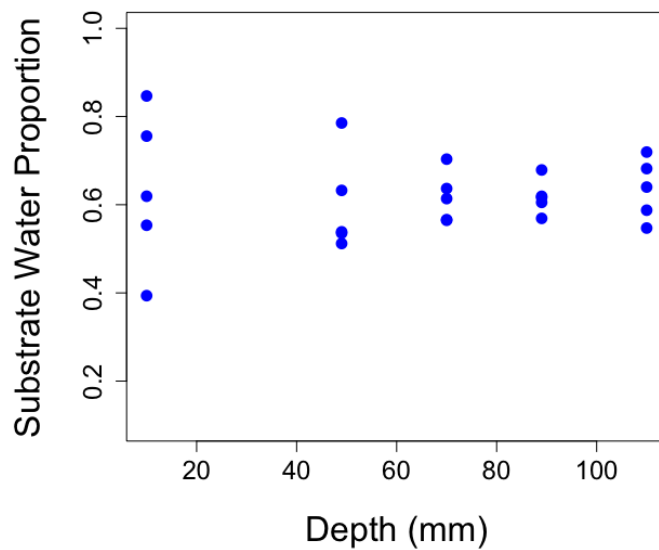
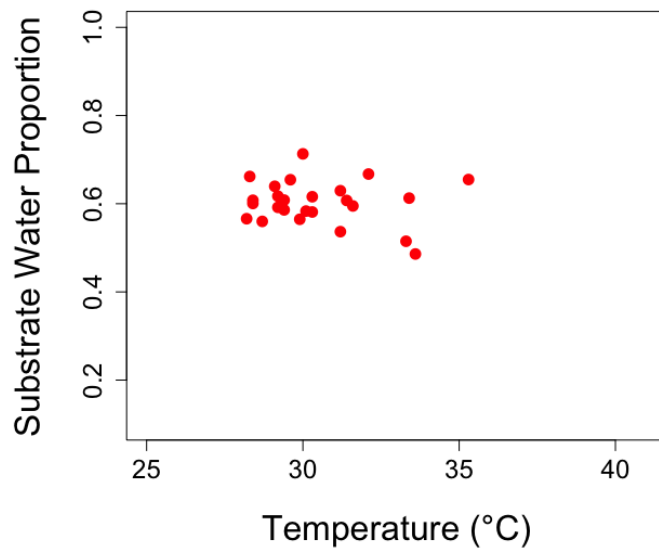
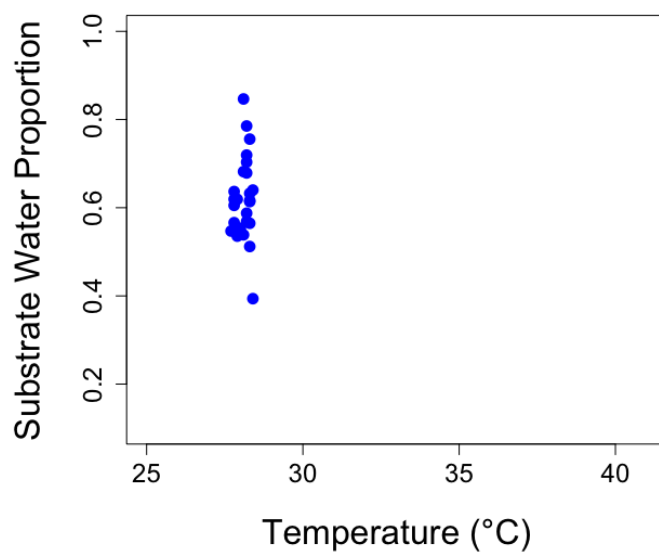


Figure 7. a) Heated and b) Ambient gradient substrate moisture (proportion of water in substrate) plotted against substrate temperature ($^{\circ}\text{C}$) when thermostat was set to 40.6°C and the lining was present (the most common condition across data collection). Moisture in the Heated gradient did not change with temperature, and temperature in the Ambient gradient did not vary enough to establish a relationship between moisture and temperature.

a



b



Statistical Analysis: Nesting Sites

I omitted one cage from the study (containing three females and one male) because no oviposition occurred during acclimation. There was also one female mortality (with an unknown cause of death) during acclimation, so that lizard was also omitted from the study.

To characterize the available conditions in the Heated and Ambient Boxes between which the females could choose for their oviposition sites, I conducted paired t-tests comparing the surface temperatures of the Ambient and Heated nesting boxes, as well as paired t-tests comparing the base temperatures of the Ambient and Heated Nesting Boxes.

I conducted a chi-square test to determine if females exhibited a preference for oviposition in the Heated or Ambient Boxes. I conducted a Levene's Test to test for unequal variances in the Heated and Ambient data, in order to determine whether non-parametric analyses were necessary to accurately describe the relationship between nesting box treatment and nesting site temperature. From the results of the Levene's Test ($F_{1,79} = 4.18$, $p = 0.044$), I chose to perform a Kruskal-Wallis test to examine the association between nesting box treatment and nesting site temperature. For nesting site temperature, I also ran an ANCOVA, examining the association between nesting box treatment (Heated or Ambient) and nesting site temperature ($^{\circ}\text{C}$), and considering substrate moisture, cage number and data collection date as covariates.

For nesting site moisture, I conducted a Levene's Test on the Ambient and Heated nesting site moistures as well, to determine whether non-parametric analyses were necessary to accurately describe the relationship between nesting box treatment and nesting site moisture. The results of the Levene's Test ($F_{1,79} = 2.80$, $p = 0.098$) indicated that the variances of moisture in the two conditions did not differ. I conducted an ANCOVA, examining the relationship between nesting

box treatment and substrate moisture (proportion of water in the substrate surrounding the egg), and considering cage number and data collection date as covariates.

For nesting site depth, I conducted a Levene's Test comparing the depths (mm) in the Heated and Ambient Boxes, to determine whether non-parametric analyses were necessary to accurately describe the relationship between nesting box treatment and nesting site depth. From the results of the Levene's Test ($F_{1,79} = 9.44$, $p = 0.0029$), I chose to conduct a Kruskal-Wallis test to consider the influence of nesting box treatment on nesting site depth. I also conducted ANCOVAs, examining the relationship between nesting box treatment and nesting site depth (mm), and considering cage number and data collection date as covariates.

Results

Across 14 egg checks in a period of 28 days, 32 female lizards in 13 cages laid 81 eggs. The number of nesting sites in each cage ranged from 2-15 (Table 1).

Females were offered a distinct set of nesting site conditions between the Heated and Ambient Boxes. Both surface and base temperatures in the Heated Boxes were warmer than those in the Ambient Boxes (surface temperatures: $t_{181} = -25.83$, $p < 2.2 \times 10^{-16}$; Fig. 8; base temperatures: $t_{181} = -38.20$, $p < 2.2 \times 10^{-16}$; Fig. 9). However, despite these distinct set of temperature conditions offered, females did not exhibit a preference between nesting sites in the two boxes ($\chi^2 = 1$, $p = 0.31$), as there was not a significant difference in the number of eggs laid in the Heated ($n = 36$) and Ambient Boxes ($n = 45$).

There was a difference between not only the set of temperature conditions offered to ovipositing females, but also the temperatures of the nesting sites in the Heated and Ambient nesting boxes. Nesting site temperatures were more variable in the Heated Box compared to the Ambient Box ($F_{1,79} = 4.18$, $p = 0.044$; Fig. 10). Nesting site temperatures in the Heated Boxes were also warmer than those in the Ambient Boxes (Kruskal-Wallis $\chi^2 = 0.97$, $p = 6.06 \times 10^{-13}$; Fig. 10). Both date ($F_{11,55} = 7.68$, $p = 7.80 \times 10^{-8}$) and cage number ($F_{12,55} = 5.01$, $p = 1.51 \times 10^{-5}$) were significant covariates, but moisture was not ($F_{1,55} = 0.16$, $p = 0.69$). Despite these differences in nesting site temperatures, females generally avoided temperatures above 31°C in both nesting boxes (with the exception of one outlier at 33.2°C in the Heated Box), and 80 of the 81 nesting sites in either the Heated or Ambient Box were cooler than 31.5°C (Fig. 10).

There was no difference in variability between the Heated and Ambient nesting site moistures ($F_{1,79} = 2.80$, $p = 0.098$; Fig. 11). Mean nesting site moisture also did not differ between

the Heated and Ambient Boxes ($F_{1,56} = 3.42$, $p = 0.070$; Fig. 11). Date ($F_{11,56} = 13.59$, $p = 3.70 \times 10^{-12}$) was a significant covariate of moisture but cage number was not ($F_{12,56} = 1.67$, $p = 0.098$).

Nesting site depths were more variable in the Heated Box in comparison to the Ambient Box ($F_{1,79} = 9.44$, $p = 0.0029$; Fig. 12). In the Heated Box, 50% of the nesting sites were between 34 and 48 mm, whereas in the Ambient Box 50% of the nesting sites are between 28 and 61 mm (Fig. 12). There was no difference in nesting site depths between the Heated and Ambient Boxes (Kruskal-Wallis $\chi^2 = 0.97$, $p = 0.33$; Fig. 12). Nesting site moisture ($F_{1,55} = 0.23$, $p = 0.64$), and date ($F_{11,55} = 0.51$, $p = 0.89$) were not significant covariates of depth. However, cage number ($F_{12,55} = 6.20$, $p = 9.56 \times 10^{-7}$) was a significant covariate of depth.

Table 1. Number of eggs laid in the Heated (red) and Ambient (blue) Boxes across days and cages.

		Cage Number												
Day of Data Collection		1	2	3	4	5	6	7	8	9	10	11	12	13
	1	0 1	0 0	0 0	1 0	0 0	0 0	0 0	1 0	0 0	0 1	0 0	0 0	2 0
	2	0 0	0 0	0 0	0 0	0 0	0 0	1 0	0 1	0 0	0 0	0 0	0 0	1 0
	3	1 0	0 0	0 0	0 0	0 0	0 1	0 0	0 0	1 2	0 0	0 0	1 0	1 0
	4	0 0	0 1	0 0	0 0	0 0	1 0	1 1	0 0	0 0	0 1	1 0	0 1	1 0
	5	1 2	0 0	0 1	0 0	0 2	0 1	0 0	0 0	0 2	0 1	0 0	0 0	0 1
	6	0 0	0 1	0 0	0 1	0 0	0 0	0 0	0 0	0 1	0 1	0 0	0 0	0 0
	7	0 0	0 1	1 1	1 0	1 0	1 1	0 0	0 0	0 1	0 1	0 0	0 1	2 1
	8	0 0	0 0	0 0	0 0	0 0	0 0	0 2	0 0	0 0	0 1	0 0	0 0	0 1
	9	1 0	1 0	0 1	0 0	0 0	1 0	0 1	0 0	0 0	1 0	1 0	1 0	2 0
	10	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	1 0	0 1	0 0	0 0	0 1
	11	0 0	0 0	1 0	0 0	0 0	0 0	0 0	0 0	0 0	0 1	0 0	0 1	0 0
	12	1 0	1 0	0 0	0 0	0 1	1 1	0 0	0 0	0 1	0 1	0 0	0 0	2 0

Figure 8. Surface layer temperatures (median \pm 95% CI) in the Heated Boxes (n = 182) were warmer than those in the Ambient Boxes (n = 182).

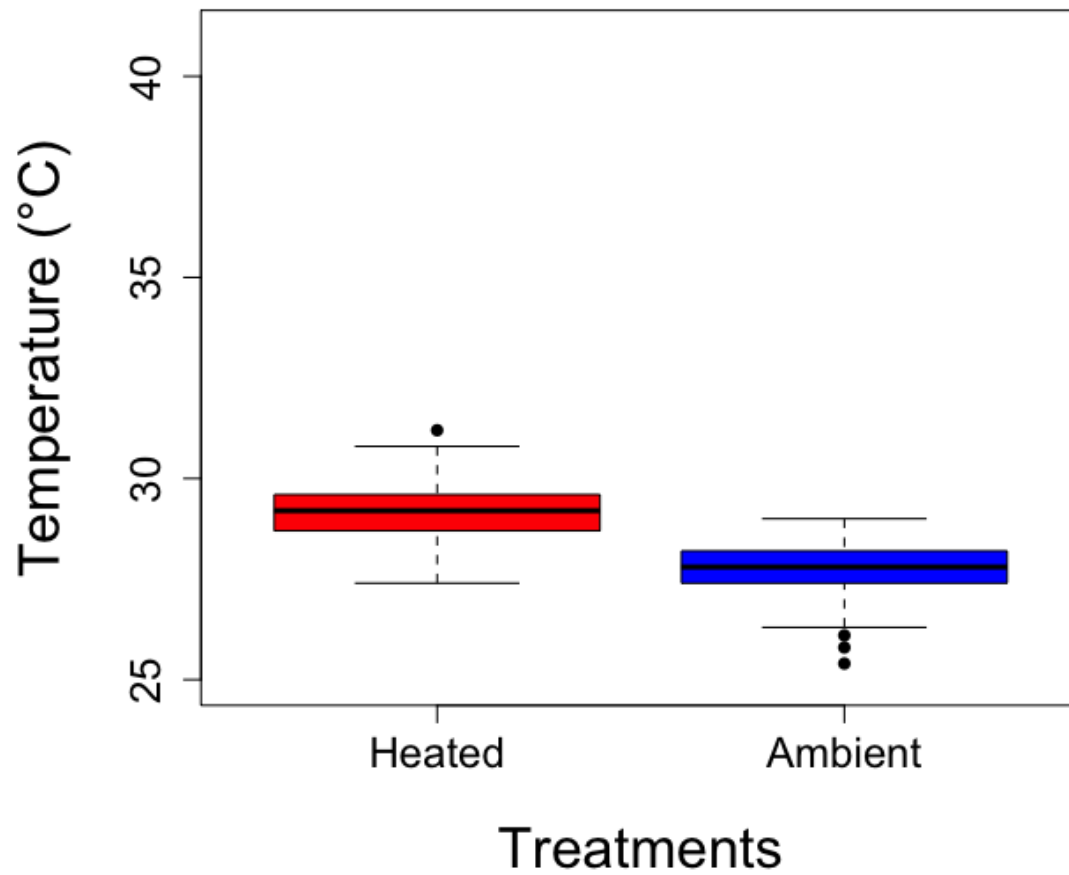


Figure 9. Base layer temperatures (median \pm 95% CI) in the Heated Boxes (n = 182) were warmer than those in the Ambient Boxes (n = 182).

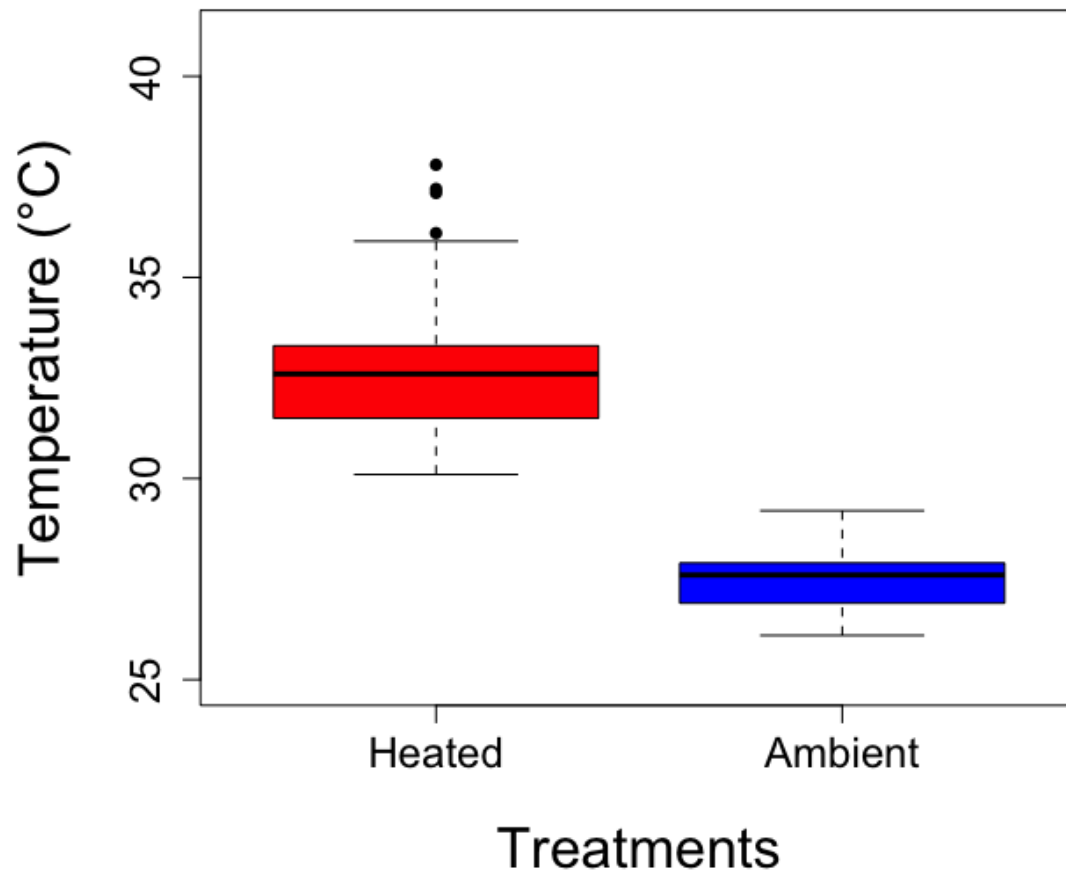


Figure 10. Nesting sites (median \pm 95% CI) in the Heated Box (n = 36) were warmer than those in the Ambient Box (n = 45).

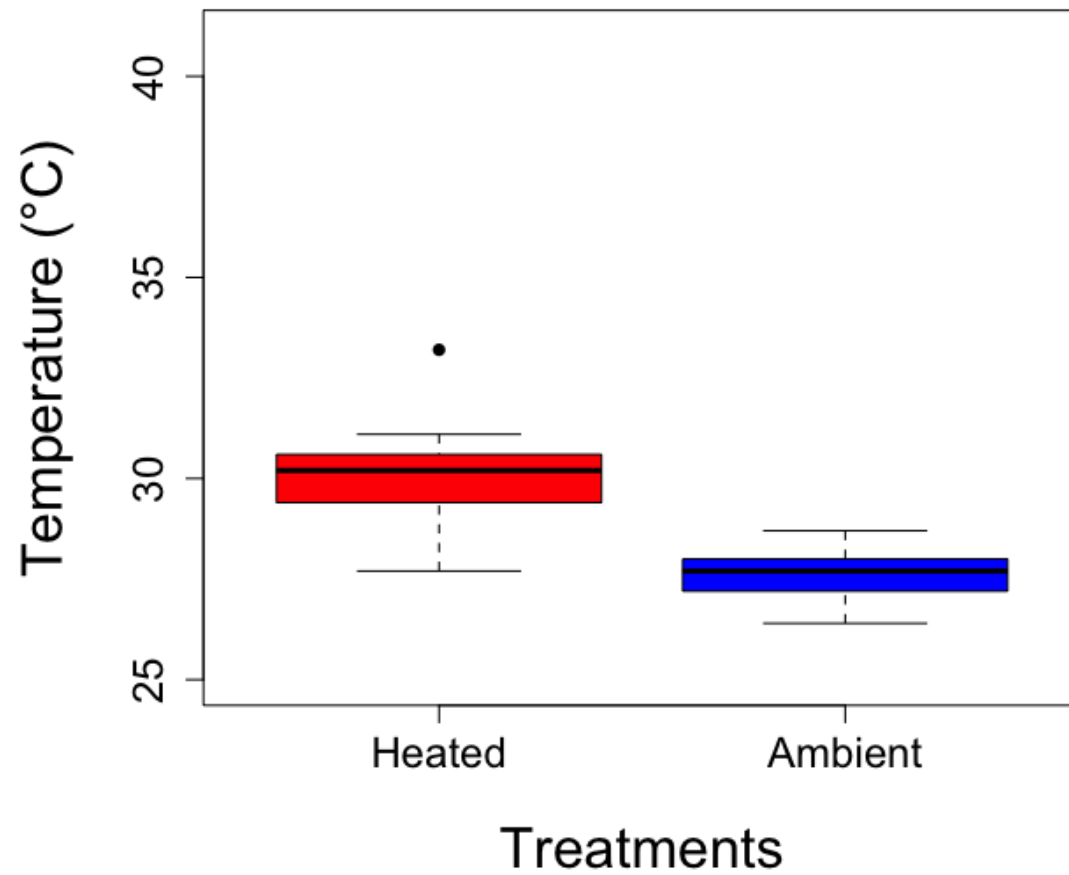


Figure 11. Substrate moistures (median \pm 95% CI) did not differ between nesting sites in the Heated (n = 36) and Ambient (n = 45) Boxes.

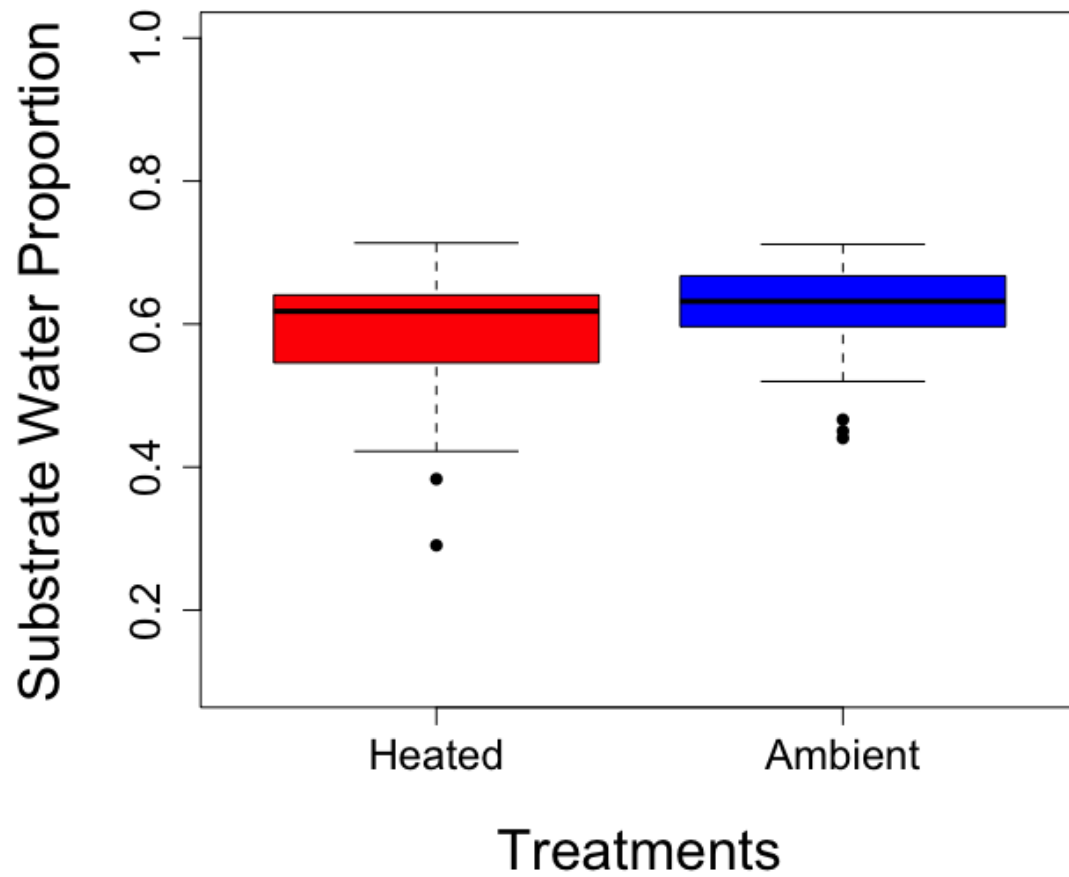
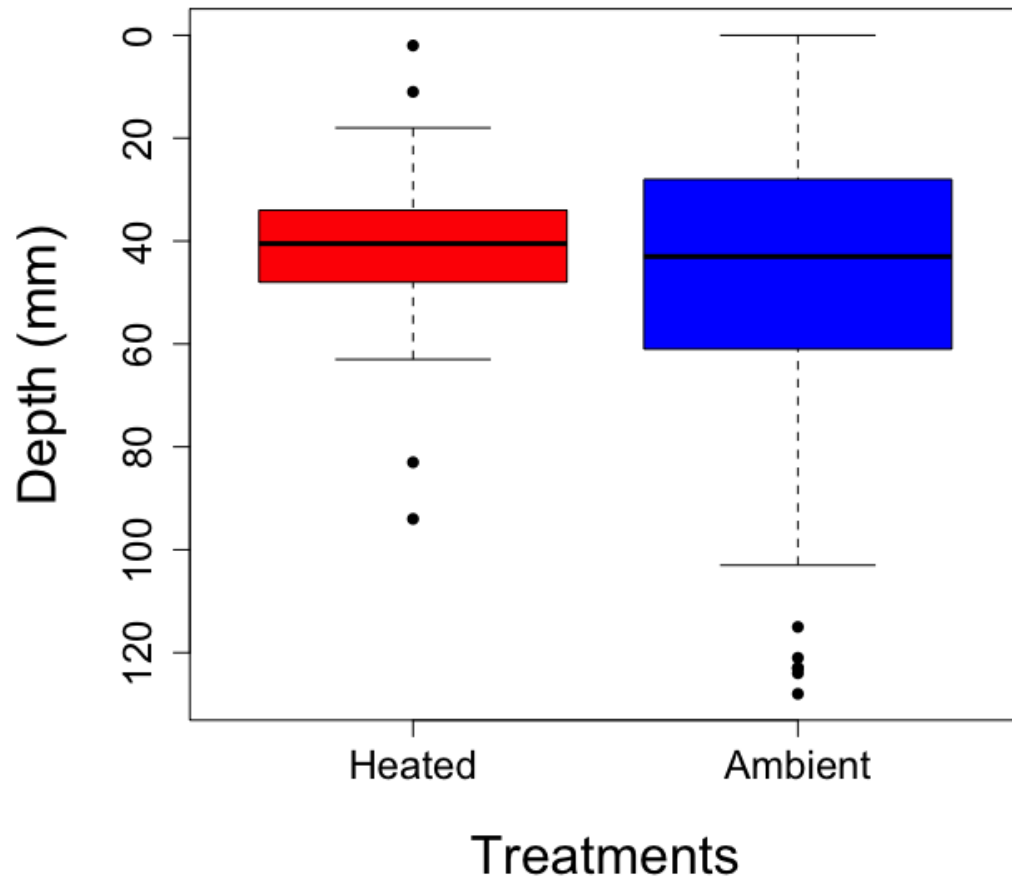


Figure 12. Nesting site depths (median \pm 95% CI) in Heated (n = 36) and Ambient (n = 45) Boxes did not differ, but depths in the Ambient Box were more variable than those in the Heated Box.



Discussion

My study sought to examine the extent to which temperature impacts female nesting site choice in brown anole lizards. Females were offered a distinct set of temperature conditions between the Heated and Ambient Boxes within a cage, and the range of these available temperatures are similar to those that the wild-caught brown anole lizards and other oviparous ectotherms would experience in the field. While nesting site temperatures fluctuate with time of day and year (Huang & Pike, 2011; Hall & Warner, 2018), Puerto-Rican crested anoles in Florida chose field nesting sites ranging from 25-26°C in a forest habitat, and 26-29°C in an urban habitat between May and September (Hall & Warner, 2018). In New Jersey, for about eight hours of the day, nest temperatures averaged greater than 28°C in the eastern fence lizard (*Sceloporus undulatus*) between June and August (Angilletta, Sears & Pringle, 2009). In Taiwan, over the course of the day, nesting site temperatures of the long-tailed sun skink averaged from approximately 27-30°C in natural habitats, and approximately 28-31°C in artificial habitats between May and August (Huang & Pike, 2011). Both the range of available nesting site temperatures and the chosen nesting site temperatures in the present study mirror those of oviparous ectotherms in the field.

Despite this clear difference in available nesting site conditions, females did not exhibit a preference for the Heated or Ambient Box. A possible explanation for this result is that females have a somewhat wide range of acceptable nesting site temperatures, and both the Heated and Ambient Boxes spanned that range, at different depths. Brown anoles can successfully develop at temperatures ranging between 26-30°C (Warner et al., 2012), and survive temperatures ranging 27-33°C (Sanger et al., 2018). Of the 81 nesting sites in my experiment, 80 were between 26 and

31.5°C, with one nesting site at 33.2°C. Therefore, the nesting sites in both the Heated and Ambient Box are consistent with temperatures under which brown anole embryos can successfully develop.

Nesting sites in the Heated Box were warmer than those in the Ambient Box. When offered warm conditions, females often chose to oviposit at these warmer temperatures. A possible explanation for this result is that some warmth is beneficial for development. Warner et al. (2012) found that increasing incubation temperature from 27°C to 30°C can speed up the rate of development in brown anole lizards, and Huang & Pike (2011) found that at temperatures lower than 32°C, hatching success rates were high in the long-tailed sun skink.

While females did select warm nesting sites in the Heated Box, females avoided laying eggs at temperatures above 31.5°C, and no nesting site was ever warmer than 33.2°C. The literature is consistent with these results, as fitness consequences arise at 32°C in the southern alligator lizard (Telemeco, 2014), at 36°C in brown anole lizards (Sanger et al., 2018), and above 34°C in the painted turtle (Telemeco et al., 2013). Kamel & Mrosovsky (2004) suggested that female nesting site choice in reptiles is under strong selection. My findings are consistent with the literature; females avoided nesting sites that would provide dangerous conditions for development. The results of my study also support the hypothesis that female nesting site choice is under selection.

One of the challenges of this study was that it was difficult to accurately measure the base layer temperatures of the Heated Boxes. The heating mats exhibited variability between days and cages, even when they were controlled by thermostats all set at the same temperature. Further, base layer temperatures were not uniform within the Heated Boxes. A temperature taken at the edge of the base layer could be several °C cooler or warmer than a temperature taken at the middle of the base layer of the same box. A future study with a more precise way to measure base layer temperatures would be useful. It is also worth noting that both date of data collection and cage

number were significant covariates. This means that conditions available, or nesting site temperature preferences may have changed depending on the day, and depending on the group of lizards involved. This indicates that personality, instinct or experience may play a role in choosing a nesting site temperature on a given day.

Females were not offered a distinct set of moisture conditions between the Heated and Ambient Boxes, and there was no difference in nesting site moistures between the Heated and Ambient Boxes, as distinguishing between substrate moisture was not the primary goal of this study. Date of data collection was a significant covariate of moisture, which indicates that depending on the nesting day, we may have used slightly drier or slightly wetter substrate in the nesting boxes. However, this same substrate would have been placed in both the Heated and Ambient Boxes of all cages. Therefore, in this experiment, substrate moisture did not drive female nesting site choice. Nesting site moisture can impact development, as nesting sites that are too dry can desiccate anole eggs and kill the embryos, and nesting sites that are too moist can damage the embryos as a result of high osmotic pressure on the egg (Sanger et al., 2008). Further, nesting sites with optimal moisture conditions (75% moisture) can yield high fitness offspring, and as a result, substrate moisture conditions can influence female nesting site choice (Reedy, Zaragoza & Warner, 2012).

Between the Heated and Ambient Boxes, females were offered the same depth conditions. Average nesting site depths also did not differ between the Heated and Ambient Box. In the field, lizards may choose deeper nesting sites to avoid egg predators (Doody et al., 2015) or dry conditions near the surface (Angilletta, Sears & Pringle, 2009). However, in this study, moisture was uniform with depth, so that factor would not have influenced nesting site choice, and an instinct to avoid egg predation should not change between the Heated and Ambient Boxes. While

there was no difference between the means of the Heated and Ambient Boxes, the nesting sites in the Ambient Box were more variable than those in the Heated Box. A possible explanation for this result is that females laying eggs in the Heated Box were constrained by the available range of acceptable temperatures. At certain depths, the available temperatures would be dangerous for offspring development, and it is clear that the females avoided these nesting sites. In the Ambient Box, temperature was consistent with depth, so females were not constrained by the available range of acceptable temperature conditions. Females could lay their eggs at any depth, and still choose a nesting site beneficial for offspring development.

The experimental design of this study does create limitations for interpreting nesting site depth choices. In this study, the warmest temperatures in the Heated Box were near the base of the box, and the coolest temperatures were near the surface. In the Ambient Box, temperature did not change with depth. Doody et al. (2015) found that in Western Australia, during the hottest part of the year, substrate temperatures are warmest and most variable near the surface. It is likely that the conditions that these wild-caught females would be accustomed to in the field are more similar to those reported in Doody et al. (2015), as the substrate surface would be warmed by the sun. During this study, females ovipositing in the Heated Box experienced the opposite of what they would experience in the field. Females in the field would also be unlikely to encounter a range of nesting site conditions completely uniform in temperature, like the Ambient Box. Given that cage was a significant covariate, and mean depth did not differ between the Heated and Ambient Boxes, it is entirely possible that the female nesting depth choices in this experiment reflect differences in personality between the females in different cages, and females may be influenced more by experience or instinct than by nesting site quality in this case.

The experimental design also limited interpretations of nesting site choice in that multiple females were housed in each cage. We were unable to determine which females laid which eggs in each cage, or how many eggs each female laid, and this could have given us useful information regarding the repeatability of female nesting site behaviors. In green anole lizards, Propper et al. (1991) observed that females sometimes began the nesting site sequence, but did not complete it, indicating that the females determined the nesting site to be unsuitable. My experimental design did not allow me the opportunity to observe rejected nesting sites, and measuring those conditions could have been informative.

After burying eggs, green anole lizards abandon eggs, and nest guarding is not observed (Propper et al., 1991). To the best of my knowledge, brown anoles also do not engage in nest guarding behaviors. Anole lizards also lay single-egg clutches (Cox & Calsbeek, 2010). However, nesting behaviors can be impacted by social interactions with other females, and we were unable to account for that. In *Anolis* species, females may demonstrate territoriality and defend resources from females of a similar size (Edwards & Lailvaux, 2013). In green anole lizards, limited resources can lead to dominant-subordinate relations in cohabitating, captive females (Andrews & Summers, 1996). Andrews & Summers (1996) found that in cohabitating females, one of each pair displaced its cagemate, and female social status may indicate the importance of access to a mate. In addition, ovarian and oviductal growth can be inhibited in small females housed in multi-female groups (Summers, Suedkamp & Grant, 1995). In my study, 2-3 females were housed with one male, so it is possible that these dominant-subordinate relationships may have formed and restricted access to resources such as the nesting boxes for the subordinate female. In the future, a study housing only one female in each cage may prevent social interactions from influencing nesting site outcomes.

In my study, eggs were collected a few days after oviposition and then frozen, which meant that I was unable to interpret the long-term impact of nesting site choice on post-hatching fitness. Mitchell, Janzen & Warner (2018) call for more longitudinal studies quantifying the effects of embryonic environment on adult phenotype and fitness. Nesting site temperature can have far-reaching ontological effects, as in reptiles, thermal extremes experienced during development can lead to skeletal abnormalities that persist into adulthood and impact performance (Mitchell, Janzen & Warner, 2018). A future study could collect the eggs laid in Heated and Ambient Boxes, and then examine the impacts of these temperatures on juvenile phenotype and adult fitness.

While females may know which nesting sites to choose, and which nesting sites to avoid, this trait will not benefit them if the necessary conditions for optimal development are no longer present. As climate change continues to progress, large scale environmental changes will result in habitat loss for lizards and other reptiles (Gibbons et al., 2000). Conditions that were once available may not be available in the coming years. On a smaller scale, even if habitats are not lost entirely, optimal nesting site conditions may no longer exist in a species' current range. Huang & Pike (2011) found that nesting site temperatures had increased over the course of several years, but the female long-tailed sun skinks continued to choose those nesting sites. In the coming years, climate change could have serious implications for the biodiversity of oviparous ectotherm species within their current ranges.

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