ORIGINAL ARTICLE

Natural nest substrates influence squamate embryo physiology but have little effect on hatchling phenotypes

Joshua M. HALL, 🆔 Jocelyn MIRACLE, 🆔 Cindy D. SCRUGGS 🆔 and Daniel A. WARNER 🆔

Department of Biological Sciences, Auburn University, Auburn, Alabama, USA

Abstract

Vertebrate embryos require access to water; however, many species nest in terrestrial habitats that vary considerably in moisture content. Oviparous, non-avian reptiles have served as models to understand how environmental factors, like moisture availability, influence development because eggs are often exposed to prevailing environments in the absence of parental care. Though much research demonstrates the importance of water absorption by eggs, many ecological factors that influence moisture availability in natural nests have received little attention. For example, the type of substrate in which nests are constructed is understudied. We experimentally incubated eggs of the brown anole lizard (*Anolis sagrei*) in 2 naturally occurring nest substrates that were treated with varying amounts of water to determine how natural substrates influence development at different moisture concentrations. One substrate consisted of sand and crushed seashells and the other was mostly organic material (i.e. decayed plant material). Both are common nesting substrates at our field site. When controlling for water uptake by eggs, we found that egg survival and hatchling phenotypes were similar between substrates; however, embryos developed more quickly in the sand/shell substrate than the organic substrate, indicating substrate-specific effects on embryo physiology. These results demonstrate that different natural substrates can result in similar developmental outcomes if the water available to eggs is comparable; however, some aspects of development, like developmental rate, are affected by the type of substrate, independent of water availability. Further study is required to determine how natural substrates influence embryo physiology independent of water content.

Key words: developmental plasticity, egg incubation, hydric conditions, nesting behavior, nest-site selection

INTRODUCTION

Vertebrate evolution is characterized by many important events, not least of which is the transition from water to land. One of the requirements of this transition to terrestrial habitat was an increase in desiccation tolerance.

Indeed, many terrestrial vertebrates require a life in proximity to water, and this may be due to the moisture needs of developing embryos in addition to the needs of later life stages (Heatwole 1961; Martin 1999; Mitchell 2002). Terrestrial environments are characterized by a diversity of microhabitats that vary in shade cover, substrate composition, moisture content, and temperature among other factors. Each of these can influence water absorption into the egg or result in desiccation (Ackermann & Lott 2004). Therefore, a major challenge faced by the first terrestrial vertebrates, and still facing many oviparous vertebrates

Correspondence: Joshua M. Hall, Department of Biological Sciences, Auburn University, 101 Rouse Life Sciences Building Auburn, Auburn, Alabama 36849, USA.
Email: halldevoeco@gmail.com

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today, is locating nest sites that provide adequate moisture (Martin & Carter 2013).

The evolution of the amniotic egg has played an important role in vertebrate evolution by enhancing successful embryo development in relatively dry, terrestrial environments (Gray 1928; Packard & Packard 1980). Regardless, water uptake from the environment is still important for eggs of many amniotes. For example, many non-avian reptiles (henceforth "reptiles") have pliable or parchment-shelled eggs which absorb water from the nest environment, and such absorption has important effects on embryo physiology, survival, and hatchling phenotypes (Packard *et al.* 1977, 1992; Miller 1993; Warner *et al.* 2011; Bodensteiner *et al.* 2015; Brusch & DeNardo 2019). Water is absorbed both passively and actively by eggs (Packard *et al.* 1977; Warner *et al.* 2011); thus, nesting reptiles must select oviposition sites with sufficient moisture to support development. This is particularly important because eggs of many species must extract moisture from the surrounding environment to survive (Cunningham & Hurwitz 1936; Packard *et al.* 1977). As a result, reptiles have evolved various strategies to control moisture availability during development via nesting behavior or other means (e.g. egg brooding; Lourdais *et al.* 2007).

The first reports of reptile eggs absorbing water were made at the turn of the 20th century (e.g. Brimley 1903); however, until the late 1970s, most observations were made in laboratory incubation experiments, while field observations were comparatively rare (Packard *et al.* 1977). Subsequent field studies demonstrated how substrate moisture influences development in the wild (e.g. Bodensteiner *et al.* 2015), revealing that a diversity of nest microhabitat variables influence water uptake by eggs (e.g. temperature, shade cover, relative humidity). The effects of many such ecological variables, however, are still not well understood. For example, the type of substrate in which eggs incubate has received relatively little attention (Mitchell & Janzen 2019). Several studies have examined the influence of substrate composition on nest-site choice and/or egg development and survival by quantifying substrate composition of maternally selected nests in the field (e.g. Mortimer 1990; Trivalairat *et al.* 2016; Tornabene *et al.* 2018; Velázquez-Rodríguez *et al.* 2019; Erickson *et al.* 2020). Such observational field studies, however, cannot pinpoint substrate-specific effects, *per se*, due to the confounding abiotic and biotic conditions that characterize natural nests. For this, experimental manipulations are necessary. Although several studies compare the effects of natural and artificial (e.g. vermiculite) substrates on development (e.g. Packard

et al. 1987; Dmi'el *et al.* 1993; Pilcher 1999; Saidapur *et al.* 2002; Makowski *et al.* 2008), very few have manipulated natural substrates (turtles—Milton *et al.* 1997; Rusli & Booth 2018; Mitchell & Janzen 2019; Stewart *et al.* 2020; tuatara—Refsnider *et al.* 2010).

Although such studies are relatively uncommon, the few that have examined these effects vary considerably in the results they observe. For example, Milton *et al.* (1997) tested the effects of 2 types of naturally occurring beach sand on sea turtle (*Caretta caretta*) development and found no substrate-specific moisture effects but did find temperature differences that potentially impact embryo development. Makowski *et al.* (2008), conversely, found no differences between turtle eggs incubated in natural versus artificial (i.e. cullet) sand. Mitchell and Janzen (2019) incubated eggs of *Chrysemys picta*, a turtle with temperature-dependent sex determination, in artificial nests constructed in loam, sand, and gravel. They found that hatchling sex ratios differed across nests due to substrate-specific hydric and thermal environments even though nests in each substrate experienced the same prevailing weather conditions.

To our knowledge, no experimental manipulation of natural substrates has been conducted with squamate reptiles (i.e. snakes and lizards). Indeed, descriptions of squamate nest environments and their effects on development are rare compared to other reptiles (Ackerman & Lott 2004) probably due to the relative difficulty in locating squamate nests. A few studies have incubated squamate eggs on various substrates; however, the goals of these studies were physiological, rather than ecological, and substrates did not reflect those of natural nests (e.g. sand, vermiculite, cotton wool; Dmi'el *et al.* 1993; Saidapur *et al.* 2002). To our knowledge, only one study has manipulated natural squamate nest substrates in the field (DeSana *et al.* 2020); however, this study focused on egg depredation and did not measure substrate-specific effects on development.

To understand how natural nest substrates influence squamate development, we incubated eggs of the brown anole lizard (*Anolis sagrei* Duméril & Bibron, 1837) in 2 substrates taken from the field. At our study site, these lizards are densely populated on small spoil islands in the Intracoastal Waterway of Florida, USA. Island substrates primarily consist of broken seashells and sand; however, due to years of plant growth and leaf drop, some regions of the islands have dark, organic soil. Because eggs are found in both types of substrate (Pruett *et al.* 2020), we designed an experiment to examine the consequences of these substrates on embryo developmental rate, egg survival, and hatching morphology and performance since
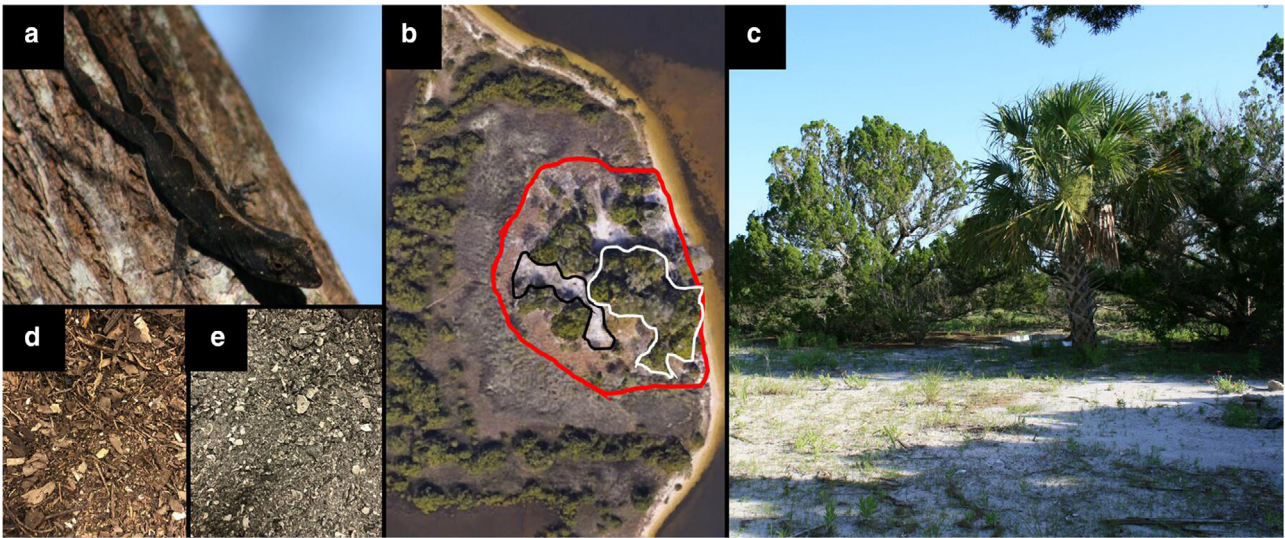
Figure 1 Representative photos of (a) a female brown anole (*Anolis sagrei*), (b) aerial view of the substrate collection island, (c) ground view of substrate collection island, (d) organic substrate, and (e) sand/shell substrate. In panel (b), the area inside the red circle is the portion of the island that is most densely populated with lizards. The area within the black line is an example of open canopy habitat where substrate is primarily sand and crushed shell. The area inside the white line is an example of closed canopy habitat with dark, organic substrate. Panel (c) shows the ground view of the same open and closed canopy sites outlined in panel (b).

these factors are influenced by incubation environments (Reedy *et al*. 2013; Pearson & Warner 2016; Pruett *et al*. 2020) and may correlate with fitness. Few studies relate natural substrates to developmental trajectories, and these have mixed results (see above); therefore, we abstain from making a priori hypotheses and predictions. Rather, we present 2 goals: (1) to quantify the effects of substrate with variable levels of hydration, and (2) to determine if the 2 substrates can have equivalent developmental effects depending upon the level of substrate hydration. We collected each substrate, treated them with various volumes of water, and incubated eggs in each substrate by moisture combination. We carefully monitored water uptake by eggs and report resulting effects on egg physiology and survival, as well as hatchling morphology and performance. Because maternally chosen nest environments influence offspring development and survival in diverse animal taxa (Penn & Brockmann 1994; Machado & Oliveira 2002; Visscher 2007; Mainwaring *et al*. 2017), our study provides insight into the importance of this behavior in habitats with substrate heterogeneity. Additionally, our study is a novel assessment of the effects of natural nest substrates on egg survival and hatchling phenotypes and advances our understanding of embryo development in the wild.

MATERIALS AND METHODS

Study species and site

The brown anole (*A. sagrei*; Fig. 1a) is a relatively small (≈2–5 g), subtropical lizard that is native to the islands of the West Indies; however, non-native populations are established across the world (Hulbert *et al*. 2020). Females lay a single egg clutch every 4–14 days across an extended reproductive season (Hall *et al*. 2020). Eggs are deposited in shallow nests (<5 cm) in loose soil or on the soil surface beneath cover objects (e.g. rocks, palm fronds, logs) (Delaney *et al*. 2013; Tiatragul *et al*. 2019; Pruett *et al*. 2020). Eggs have a parchment-like shell and must draw water from the surrounding substrate during incubation (Warner *et al*. 2011). Thus, because substrate moisture varies across habitats (e.g. open vs closed canopy) and soil types (Pruett *et al*. 2020), nest-site choice is an important determinant of egg survival and hatchling phenotypes (Reedy *et al*. 2013).

Our study population, which is outside the native range, inhabits several spoil islands located within the Guana Tolomato Matanzas National Estuarine Research Reserve near Palm Coast, Florida, USA. Though habitat structure varies across islands, each island typically

consists of a central area of dense tree canopy (mostly cedar and palm trees) with open canopy areas interspersed between trees and around the periphery (Fig. 1b,c). Due to regular tidal flooding, most lizards occupy the central portion of each island (Fig. 1b, inside the red line).

Experimental design

We collected substrate from open and closed canopy habitats on a spoil island in the Matanzas River (29.6233842, −81.2108003; WGS84) (Fig. 1b,c). Closed canopy habitats consist of dark, organic soil (henceforth "organic substrate"; Fig. 1d), while open canopy habitats primarily consist of sand and broken seashells (henceforth "sand/shell"; Fig. 1e). Females nest in each substrate (Pruett *et al*. 2020). Although some locations on the island consist of a mixture of substrates, we collected samples of the most extreme substrate types (i.e. ≈100% organic substrate and 100% sand/shell) and desiccated samples to constant weight by spreading each substrate in a thin layer across a lab bench and blowing air over the surface with a fan. We did not use heat for drying (e.g. drying oven) because it might alter important abiotic (i.e. water retention capacity) or biotic (i.e. microbial communities) aspects of the soil. Once dry, we created 4 moisture treatments for each substrate. Preliminary trials of mixing the substrates with water revealed that water retention capacity differs remarkably between substrates. During these trials, we mixed substrates with water and qualitatively assessed moisture content by touch and sight with the purpose of determining water concentrations for each substrate that had similar moisture availability to eggs. This resulted in concentrations of 1%, 5%, 10%, and 15% water content for the sand/shell substrate and 10%, 30%, 50%, and 70% water content for the organic substrate. These are percent water content by mass (i.e. water mass divided by total mass × 100). These moisture levels encompass the full range of soil moisture contents observed at our field site (1–60%; Pruett *et al*. 2020). We determined the water potential of each substrate using a Wescor Water Potential System (Psypro; Wescor, Inc.) and a Wescor C-52 sample chamber psychrometer according to the manufacturer's instructions.

We collected lizards (*n* = 60 females; *n* = 12 males) from a different island approximately 16.3 km north of where substrates were collected (29.763429, −81.257317). In 2011 and 2015, the source islands for the substrates and lizards, respectively, were cleared of the existing lizard population (as best as possible) and reseeded with lizards from the same source population. Therefore, our 2 experimental islands consist of lizards with similar genetic origin and likely respond to incubation environments in similar ways. Regardless, using lizards from a separate island isolated the effects of substrates, *per se*, from potential population-specific effects. Lizards were housed indoors in ReptiBreeze screen cages (*n* = 12; 46 × 46 × 91 cm) in a 5:1 female:male ratio. Cages were illuminated with a 14:10 h light:dark cycle provided by Reptisun 5.0 UVB bulbs (Zoo Med Inc.) and plant grow bulbs (model F40; General Electric Co.). Each cage had one vertical shaft of bamboo nearly equal to the height of the cage with 3–4 perches (tongue depressors) projecting horizontally at different heights. This was wrapped with artificial plants to provide additional habitat structure. Reptile cage carpet (Zoo Med Inc.) was the floor substrate and a plastic container filled with moistened potting soil was provided for egg-laying. We provided each cage with 18–20 crickets twice weekly (dusted with vitamins and calcium) and misted them daily with water. Ambient temperatures fluctuated between 25°C and 28°C; however, light sources provided warmer basking areas, but basking temperatures were not recorded.

We checked nest pots for eggs (*n* = 350 total eggs) twice weekly (each Monday and Thursday) and randomly allocated them among 8 treatments (2 substrates, each with 4 moisture concentrations). To incubate eggs, we filled 60-mm petri dishes approximately half-full with a moistened substrate, made a depression in the substrate with our finger, and placed the egg in the depression. Eggs are often found beneath cover objects (e.g. rocks, leaves, palm fronds) in the field, but are atop the substrate (i.e. not buried) (Pruett *et al*. 2020). Additionally, eggs generally absorb water vapor released from the substrate rather than directly from the substrate (Ackerman *et al*. 1985). Petri dishes were sealed with parafilm to prevent moisture loss and incubated at a repeating sine wave with mean of 26.3 and amplitude of 2.4°C, which mimics nest temperatures in the field (Hall & Warner 2021). Twice during incubation, we removed the parafilm so we could re-measure egg mass (see "Measuring water uptake" below). This ensured that oxygen levels were normoxic throughout development. Moreover, on day 14, we placed eggs into petri dishes with freshly mixed substrate, which replaced any moisture that may have been lost through the parafilm. All eggs were incubated in a single incubator (Memmert IPP55 Plus).

Measuring water uptake

To quantify water uptake across development, we measured the mass of each egg at the time of collection (i.e. oviposition) and on day 14 and day 28 of incubation.

On day 14, eggs were removed from their petri dishes, reweighed, provided with fresh substrate, and returned to the incubator. On day 28, eggs were again removed and reweighed; however, each egg was then placed in a petri-dish half-filled with vermiculite with a water potential of −150 kPa (i.e. our standard incubation medium) to control the hatching environment across treatments. This was done to ensure that substrate-specific effects were due to the developmental environment and not the hatching environment. For example, reptiles often ingest substrate after hatching, which could influence whole body mass or water content as well as other factors like the gut microbiome (see Terebiznik et al. 2020). Hatchling performance, growth, and survival could be likewise affected. Additionally, anoles, like many reptiles, exhibit environmentally-cued hatching which could occur while checking the incubators for hatchlings if eggs are jostled and rub against a coarse substrate (i.e. a "physical disturbance"; see Doody 2011). The probability of early hatching may be substrate-specific. We avoided these confounding factors by placing all eggs in a standardized substrate prior to hatching. Mean incubation period was 35.6 days (range = 30–43 days); thus, day 14 and day 28 represent approximately 40% and 80% development completed, respectively. Most water absorption occurs prior to 80% of development being completed (Warner & Chapman 2011). We checked the incubator daily for hatchlings.

Measuring egg and hatchling variables

To assess treatment effects on egg physiology, we calculated a developmental rate for each egg that successfully hatched ($n = 245$) by taking the reciprocal of the incubation period (number of days from oviposition to hatching) and multiplying by 100 (i.e. percent development per day; Taylor et al. 2020). To assess treatment effects on hatchling morphology, for all hatchlings ($n = 245$), we measured hatchling body mass (to 0.0001 g) with an electronic balance and snout-to-vent length (SVL; to 0.01 mm) with a digital caliper. To assess treatment effects on hatchling performance, most hatchlings ($n = 176$) were run on a circular racetrack to measure endurance (described in the next paragraph). The remaining hatchlings ($n = 75$) were euthanized by decapitation immediately after hatching to assess water mass and dry body mass. Dry mass was the final carcass mass after drying in the oven. To calculate water mass, we subtracted the carcass mass after drying from the carcass mass before drying in the oven. Notably, no eggs survived in the driest treatment for each substrate; thus, for each substrate by moisture combination, we had approximately 40, 40, 30,

and 12 individuals to assess treatment effects on egg physiology, hatchling morphology, hatchling endurance, and wet/dry hatchling body mass, respectively. See Table 1 for total sample sizes per treatment and Table S1, Supporting Information, for sample sizes available for each analysis.

Our racetrack consisted of 2 circles of aluminum flashing set atop a wooden panel. The outer and inner pieces of flashing were 1.35 and 1.06 m in circumference, respectively. We heated the racetrack from underneath to maintain preferred body temperatures of lizards. A thermocouple recorded the surface temperature during each trial (range = 31°C to 36.4°C; mean = 33.8°C). After hatching, each lizard was placed in a cage (13 × 21 × 17 cm) that included reptile carpet as a substrate and several plastic leaves as cover. Temperature and light cycles were as previously described. Hatchlings were raced within 3 days of hatching and were provided with water but no food. For racing, lizards were placed individually in 50-mL centrifuge tubes wrapped with duct tape to prevent them from seeing out (to reduce stress). Tubes were set on the racetrack for 45 min so lizard body temperature could equilibrate with the racetrack. Lizards were quickly placed on the racetrack, one at a time, and chased in a circle using a paintbrush until exhaustion, which was determined when the experimenter tapped the lizard on the base of the tail 5 times without it moving. We calculated the total distance each lizard ran by multiplying the number of laps each lizard ran by the circumference of the center of the racetrack (1.21 m). Each hatchling was raced once. Some hatchlings, however, refused to run or escaped prior to being placed on the track; they were given 5–10 min to rest and were raced again.

Statistical analyses

We used mixed-effects models to analyze treatment effects on 3 types of variables: (1) egg water uptake, (2) egg physiology (i.e. developmental rate) and survival, and (3) hatchling morphology (SVL, body mass, dry and wet carcass mass) and performance (i.e. endurance).

To consider how treatments influenced egg water uptake early and late in development, we analyzed egg mass separately for day 14 and day 28. Due to substrate-specific water retention properties, percent substrate moisture was not comparable between substrates. For example, the wettest sand/shell and organic substrate treatments consisted of 15% and 70% water, respectively. Therefore, to estimate treatment effects on egg water uptake, we considered each substrate by moisture concentration to be a categorical variable (i.e. 8 treatments). We performed 2 general linear mixed effects models: one with egg mass

Table 1 Water content, water potential, water uptake, egg survival, and sample size for each treatment

Substrate	Moisture (%)	Water Potential (kPa)	Water uptake (mg/day)	Egg survival (%)	Sample size
Organic	10	−7120	—	0	35
Organic	30	−100	7.64 (0.44)	89.1	46
Organic	50	−40	9.41 (0.40)	91.5	47
Organic	70	−33	12.28 (0.76)	77.8	45
Sand/shell	1	−7416	—	0	34
Sand/shell	5	−117	7.77 (0.43)	83.7	43
Sand/shell	10	−33	8.72 (0.39)	95.7	46
Sand/shell	15	−30	9.72 (0.39)	93.6	47

Standard error of water uptake is given in parentheses.

at day 14 (i.e. early development) and one with egg mass at day 28 (i.e. late development) as the response variable. In each analysis, the starting mass (i.e. mass at day 1 or mass at day 14), categorical treatment, and their interaction were fixed effects. Cage ID (i.e. the cage from which the egg was collected) was the random effect.

To analyze developmental rate, we used a general linear mixed effects model that included egg water uptake as a continuous variable, substrate type (sand/shell vs organic substrate), and their interaction as fixed effects. Initial egg mass was a covariate and cage ID was a random effect. To calculate a measure of water uptake for each egg, we subtracted the egg mass at oviposition from the egg mass at day 28 and divided by 28 (i.e. water uptake in mg/day). Thus, this analysis tests for an effect of substrate while controlling for moisture differences among our treatments via egg water uptake. We analyzed egg survival with a generalized linear mixed effects model with a binomial distribution and used the same fixed effects as for developmental rate; however, initial egg mass was removed due to lack of statistical significance ($P > 0.05$). For egg survival, we could not calculate a water uptake value for eggs that died. Therefore, we assigned each egg the mean water uptake value for its treatment and used those values (Table 1) in the analysis. A notable caveat is that eggs that died may have had water uptake values markedly different from those that survived; therefore, this analysis should be interpreted as how egg survival correlates with water uptake within the range of values that embryos could tolerate.

For hatchling morphology (SVL, body mass, dry and wet carcass mass) and endurance, we used general linear mixed effects models. Fixed effects were water uptake (as a continuous variable), substrate, and their interaction. Initial egg mass was a covariate for SVL and body mass,

but hatchling SVL was a covariate for endurance. Total wet body mass was a covariate for both wet and dry carcass mass. Cage ID from which the egg was collected was a random effect in each analysis; however, experimenter ID was an additional random effect in the analysis of endurance.

For each analysis, we compared models with and without the random effect via likelihood ratio test to see if we could remove the random effect and simplify the analysis. Next, we considered that the effect of water uptake might be curvilinear (i.e. have an optimum; Bodensteiner *et al*. 2015) by comparing a model that included water uptake as a linear term and a model that included water uptake as a second-degree polynomial (i.e. linear + quadratic) with a likelihood ratio test. In both comparisons, if an additional parameter (i.e. the random effect or the quadratic term) significantly improved model fit ($P < 0.05$), it was retained. Otherwise, it was removed. Results of these comparisons are in Tables S2 and S3, Supporting Information.

Finally, after analyzing the data, we noticed the wettest organic substrate treatment (70% water) resulted in substantially greater water uptake than the other treatments. Therefore, when we observed statistically significant effects of substrate type, we reduced the dataset to include only treatments that resulted in comparable water uptake (organic substrate with 30% and 50% water and sand/shell substrate with 5% and 15% water; Table 1) and reanalyzed the data. This ensured that our wettest organic substrate treatment did not substantially influence results. Moreover, 6 eggs from the wettest organic substrate treatment had unusually high values of water uptake (see Results). These 6 eggs also exhibited substantially slower developmental rates compared to other eggs (Fig. S1, Supporting Information). Therefore, we reanalyzed our

data without these 6 outliers to consider how these eggs might influence our results. Some analyses are qualitatively different when removing these outliers (see Results). Nevertheless, these 6 eggs successfully hatched. Therefore, we include them in the results presented in the main text while results without the outliers are in the supplement.

All analyses were performed in R (R Core Team 2018; version 4.0.0). We visually inspected model residuals to assess assumptions of statistical tests. Nonsignificant interaction terms were removed from final models. Linear estimates (i.e. Betas) were generated with the "summary" function and post hoc pairwise comparisons were made with the "emmeans" package (Lenth *et al*. 2018). For post hoc comparisons, *P* values were adjusted for false discovery rate to reduce the probability of type I errors. Mixed effects models were performed with the "lme4" (Bates *et al*. 2007) package, and we used the "lmerTest" package (Kuznetsova *et al*. 2017) to generate *P* values via Satterthwaite approximation for denominator degrees of freedom.

RESULTS

Water uptake

Water potentials were comparable among the sand/ shell and organic substrates (Table 1; Fig. 2). Egg mass at day 14 scaled positively with egg mass at oviposition ($F_{1,226.7} = 293.3$; $P < 0.0001$): for every 1 mg increase in initial egg mass, egg mass at day 14 increased by 1.3 mg (± 0.07 SE). Moreover, egg mass at day 14 varied according to treatment ($F_{6,250.7} = 25.4$; $P < 0.0001$): generally, greater substrate moisture content resulted in greater egg mass at day 14 for both substrate types; however, the eggs in the wettest organic substrate treatment (70% moisture) had the greatest increase in mass (Fig. 3a).

Egg mass at day 28 showed relationships consistent with those at day 14: egg mass at day 28 scaled positively with mass at day 14 ($F_{1,249} = 273.7$; $P < 0.0001$) and varied according to treatment ($F_{5,249} = 10.6$; $P < 0.0001$). For every 1 mg increase in day 14 egg mass, egg mass at day 28 increased by 1.3 mg (± 0.07 SE). Moreover, greater substrate moisture content resulted in greater egg mass at day 28 for both substrate types; however, eggs in the wettest organic substrate treatment (70% moisture) had the greatest increase in mass (Fig. 3b).

Mean rates of water uptake and water potentials for each treatment are provided in Table 1; however, water uptake could not be estimated for the driest treatment in each substrate type due to 100% egg mortality. Despite similar water potentials, water uptake varied more across the or-
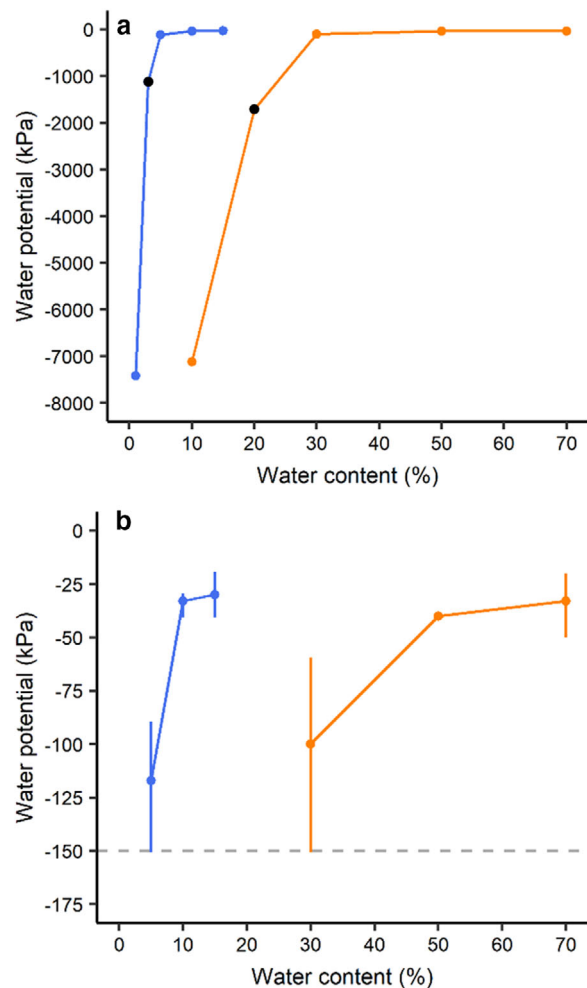

Figure 2 Water potentials for the sand/shell (blue) and organic (orange) substrates. (a) Retention curves for each substrate. The blue and orange circles show water potentials for the moisture concentrations used in our study. The black circles show data for additional samples used to characterize the retention curves. (b) Water potentials for the moisture concentrations in our study that resulted in successful development (5%, 10%, and 15% water for sand/shell [blue]; 30%, 50%, 70% water for organic [orange]). Circles show the mean of 3 samples taken for each substrate/moisture combination. Bars show the maximum and minimum water potential of the samples. The horizontal dashed line denotes the water potential of our standard incubation medium (−150 kPa vermiculite).

ganic substrate treatments than the sand/shell treatments. This was driven by relatively high water uptake by eggs in the wettest organic substrate treatment (70% moisture). Treatments of intermediate moisture content were similar between substrates with respect to water uptake (Table 1; Fig. 3). Thus, 30% and 50% moisture content in organic
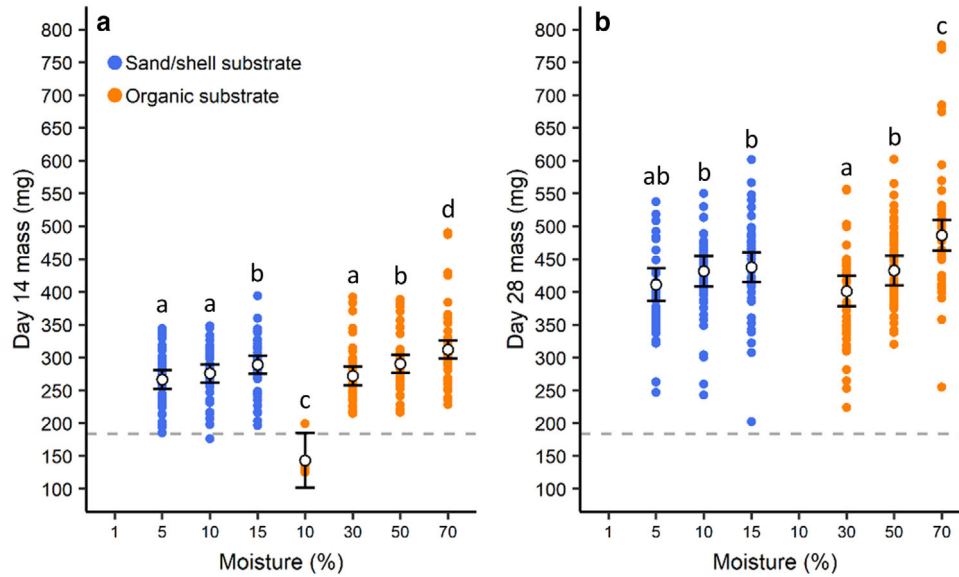
Figure 3 Egg mass at day 14 (a) and day 28 (b) of development. Open circles show the estimated marginal means (i.e. least squares means) for each treatment after adjusting for initial egg mass (using the "emmeans" package). Bars show the upper and lower 95% confidence limits for the estimated marginal means. The dashed horizontal line denotes the mean initial mass (i.e. at oviposition) of all eggs (184.04 mg). Lowercase letters denote statistical differences among groups after adjusting for false discovery rate. No eggs survived to day 14 in 1% water content in sand/shell and no eggs survived to day 28 in the 1% water content of sand/shell or the 10% water content in the organic substrate. Estimated marginal means and statistical results of all contrasts are in Tables S4 and S5, Supporting Information.

substrate resulted in rates of water uptake similar to 5% and 15% moisture content in sand/shell.

Egg physiology and survival

Eggs in the driest treatment for each substrate experienced 100% mortality. Most of this mortality occurred prior to day 14 of incubation (see Fig. S2, Supporting Information). Eggs in these treatments were shriveled and did not exhibit noticeable fungal or bacterial growth, indicating they died from desiccation rather than other causes (e.g. infection). Indeed, when adequate moisture is available, virtually all dead eggs exhibit obvious fungal and bacterial growth (personal observation). For the remaining treatments, egg survival was relatively high ($\geq$75%; Table 1). Regardless, we observed a significant substrate by water uptake interaction for egg survival (Table 2). By analyzing the data per substrate type, we observed that egg survival increased with water uptake for sand/shell ($\beta = 0.69 \pm 0.43$ SE), but decreased in the organic substrate ($\beta = -0.22 \pm 0.13$ SE) (Fig. 4); however, these post hoc results were not statistically significant ($z = 1.6$; $P = 0.11$; $z = -1.7$; $P = 0.09$, respectively). Because the wettest organic substrate treatment had much greater wa-

ter uptake than other treatments (and the lowest egg survival; Fig. 4; Table 1), we reduced the dataset to include only treatments with comparable water uptake (5% and 15% sand/shell; 30% and 50% organic substrate; Table 1) and repeated the analysis. The interaction term was no longer significant ($\chi^2_1 = 0.50$; $P = 0.48$); moreover, there were no statistically significant effects of substrate ($\chi^2_1 = 0.24$; $P = 0.63$) or water uptake ($\chi^2_1 = 1.92$; $P = 0.17$) on egg survival. Therefore, substrate-specific effects on egg survival were driven by the wettest organic substrate treatment (70% moisture).

Developmental rate was greater for eggs incubated in sand/shell than the organic substrate ($\beta = 0.059 \pm 0.02$; $t_{1,227.3} = 3.0$; $P = 0.003$) and for eggs with intermediate levels of water uptake (linear term $= 0.036 \pm 0.01$; $t_{1,229.7} = 2.7$; $P = 0.007$; quadratic term $= -0.0024 \pm 0.0006$; $t_{1,230.3} = -3.9$; $P = 0.0001$) (Fig. 5a; Table 2). Moreover, developmental rate increased with initial egg mass ($\beta = 0.00096 \pm 0.0004$; $t_{1,213.7} = 2.4$; $P = 0.018$). Notably, when we removed the 6 outliers (see Fig. 5), the substrate effect was still observed; however, the effect of water uptake on developmental rate was no longer statistically significant ($P = 0.93$; Table S6 and Fig. S3a, Supporting Information). To better evaluate the substrate

Table 2 Results of substrate, water uptake, and their interaction on egg and hatchling variables

Response	Covariate	Substrate	Water uptake	Interaction	Covariate used
Egg survival		$\chi^2_1 = 3.9$; $P = 0.047$	$\chi^2_1 = 2.9$; $P = 0.08$	$\chi^2_1 = 4.5$; $P = 0.035$	—
Developmental rate[‡¥]	$F_{1,214} = 5.6$; $P = 0.019$	$F_{1,227} = 9.2$; $P = 0.003$	$F_{2,233} = 12.9$; $P < 0.0001$	—	Initial egg mass
Hatchling SVL[¥]	$F_{1,238} = 63.3$; $P < 0.0001$	$F_{1,238} = 0.14$; $P = 0.71$	$F_{2,238} = 11.3$; $P < 0.0001$	—	Initial egg mass
Hatchling mass[‡¥]	$F_{1,217} = 185.8$; $P < 0.0001$	$F_{1,223} = 3.0$; $P = 0.086$	$F_{2,229} = 4.9$; $P = 0.008$	—	Initial egg mass
Hatchling endurance[†]	$F_{1,156} = 4.1$; $P = 0.045$	$F_{1,154} = 0.5$; $P = 0.50$	$F_{1,154} = 0.1$; $P = 0.94$	—	Hatchling SVL
Hatchling water mass[‡]	$F_{1,70} = 4878$; $P < 0.0001$	$F_{1,66} = 0.9$; $P = 0.36$	$F_{1,68} = 4.5$; $P = 0.037$	—	Wet body mass
Hatchling dry mass[‡]	$F_{1,70} = 440$; $P < 0.0001$	$F_{1,66} = 0.9$; $P = 0.36$	$F_{1,68} = 4.5$; $P = 0.037$	—	Wet body mass

Bold type denotes statistical significance ($P < 0.05$); [‡]Cage ID was a random effect; [†]experimenter ID was a random effect; [¥]water uptake was modeled curvilinearly (i.e. linear + quadratic).
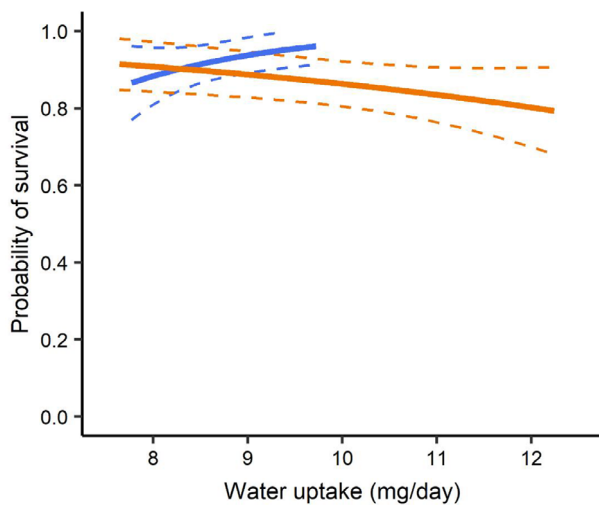

Figure 4 Egg survival in sand/shell (blue) and organic (orange) substrates. Solid lines show the model estimates of survival and dotted lines show the 95% confidence intervals. Data from the 2 driest treatments are not included because they exhibited 100% mortality.

effect, we reduced the dataset to include only treatments with comparable water uptake (5% and 15% sand/shell; 30% and 50% organic substrate; Table 1) and repeated the analysis. Water uptake was not statistically significant ($F_{2,156} = 0.13$; $P = 0.92$); however, the substrate effect remained statistically supported ($F_{1,149} = 9.1$;

$P = 0.003$). Thus, eggs developed more quickly on sand/shell than organic substrate independent of water uptake. The difference in developmental rates between substrates equates to approximately a 1-day greater incubation period for organic substrate versus sand/shell (raw means of 36.15 and 35.10 days for organic substrate and sand/shell, respectively).

Hatchling morphology and performance

Neither substrate type nor its interaction with water uptake had an effect on hatchling SVL (Table 2). Hatchling SVL increased with initial egg mass and with water uptake (Table 2). For every 1 mg increase in initial egg mass, hatchling SVL increased by 0.02 mm ($\pm$ 0.002 SE; $t_{1,238} = 8.0$; $P < 0.0001$); however, the relationship with water uptake was curvilinear (linear term = 0.38 $\pm$ 0.09; $t_{1,238} = 4.4$; $P < 0.0001$; quadratic term = $-0.01 \pm$ 0.004; $t_{1,238} = -3.6$; $P = 0.0004$) such that high levels of water uptake did not increase SVL compared to intermediate levels (Fig. 5b).

Results for hatchling body mass were like those of SVL; body mass increased with initial egg mass and with water uptake (Table 2). For every 1 mg increase in initial egg mass, hatchling mass increased by 0.68 mg ($\pm$ 0.0.05 SE; $t_{1,217} = 13.6$; $P < 0.0001$); moreover, the relationship with water uptake was curvilinear (linear term = 5.0 $\pm$ 1.6; $t_{1,225} = 3.1$; $P = 0.003$; quadratic term = -0.21 ± 0.08; $t_{1,226} = -2.7$; $P = 0.008$) such that high
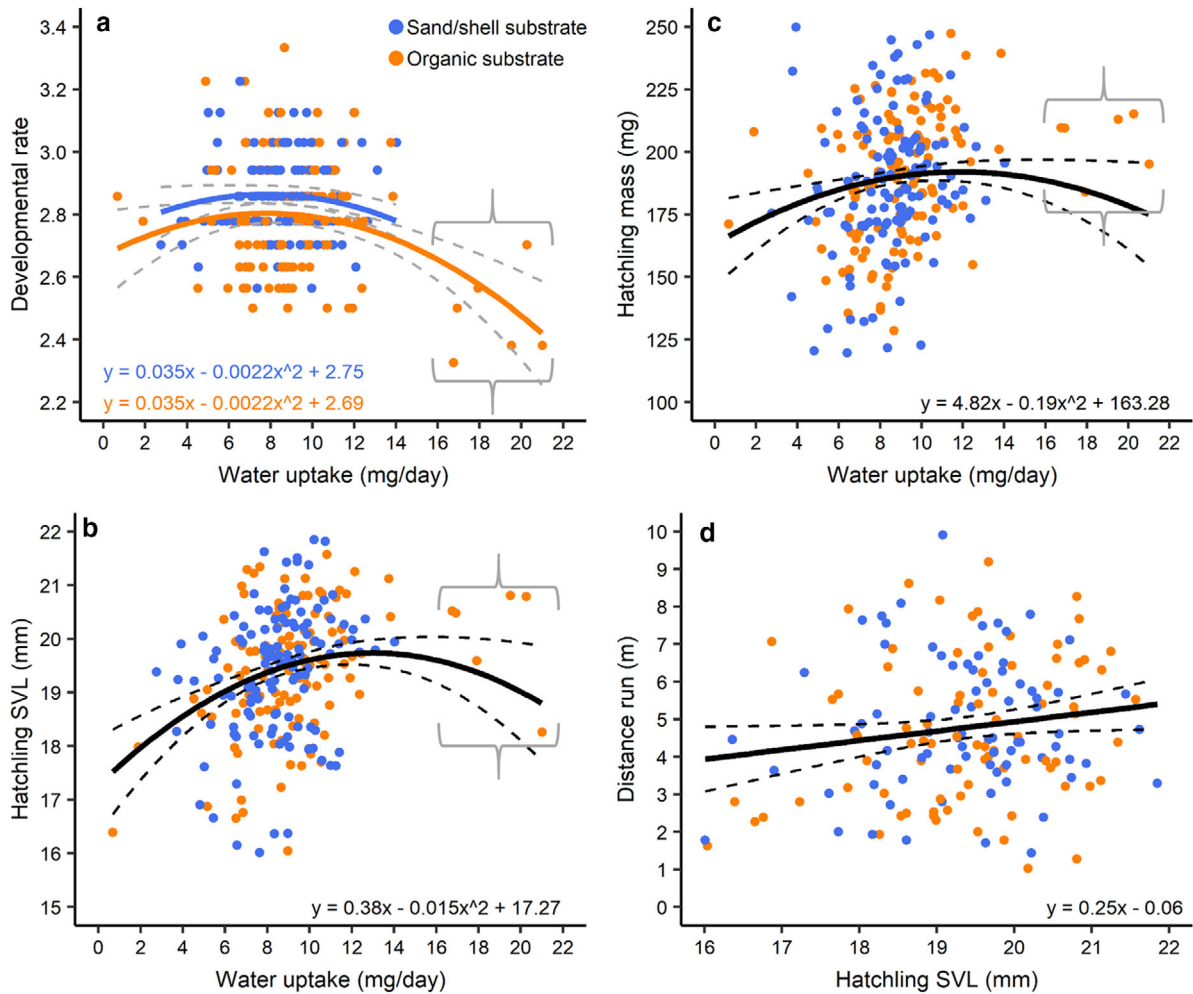
Figure 5 Effect of water uptake during development on (a) developmental rate, (b) hatchling SVL, and (c) hatchling body mass. (d) The relationship between hatchling endurance and hatchling body size (SVL). The gray brackets denote the 6 outlying eggs. See Table S6 and Fig. S3, Supporting Information, for results excluding these eggs. Solid orange and blue lines show model fits for organic and sand/shell substrates, respectively (a). Broken gray lines show the 95% confidence intervals of model fits (a). Solid and broken black lines denote the model fit and 95% confidence intervals in (b–d).

levels of water uptake did not increase body mass compared to intermediate levels (Fig. 5c). Removing the 6 outliers revealed that water uptake positively influenced SVL and body mass linearly, rather than curvilinearly (i.e. the quadratic term did not improve model fit; Table S6 and Fig. S3b,c, Supporting Information).

Neither substrate, water uptake, nor the interaction influenced hatchling endurance (Table 2); however, for each 1 mm increase in hatchling SVL, endurance increased by 0.26 m ($\pm$0.13 SE; $t_{1,156} = 2.02$; $P = 0.045$) (Fig. 5d). Thus, the largest hatchlings (22 mm) ran approximately 1.5 m farther than the smallest hatchlings (16 mm). Importantly, substrate and water uptake effects were not sta-

tistically significant even when SVL was excluded from the model ($P > 0.45$); thus, increases in SVL due to water uptake did not equate to increased hatchling performance. After removing the outliers, however, the positive trend between SVL and endurance was marginally nonsignificant ($P = 0.088$; Table S6 and Fig. S3d, Supporting Information).

Finally, hatchling carcass water mass declined by 0.26 mg ($\pm$0.12 SE; $t_{1,68} = -2.1$; $P = 0.037$) for each 1 mg/day increase in water uptake. Conversely, hatchling dry mass increased by 0.26 mg ($\pm$0.12 SE; $t_{1,68} = 2.1$; $P = 0.037$) for each 1 mg/day (Fig. 6). Importantly, because total body mass was used as a covariate, these
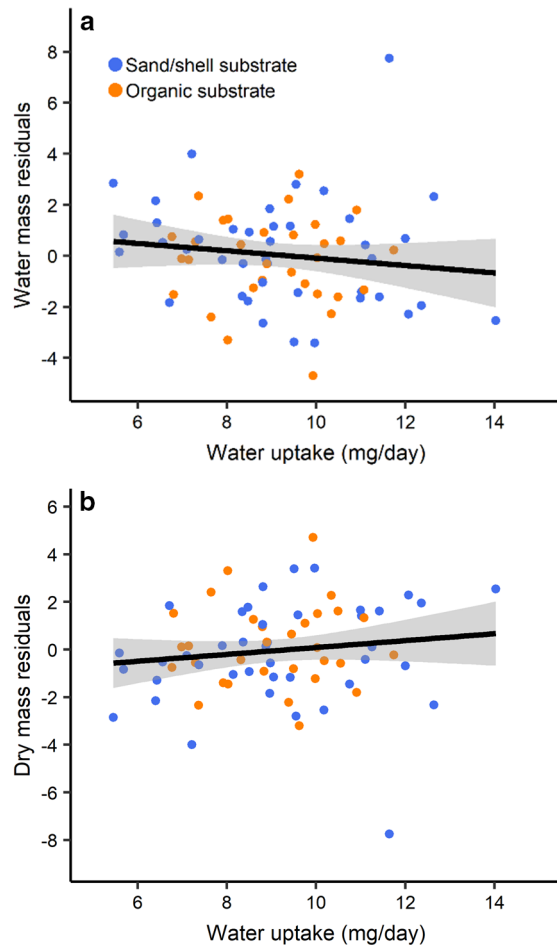
Figure 6 Residuals of water mass (a) and dry body mass (b) of hatchlings. Residuals were calculated by regressing either water mass or dry mass on total body mass. Solid lines denote the model fit and the gray area is the 95% confidence intervals.

results should be interpreted as increases or decreases relative to body size (i.e. absolute water mass increased with increasing levels of water uptake). Neither water mass nor dry mass were significantly influenced by substrate type or the interaction term (Table 2). After removing the outliers, the same trends were observed, but they were no longer statistically significant (Table S6, Supporting Information). See Table S1, Supporting Information, for the raw mean, standard deviation, and sample size of each phenotype on a per treatment basis.

DISCUSSION

The type of nest substrate can have important effects on egg survival and embryo development in a diversity of animals (birds—Cooney & Watson 2008; de

Zwann & Martin 2018; fish—Wisenden *et al*. 2009; amphibians—Seymour & Roberts 1991; Mitchell & Seymour 2003; reptiles—Mitchell & Janzen 2019; spiders—Morse 1990), but relatively few studies have considered this important aspect of the nest environment. We incubated squamate eggs in 2 natural nest substrates at various water concentrations. The driest treatments resulted in total egg mortality, but eggs survived equally well at wetter treatments, indicating that embryos will successfully develop so long as a minimum threshold of moisture is available. Greater substrate moisture resulted in greater water absorption by eggs and larger hatchling body size, potentially due to increased efficiency of yolk conversion to body mass. This increase in body size, however, had minimal effects on hatchling performance. Unlike water availability, the type of substrate (sand/shell vs organic substrate) had minimal effects on egg survival and hatchling phenotypes; however, eggs developed faster in sand/shell than in the organic substrate, indicating that natural substrates can influence egg or embryo physiology. The mechanism for substrate-specific developmental rates is unknown, which indicates that there is still much to learn about the effects of natural nest habitats on vertebrate embryo development.

Effects of substrate

Although percent water mass differed substantially between substrates, water potentials and water uptake by eggs were comparable between substrate types, indicating that the 2 substrates can have equivalent developmental effects depending upon the level of hydration. The one notable exception is that the wettest organic substrate treatment (70% moisture) resulted in greater water uptake than other treatments. Due to the relatively high water retention of this substrate, there may be a greater possible range of moisture concentrations compared with the sand/shell substrate. Regardless, substrate had no meaningful effect on most phenotypes (Table 2). Although we observed a substrate effect on egg survival, this effect was driven by the wettest organic substrate treatment. Intriguingly, this treatment had similar water potential to the wettest sand/shell treatment, indicating that aspects of the substrate other than hydration influenced egg survival. For example, it is possible that fungal/microbial communities differed among the substrates and influenced development. Regardless, substrate effects on egg survival may have little biological importance. For example, mean survival was 85.6% and 92.0% for organic and sand/shell substrates, respectively (excluding the driest treatments), and these rates are comparable to

studies that incubate eggs at standard moisture conditions (i.e. −150 kPa water potential vermiculite; Hall & Warner 2019: 85.0–95.0% survival; Hall & Warner 2020: 85–100% survival). Moreover, DeSana *et al.* (2020) reported survival of 70–100% for eggs incubated in natural substrates in the wild. Their study was conducted on the same island from which our substrates were collected, but they did not monitor eggs over the entire incubation period. Finally, Pruett *et al.* (2020) observed rates of egg survival from 43.3% to 93.3% when eggs were incubated on a nearby island and moisture was controlled across nests. Therefore, excluding the driest treatments, both substrates support high hatching success across a range of moisture concentrations.

Intriguingly, the only significant effect of substrate was on developmental rates: sand/shell increased developmental rates compared to the organic substrate. A notable caveat is that because we only checked for eggs twice weekly, oviposition dates, and, thus, incubation periods, are only accurate to 4 days, which is greater than the mean difference between substrates (i.e. ≈1 day difference). Our large sample sizes ($n = 119, 126$ eggs for organic and sand/shell substrates, respectively), however, improve the accuracy of our estimates of developmental rates. The source for the difference in developmental rates is not obvious because water potentials were similar among substrates; however, the difference persisted even when we compared substrates with similar values of egg water uptake. A few studies report substrate-specific developmental rates, but there is no clear trend. Eggs of the diced water snake (*Natrix tessellata*) take longer to hatch on sand than vermiculite, though the average difference was less than 1 day (Dmi'el *et al.* 1993). Turtle eggs (*Chrysemys picta*) hatch sooner when incubated in sand versus gravel, despite similar temperatures (Mitchell & Janzen 2019), resulting in a mean difference of 2.7 days in the incubation period. These studies indicate that some aspect of the substrate can influence egg or embryo physiology independent of water uptake and temperature.

We provide some potential explanations for the effect of substrate on developmental rate. First, substrates may differ in the number and diversity of microbes that potentially harm eggs (e.g. bacteria, fungal spores). Fungal infections can reduce egg survival and the incubation period, especially in very wet substrates (Tracy 1980; Moreira & Barata 2005). Second, substrate-specific oxygen levels could slow or speed developmental rates. For example, organic substrates contain numerous microbes which may compete with eggs for oxygen (Ackerman & Lott 2004). Germinating plants could also compete with eggs for oxygen. Indeed, we occasionally found small

seedlings growing in petri dishes with organic substrate but not sand/shell. Though seedlings were relatively uncommon, in conjunction with microbes, they could potentially have lowered oxygen levels in our organic substrate treatment, which could retard development (Liang *et al.* 2015). Moreover, effects of low oxygen and disease could compound or interact and may explain why egg survival was reduced in our wettest soil treatment.

Interestingly, the wettest treatments in each substrate had similar values of water potential but differed substantially in how much water eggs absorbed (Table 1). This bolsters our conclusion that substrate properties other than water availability influenced developmental physiology. Ultimately, several studies have observed substrate effects on developmental rates and hatchling phenotypes, but no study has unearthed the mechanisms (e.g. Packard *et al.* 1987; Dmi'el *et al.* 1993; Mitchell & Janzen 2019). These results illustrate (1) gaps in our knowledge of how natural nest environments influence development and (2) the importance of using ecologically relevant substrates in studies of developmental plasticity.

Effects of water uptake

In general, water uptake increased with greater levels of substrate moisture, which is in congruence with past research (Gordon 1960; Ackerman & Lott 2004; Warner & Chapman 2011; Warner *et al.* 2011). For many species, the effect of substrate moisture on development exhibits an optimum: egg survival is greatest at intermediate moisture concentrations but relatively low in dry or extremely wet soils (Tracy 1980). Optimal water potentials for incubation are probably between −50 and −200 kPa (Booth 2004). Most of our treatments were close to this range, and egg survival was relatively high across those treatments. The minimum water potential for development varies across species, but some squamates can develop in conditions as dry as −1500 kPa (Muth 1980). Our 2 driest treatments exhibited much lower water potentials (< -7000 kPa), and it is unlikely that any species producing parchment-shelled eggs could have survived incubation in these conditions. In retrospect, these dry treatments may have little ecological relevance; however, eggs are occasionally found in substrates with 1–10% moisture content in the field. Although Pruett *et al.* (2020) observed such low moisture concentrations in nests, they did not report the type of nest substrate (sand/shell vs organic), nor did they monitor substrate moisture over time. Therefore, eggs could survive in substrates with 1–10% moisture content if (1) they are incubating

in substrates that are predominantly sand/shell vs organic substrate or (2) these dry conditions are temporary.

Importantly, our 2 wettest treatments were so saturated that adding additional water would have resulted in standing water in the petri dishes (which would almost certainly limit oxygen diffusion across the eggshell). Therefore, we consider these treatments to be near the maximum possible moisture content of field substrates, which is further supported by our measures of water potential (Fig. 2). Yet, egg survival was still relatively high in these conditions (i.e. no practical optimum). Reedy *et al.* (2013) also did not find optimum substrate moisture for *A. sagrei* eggs, but rather showed that dry treatments vastly reduce egg survival, and survival increases relatively linearly with moisture content. *Anolis sagrei* is a notorious island colonizer, and eggs are robust to high moisture levels: even total immersion in water for up to 6 h has no apparent effect on hatching success (Losos *et al.* 2003). Complete inundation by water may be a common selection pressure for this species as eggs potentially float to Caribbean islands on flotsam and hurricane storm surge often inundates small islands inhabited by lizards (Losos *et al.* 2003). Thus, in the wild, egg survival may be relatively high even in very wet substrates. Finally, although egg survival is high across most moisture concentrations, survival rapidly declines in drier conditions (i.e. a threshold effect). Due to their large surface area to volume ratio, the small eggs of *A. sagrei* have little buffer against dry conditions (Ackerman *et al.* 1985). Interestingly, females prefer to nest in the wettest substrates available in both the lab (Reedy *et al.* 2013) and field (Pruett *et al.* 2020), rather than selecting intermediate moisture levels. This "wetter is better" nesting behavior may buffer embryos from occasional dry periods in the wild (Ackerman & Lott 2004).

Effects of water uptake on hatchling morphology were as expected, as relatively wet substrates typically result in larger hatchlings (Reedy *et al.* 2013; Pruett *et al.* 2020). Our data show that this effect is linear across moderate levels of moisture (Table S6 and Fig. S3, Supporting Information); however, extremely wet substrates do not appreciably increase body size compared to moderate moisture levels (Fig. 5). High substrate moisture likely enhances body size by increasing the moisture content of hatchlings and enhancing the efficiency of yolk conversion to somatic tissue (Packard *et al.* 1992). Indeed, we observed that greater levels of water uptake resulted in greater absolute water content of hatchlings and greater absolute dry hatchling mass (Table S1, Supporting Information); however, greater water uptake resulted in reduced water mass and more dry mass relative to body size (Fig. 6). Interestingly, greater body size due to water

uptake did not increase hatchling performance, even though performance correlates with body size (Fig. 5d). Of course, other aspects of performance that were not measured here (e.g. sprint speed; desiccation tolerance) may have been influenced by water uptake. In addition, larger body size at hatching is often assumed to correlate with increased survival probability, but this is not always true (Belinsky *et al.* 2004). For *A. sagrei*, it is not clear if greater hatchling body size increases survival probability. Pearson and Warner (2018) found no relationship between hatchling body size and survival in the wild; however, survival was low (<10%), which reduces the power for assessing a body size-survival relationship.

Finally, 6 eggs (i.e. outliers) had unusually high water uptake and slow developmental rates. When including these outliers, developmental rate decreases with water uptake. Indeed, embryo developmental rate sometimes decreases with water uptake resulting in hatchlings with greater body size and lower residual yolk mass (Packard & Packard 1980). Conversely, longer incubation periods result in increased water uptake due to additional time to absorb moisture; therefore, it is difficult to determine whether increased water uptake reduced rates of development or vice versa. Regardless, including and excluding these outliers during analysis demonstrate that moisture-induced increases in body size are relatively linear over a wide range of substrate moisture levels but have an upper limit (see also Bodensteiner *et al.* 2015).

Ecological relevance

Although many properties of our 2 substrates differ, their capacity to retain water may be the most important factor for eggs. We controlled water content between substrates by using water uptake as a covariate in the analyses; however, in the field, the sand/shell substrate is likely drier than the organic substrate. First, sand-based substrates have lower water retention than organic-based soils (Gupta & Larson 1979). Consequently, even if the same absolute volume of water is present in these 2 substrates, the water available to the eggs will not be equal. Moreover, moisture levels will differentiate due to substrate-specific rates of evaporation and drainage. Second, at our study site, the portion of these substrates available for nesting (i.e. <5 cm depth) is found in vastly different microhabitats: sand/shell is usually in the open, lacking shade cover and the organic substrate is found beneath vegetation and shaded. Substantial temperature differences between these habitats (Pearson & Warner 2016; Pruett *et al.* 2020) will influence evaporative water loss. For these reasons, our results should not be

interpreted as substrate-specific effects of eggs in field nests, but rather as (1) effects of substrate while controlling for egg water uptake (due to moisture variation) and (2) the effect of water uptake on development while incubating in natural substrates. We do not know if females prefer to nest in one substrate or the other; however, they generally lay eggs in cool, shaded microhabitats (Pruett *et al*. 2020), indicating that more eggs are laid in the organic substrate than sand/shell. Indeed, while drying substrates, we found several hatched eggshells in the organic substrate and none in sand/shell; however, detection probability may be lower in sand/shell since the broken seashells are similar in size and color to lizard eggshells.

CONCLUSIONS

Many nest characteristics can influence the water available to vertebrate embryos. The type of nest substrate has received little attention, particularly for squamates. We found that nest substrate had little effect on hatchling phenotypes when controlling for moisture; therefore, with respect to hatchling body size and performance, the most important aspect of the substrate may be its water retention qualities. Conversely, we observed some relatively small substrate-specific effects on developmental rates and egg survival; thus, there may be substrate-specific factors other than moisture availability that are important for development. Additional study is warranted. Indeed, our knowledge of embryo responses to natural nest substrates is still relatively limited, particularly for squamates; therefore, future studies should incorporate natural substrates into lab studies of developmental plasticity when possible. This would greatly enhance our understanding of how vertebrate embryos interact with developmental environments in the wild.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflict of interest.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Raw phenotypic mean, standard deviation (SD), and sample size (N) for each treatment

Table S2 Comparison between models that did and did not include a random effect

Table S3 Comparison of models that considered water uptake to be curvilinear (linear + quadratic) vs linear

Table S4 Estimated marginal means of egg mass early (day 14) and late (day 28) in development

Table S5 Contrasts of estimated marginal means for water uptake early (day 14) and late (day 28) during development

Table S6 Results of substrate (organic vs sand/shell), water uptake, and their interaction when outliers ($n = 6$) are removed from the analysis

Figure S1 Six eggs from the wettest organic substrate treatment (70% water) were outliers with respect to water uptake.

Figure S2 Egg survival data across time.

Figure S3 Effect of water uptake on (a) developmental rate of eggs, (b) hatchling SVL, (c) and hatchling body mass when the 6 outliers are removed from the analysis (compare to Fig. 5 in the main text).

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