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Egg environments have large effects on embryonic development, but have minimal consequences for hatchling phenotypes in an invasive lizard

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Plastic responses of embryos to developmental environments can shape phenotypes in ways that impact fitness. The mechanisms by which developmental conditions affect offspring phenotypes vary substantially among taxa and are poorly understood in most systems. In this study, we evaluate the effects of thermal and hydric conditions on patterns of egg water uptake, embryonic development and yolk metabolism in embryos of the lizard *Anolis sagrei* to gain insights into how these factors shape morphological variation in hatchlings. Our 3×2 experimental design (3 thermal and 2 hydric conditions) revealed that developmental temperature has strong effects on rates of development and yolk metabolism, but the impacts of moisture were minimal. Increased water uptake by eggs under relatively wet conditions resulted in larger hatchlings with less internalized residual yolk than hatchlings from dry-incubated eggs. However, the relatively small phenotypic differences among treatments may have small fitness consequences. These results demonstrate that embryos of *A. sagrei* can tolerate a broad range of environmental conditions without substantial impacts on critical morphological traits. Such embryonic tolerances may facilitate colonization and establishment in novel environments. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **105**, 25–41.

ADDITIONAL KEYWORDS: body size – developmental plasticity – egg incubation – egg size – incubation moisture – incubation temperature – limb length – residual yolk.

INTRODUCTION

Plastic responses to developmental environments are nearly ubiquitous across taxa and create phenotypic variation upon which natural selection can operate (West-Eberhard, 2003). Although selection on environmentally induced phenotypic variation may constrain adaptive evolution, plastic responses to environmental conditions (reaction norms) can evolve like any heritable trait (DeWitt & Scheiner, 2004). Indeed, selection likely shapes embryonic responses to developmental environments so that resultant offspring phenotypes optimize fitness under prevailing environmental conditions (Stearns, 1989). Such developmental responses should be favoured by selection because they enhance both offspring and parental fitness. Accordingly, the adaptive value of developmental plasticity has received considerable attention in recent research (Ghalambor *et al*., 2007; Gilbert & Epel, 2008), but specific mechanisms that shape environmentally induced phenotypes are poorly understood in most species.

How the environment initiates or guides developmental trajectories of phenotypes varies considerably across taxa and depends upon the trait of interest and

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individual genotype (Schlichting & Pigliucci, 1995). Such genotype-specific responses have been demonstrated in a diversity of organisms (e.g. Sultan & Bazzaz, 1993; Gurganus *et al*., 1998; Tonsor & Scheiner, 2007; Chandler, 2010). In many cases, developmental environments affect gene expression, thereby initiating biochemical cascades that determine phenotypic development (Beldade, Mateus & Keller, 2011). In other cases, plastic responses at different times of development may be a consequence of physiological limitations, rather than a result of environmentally induced gene expression. By identifying the physiological effects of environmental stimuli on embryos at different times of development, we can gain a better picture of how specific conditions shape phenotypic variation.

Reptile eggs and embryos are extremely sensitive to environmental conditions during incubation (Deeming, 2004) and thus provide excellent models for studies of developmental plasticity. Because most species do not exhibit parental care, the environment that eggs experience depends on the nest microhabitat and the prevailing ambient environment. As a result, the thermal and hydric environments of nests can vary substantially over space and time (Packard *et al*., 1985; Warner, Jorgensen & Janzen, 2010) and this variation often has important fitness consequences for parents and offspring. A large body of literature illustrates that both temperature and moisture affect patterns of embryonic development and offspring phenotypes in ways that can impact fitness (e.g. Janzen, 1993; Andrews, Mathies & Warner, 2000; Warner & Shine, 2008). For example, incubation temperature influences embryonic developmental rate and can impact offspring body size, locomotor performance and social or anti-predatory behaviours (Gutzke & Crews, 1988; Elphick & Shine, 1998; Downes & Shine, 1999; Andrews *et al*., 2000; Telemeco *et al*., 2010). Moisture conditions can also affect developmental patterns and water uptake by eggs (Packard & Packard, 1988). Wet incubation substrates allow more efficient yolk metabolism than dry substrates during embryogenesis (Miller & Packard, 1992), which in turn, affects offspring body size and growth, as well as the quantity of residual yolk that is internalized prior to hatching (Packard *et al*., 1987; Overall, 1994). Unfortunately, relatively little is known about how interactive effects of temperature and moisture impact egg water uptake and embryo yolk metabolism at different stages of embryonic development, and their consequences on offspring morphology, hydration and internalized residual yolk (Morris *et al*., 1983; Packard *et al*., 1987; Phillips & Packard, 1994; Flatt *et al*., 2001).

We addressed these issues by incubating eggs of *Anolis sagrei* (brown anole lizard) under multiple thermal and hydric environments. Moreover, by sampling eggs/embryos at specific intervals during development, we quantified changes in embryo stage, embryo mass and hydration, yolk quantity and yolk water content during incubation. Additional treatments allowed eggs to hatch so that we could measure thermal and hydric effects on offspring morphology (body size as well as limb and head shape), quantity of internalized residual yolk and hydration of hatchlings; all traits that are likely important for offspring fitness (Troyer, 1987; Sinervo *et al*., 1992; Kolbe & Janzen, 2002). For example, limb length has been shown to be particularly important for individual performance, habitat use and community structure in *Anolis* lizards (reviewed in Losos, 2009) and is under selection in natural populations of *A. sagrei* (Calsbeek & Irschick, 2007). Thus, identifying the incubation environment as a source of variation in this critical trait (and others) is of interest to evolutionary ecologists. Our primary objective was to quantify the relative importance of thermal and hydric conditions during egg incubation on water and yolk regulation during development, and its subsequent effect on hatchling morphology. Because of recent colonization and successful establishment of *A. sagrei* in novel environments (Kolbe *et al*., 2004), we predicted that embryos of this species could develop under a broad range of environmental conditions, with minimal impacts on fitness-related morphology. Nevertheless, like most reptiles, thermal and hydric environments should impact rates of embryonic development and yolk metabolism in ways that shape offspring morphology.

MATERIAL AND METHODS

Lizard husbandry and experimental design

Eggs were obtained from a wild-caught captive colony of brown anoles (*Anolis sagrei*) that were collected from Ormond Beach, Florida, in October 2008. In our colony, each cage (29 cm high × 26 cm wide × 39 cm deep) housed a breeding male/female pair, and lizard care generally followed the protocol of Sanger *et al*. (2008a). Each cage contained several perches for basking and a potted artificial plant. Plant pots were filled with moist potting soil to provide females with a suitable site for oviposition. Cages were illuminated with ReptiSun 5.0 UVB bulbs (ZooMed Inc.) set on a 12-h light cycle. Additional room lights turned on 1 h before and turned off 1 h after the cage lights. Maximum room temperature reached an average of 32.1 °C (SD = 5.6) during the day and dropped to a minimum daily average of 23.8 °C (SD = 3.2) when lights were off at night. Lizards were fed crickets (dusted in a mix of vitamins and calcium) three times per week and cages were misted with water once per day.

We obtained eggs for our experiment by thoroughly searching the potting soil in each cage twice per week

(from 18 May to 30 August 2010). All eggs were immediately weighed (to 0.0001 g) and half buried in moist vermiculite in individual glass jars (59 mL) that were covered with plastic wrap (sealed with a rubber band) to prevent evaporation (glass jars without eggs were weighed at the beginning and end of a 30-day period, and average mass loss attributable to evaporation was minimal: 0.61% mass loss, range: 0.03–0.86%). Eggs were put into one of six treatments in a full 3×2 factorial design. To evaluate the effect of incubation temperature on embryonic development and offspring phenotypes, eggs were placed into one of three incubators that were set at constant temperatures of 26 °C, 28 °C or 30 °C. Within these three temperature regimes, eggs were placed into either relatively moist (–150 kPa) or dry (–450 kPa) vermiculite. Vermiculite water potential was determined using a water retention curve developed by Packard *et al*. (1987). Although thermal and hydric conditions of *A. sagrei* nests are unknown, these conditions are within the range found in potential nest sites of other lizard species in Florida (Du *et al*., 2010; Robbins & Warner, 2010). Because *Anolis* lizards produce approximately one egg per week, we kept track of treatment assignments for eggs produced by each female to ensure that we allocated eggs from single females as evenly as possible among treatments. Indeed, treatments contained eggs produced by the same female on only seven occasions (eggs from 42 females were used in this study). Eggs were rotated within each incubator three times per week to minimize potential effects of thermal gradients within incubators.

The protocol described above was used for two related experiments to evaluate thermal and hydric effects on egg water uptake, embryo yolk metabolism and offspring morphology. In the first experiment, eggs were terminally sampled either on the day of collection (day 0), day 7, day 14 or day 21 after collection. Because we checked for eggs twice per week, the day of collection was between 0 and 3 days after oviposition; hereafter we refer to the day of collection (day 0) as the day of oviposition. At each of these time periods, eggs were weighed before carefully removing the contents of the eggs. Egg contents were completely removed from the eggshell onto a weighing tray. After the embryo was separated from the yolk sac, it was gently dabbed with a Kimwipe and weighed to 0.0001 g. Embryonic stages were identified according to criteria developed for *A. sagrei* (Sanger, Losos & Gibson-Brown, 2008b) and embryos were re-weighed after being dried in an oven at 50 °C for 5 days. The remaining material within the eggshell (primarily yolk) was weighed immediately after dissection and then re-weighed after being dried in an oven at 50 °C for 5–7 days. Because of the small size of *A. sagrei* eggs and general lack of distinction among egg contents, we did not separate the yolk from the chorioallantois or the albumen. Thus, our measurements of actual yolk may be slightly inflated in our analyses; however, past studies show that lizard eggs are laid with little to no albumen (Tracy & Snell, 1985; Belinsky *et al*., 2004; Thompson & Speake, 2004). Eight eggs were sampled on the day of collection and 5–7 eggs were allocated to each temperature/moisture/sampling-day combination (total of 115 eggs).

In a second experiment, eggs were incubated under the same six treatments described above, but were allowed to hatch in order to measure treatment effects on offspring phenotype. We also repeatedly weighed eggs at 7-day intervals to evaluate treatment effects on change in egg mass (because of water uptake) during incubation. Thus, eggs were re-weighed at day 7, day 14 and day 21 after oviposition. Eggs were checked daily for hatchlings.

Immediately after hatching, offspring were measured [snout–vent length (SVL) and tail length (TL) to 0.5 mm], weighed and their sex was identified by dorsal pattern and by the presence (male) or absence (female) of enlarged scales posterior to the cloaca. After taking measurements, hatchlings were killed by decapitation and their internalized residual yolk was surgically removed (e.g. Radder *et al*., 2007). Residual yolk was weighed immediately after dissection (to 0.0001 g) and re-weighed after being dried in an oven at 50 °C for 5–7 days. We also inspected each eggshell for any yolk that was not internalized prior to hatching and weighed this yolk separately from the internalized yolk (and re-weighed after being dried as above). Each hatchling was then X-rayed and measurements of hindlimb length, forelimb length, head width and head length were taken using ImageJ software (e.g. Prosser, Hudson & Thompson, 2006). Left and right side measurements were averaged for analysis. After morphological measurements, each hatchling was placed in an oven for 5 days at 50 °C in order to measure dry carcass mass and to calculate the water content of each individual [live mass – (dry carcass mass + dry residual yolk mass)]. Sample sizes ranged from 13 to 18 hatchlings per treatment.

Statistical analyses

For the eggs that were allowed to hatch, we used logistic regressions to evaluate the effect of moisture and temperature (and their interaction) on egg survival. Mixed model analysis of variance (ANOVA) was used to evaluate the effect of incubation temperature and moisture on incubation duration (i.e. days spent in incubation treatment to hatching). Mixed-model repeated-measures analysis of covariance (ANCOVA)

was used to evaluate changes in egg mass during the incubation period. This analysis included temperature and moisture (and their interaction) as independent variables, egg mass as the repeated dependent variable (at days 0, 7, 14 and 21 after oviposition) and egg mass at day 0 as the covariate. All mixed models used maternal identity as a random effect. Although our data did not meet the sphericity assumption for repeated measures, *P*-values adjusted by the Hunyh–Feldt epsilon correction yielded the same results as our uncorrected analyses.

For eggs that were terminally sampled at different points in development, we used mixed-model ANOVAs or ANCOVAs that included incubation moisture (–150 and –450 kPa), temperature (26 °C, 28 °C and 30 °C), day (7, 14 and 21), and their interactions as fixed independent variables. All mixed-model analyses included maternal identity as a random effect. Separate ANCOVAs were used for analysis of each dependent variable because different variables often required different covariates. Dependent variables included embryo stage, embryo mass, embryo water content, yolk mass and yolk water content. The water content of embryos was calculated as the difference between wet and dry embryo mass, using wet embryo mass as a covariate. Yolk dry mass was evaluated using egg mass a covariate. The water content of egg yolk was calculated as the difference between wet and dry yolk mass, using wet yolk mass as a covariate. Results from analysis of embryo and yolk mass did not differ whether based on dry vs. wet mass, thus we report only results from analyses of dry mass. In our figures, water content of embryos and yolk is expressed as a percentage {i.e. [(wet mass – dry mass)/wet mass] × 100}.

Because developmental rate is temperature dependent in reptiles (Andrews, 2004), we performed an additional set of analyses to determine whether developmental variation (e.g. in embryo and yolk mass and water content) can be explained by incubation temperature independent of embryo stage. Thus, for these analyses, we evaluated the effects of incubation temperature corrected for embryo stage. Analyses were performed with four separate mixed-model ANCOVAs using temperature as a fixed independent variable with embryo dry mass, embryo water content, yolk dry mass and yolk water content as dependent variables; embryo stage was a covariate and maternal identity was a random effect. Embryos at stage 17 or greater (i.e. primarily advanced embryos from the 30 °C treatment at day 21) were removed from these analyses so that all three temperature treatments had an equal range of embryo stages (stages 6–16). By removing these individuals, the exponential relationship between embryo stage and mass (see Results) was linearized for parametric analysis.

Mixed-model ANCOVAs were used to evaluate the effects of incubation temperature, moisture and their interaction on offspring morphology and internalized residual yolk. For analyses of offspring SVL and body mass, egg mass at oviposition was used as a covariate. Snout–vent length was used as a covariate for analyses of tail length and body condition (i.e. mass relative to SVL). Live body mass and dry carcass mass were used as covariates in analyses of wet and dry internalized residual yolk mass, respectively. Water content of residual yolk was evaluated as the difference between wet and dry residual yolk mass, with wet residual yolk mass as a covariate. Similarly, water content of hatchlings was evaluated as the difference between live and dry carcass mass of hatchlings, using live mass as a covariate. Logistic regression was used to evaluate the effect of moisture and temperature (and their interaction) on the presence of non-internalized yolk within the eggshell. Treatment effects on limb and head measurements were initially evaluated with multivariate ANCOVA using SVL as a covariate and then followed up with individual mixed-model ANCOVAs for each trait. Maternal identity was included as a random effect in all univariate ANOVAs or ANCOVAs. We excluded sex as a factor in our analyses because preliminary results revealed that incubation temperature, moisture and their interaction did not affect offspring sex ratios (generalized linear mixed model: all P-values > 0.210), and that males and females did not differ in any phenotypic trait (mixed-model ANOVAs: all P-values > 0.141).

All variables were checked for normality and log-transformed if needed. However, because overall results did not differ between analyses of raw vs. transformed data, we present results based on analyses of raw data. Additionally, Bartlett's tests for each trait indicated that our data met parametric assumptions of homogeneity of variances. For all ANCOVAs, interactions between the main effects and covariates were retained if significant and removed from final models if non-significant (Engqvist, 2005). To account for non-independence of dependent variables in separate ANCOVAs, sequential Bonferroni corrections were used across analyses (Rice, 1989). All analyses were performed with SAS software (version 9.2; SAS Institute, 1997).

RESULTS

Treatment effects on embryonic development and egg physiology

Thermal and hydric conditions during egg incubation had strong, but different, effects on patterns of embryonic development, egg water uptake and yolk metabolism (Table 1). Incubation duration was strongly

Table 1. Effect of egg incubation temperature and moisture on embryonic development of the brown anole (*Anolis sagrei*). *P*-values in bold face are statistically significant after Bonferroni corrections. Directions of effects are described in text or can be visualized in Figures 1–4

	Covariate	Temperature	Moisture	Temperature × moisture	Day	Temperature × day	Moisture × day	Temperature × moisture × day
Live egg mass (g)*	Egg mass (g) at oviposition	$F_{2,294} = 33.4$, ***P* < 0.001**	$F_{1,294} = 30.0$, ***P* < 0.001**	$F_{2,294} = 8.3$, ***P* < 0.001**	$F_{3,294} = 566.0$, $P <$ **0.001**	$F_{6,294} = 10.9$, ***P* < 0.001**	$F_{3,294} = 6.5$, ***P* < 0.001**	$F_{6,294} = 5.0$, ***P* < 0.001**
Embryo stage†	–	$F_{2,55} = 58.0$, ***P* < 0.001**	$F_{1,55} = 0.0$, $P = 0.983$	$F_{2,55} = 0.5$, $P = 0.619$	$F_{2,55} = 411.8$, ***P* < 0.001**	$F_{4,55} = 1.1$, $P = 0.363$	$F_{2,55} = 0.4$, $P = 0.687$	$F_{4,55} = 0.2$, $P = 0.926$
Embryo dry mass†	–	$F_{2,54} = 90.0$, ***P* < 0.001**	$F_{2,54} = 1.3$, $P = 0.263$	$F_{2,54} = 3.2$, $P = 0.047$	$F_{2,54} = 294.2$, ***P* < 0.001**	$F_{4,54} = 43.2$, ***P* < 0.001**	$F_{2,54} = 1.1$, $P = 0.345$	$F_{4,54} = 1.5$, $P = 0.220$
Embryo water content (live embryo mass–embryo dry mass)†	Embryo live mass (g)	$F_{2,53} = 49.6$, ***P* < 0.001**	$F_{1,53} = 0.8$, $P = 0.368$	$F_{2,53} = 2.5$, $P = 0.090$	$F_{2,53} = 40.0$, ***P* < 0.001**	$F_{4,53} = 51.3$, ***P* < 0.001**	$F_{2,53} = 0.1$, $P = 0.943$	$F_{4,53} = 4.1$, ***P* = 0.006**
Yolk dry mass (g)†	Egg mass (g) at oviposition	$F_{2,58} = 9.2$, ***P* < 0.001**	$F_{1,58} = 0.0$, $P = 0.949$	$F_{2,58} = 0.3$, $P = 0.733$	$F_{2,58} = 31.8$, ***P* < 0.001**	$F_{4,58} = 5.8$, ***P* = 0.001**	$F_{2,58} = 3.1$, $P = 0.055$	$F_{4,58} = 1.6$, $P = 0.179$
Yolk water content (yolk wet mass–yolk dry mass)†	Yolk wet mass (g)	$F_{2,58} = 7.5$, ***P* = 0.001**	$F_{1,58} = 0.1$, $P = 0.793$	$F_{2,58} = 0.3$, $P = 0.775$	$F_{2,58} = 26.3$, ***P* < 0.001**	$F_{4,58} = 5.7$, $P <$ **0.001**	$F_{2,58} = 4.2$, $P = 0.019$	$F_{4,58} = 0.8$, $P = 0.503$

*Analyses were performed with mixed-model repeated-measures ANCOVA with maternal identity as a random effect.
†Analyses were performed with mixed model ANOVA or ANCOVA with maternal identity as a random effect. In all cases, interactions between main effects and covariates were never significant and these terms were removed from final models.

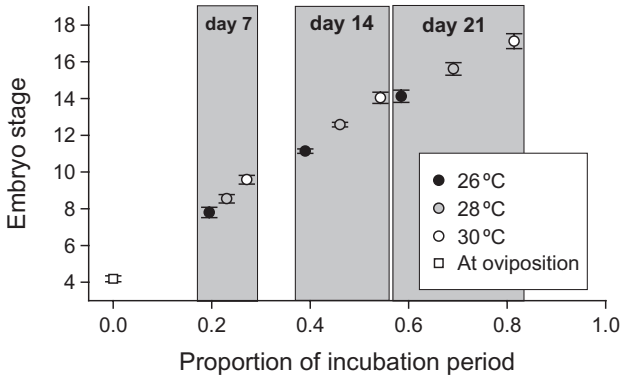

Figure 1. Effect of egg incubation temperature on embryo developmental stage at four time periods during incubation (at oviposition, day 7, day 14 and day 21). Because results were nearly identical between the dry and wet treatments (Table 1), data from both moisture treatments are combined. Embryos were staged according to the criteria of Sanger *et al*. (2008b); oviposition is around embryonic stage 4 and full-term embryos hatch at stage 19. Data are summarized as means ± 1 standard error. Eggs sampled at oviposition (day 0) were not exposed to any incubation treatment. Statistical results are reported in Table 1.

affected by temperature ($F_{2,49} = 454.2$, $P < 0.001$), but not by moisture ($F_{1,49} = 0.0$, $P = 0.918$) or the temperature by moisture interaction ($F_{2,49} = 0.2$, $P = 0.812$). Incubation duration was shortest at warm temperatures, and all three temperature treatments differed significantly from each other (mean ± SE; at 30 °C, 25.8 ± 0.19 days; at 28 °C, 30.4 ± 0.24 days; at 26 °C, 35.9 ± 0.27 days). This pattern was reflected in our analysis of the embryonic stage (Fig. 1). Warm temperatures accelerated embryonic development, as indicated by successively more advanced embryos at respectively higher temperatures at each sampling period; this pattern was similar between moisture treatments (i.e. non-significant temperature × moisture interaction; Table 1). For eggs that were not sampled during incubation, overall survival was high (92.2%) and was not influenced by incubation temperature or moisture (temperature: $\chi^2 = 0.004$, $P = 0.998$; moisture: $\chi^2 = 0.004$, $P = 0.951$; interaction $\chi^2 = 0.005$, $P = 0.998$).

All eggs gained mass during incubation, but the change in egg mass varied depending on temperature and moisture treatments (Table 1, Fig. 2). Eggs incubating under dry conditions gained less mass than those under wet conditions, and this pattern was most pronounced at 30 °C toward the end of incubation (Fig. 2C). Embryo mass increased substantially during incubation and, by day 21, embryos in the 30 °C treatment were substantially heavier than those from the 28 °C and 26 °C treatments (Fig. 3A, B). Despite increases in egg and embryo mass, the water content of embryos decreased during incubation and this decline occurred most rapidly between days 14 and 21 of incubation under our 30 °C treatment (Fig. 3C, D). Incubation moisture did not affect embryo mass or hydration (Table 1).

The patterns for yolk mass and water content (Fig. 4) were opposite to those observed for embryo mass and water content. Yolk mass declined during incubation, and embryos from successively warmer temperatures metabolized greater amounts of yolk by day 21 (Fig. 4A, B). Although the amount of yolk decreased during development, the relative water content of yolk increased (Fig. 4C, D); this increase in yolk water content varied under increasingly warmer incubation temperatures. Change in yolk mass and water content during incubation was not influenced by moisture (Table 1).

Overall, successively warmer incubation temperatures resulted in increasingly heavier embryos (with low water content) with smaller quantities of yolk (with high water content) by day 21 of incubation. However, our final analyses suggest that this variation in embryo and yolk mass and water content is largely attributable to successively more advanced embryos at warmer temperatures, rather than to a direct effect of temperature per se (Fig. 5). Indeed, in most cases, the effect of incubation temperature disappeared when analyses were corrected for embryonic stage. Incubation temperature did not explain variation in embryo water content ($F_{2,55} = 2.3$, $P = 0.749$), yolk dry mass ($F_{2,57} = 1.3$, $P = 0.273$) or yolk water content ($F_{2,57} = 0.1$, $P = 0.947$) when adjusted for embryo stage. The effect of incubation temperature on embryo mass remained significant when adjusted for embryo stage ($F_{2,55} = 3.5$, $P = 0.036$), but the differences among treatments were relatively small (mean ± SE; 26 °C: 4.9 ± 0.2 mg; 28 °C: 4.9 ± 0.2 mg; 30 °C: 4.1 ± 0.2 mg) and did not remain significant after Bonferroni correction.

Treatment effects on hatchling morphology and residual yolk

The only phenotypic effect of incubation temperature was on offspring body size (SVL and mass) and the strength of the effect depended on egg size (Table 2; Fig. 6). In most cases, larger eggs produced larger offspring, but the relationship between egg size and offspring size differed among temperature (for SVL and body mass) and moisture (for body mass) treatments (Fig. 6A, B, C). The relatively steep relationship between egg mass and SVL for hatchlings from the 26 °C treatment suggests that cool conditions produce relatively large offspring, but only for large eggs. Hatchling SVL was not influenced by egg mass in the 28 °C treatment. Variation in hatchling body

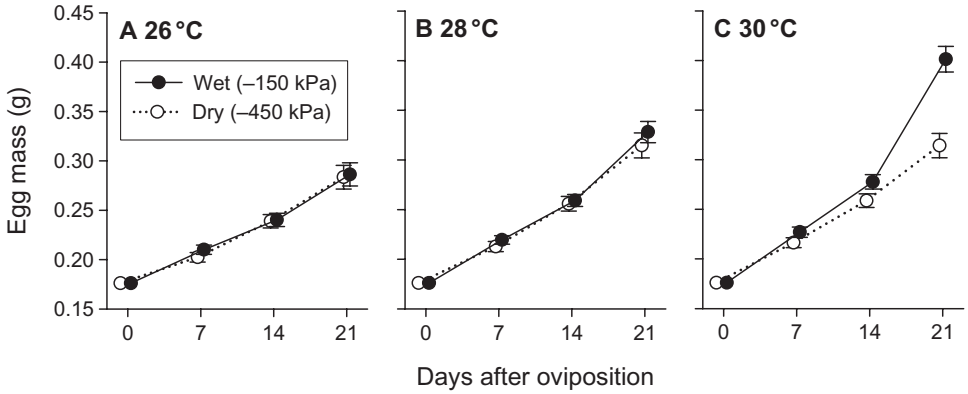

Figure 2. Effect of temperature and moisture on mass gain of *Anolis sagrei* eggs during incubation. Data are summarized as least-squares means ± 1 standard error. Each graph represents egg incubation temperatures of (A) 26 °C, (B) 28 °C and (C) 30 °C. Statistical results are reported in Table 1.

mass was influenced by interactions among temperature, moisture and egg mass (albeit this three-way interaction was not significant after Bonferroni correction; Table 2). Generally, body mass was positively related to egg mass, but this relationship was reversed under the hottest and driest incubation condition (Fig. 6C).

Overall, relatively wet egg incubation conditions (–150 kPa) produced hatchlings that were longer in SVL and in better body condition than individuals from the dry treatment (–450 kPa); this pattern was most pronounced for hatchlings from the 30 °C temperature treatment (albeit, moisture × temperature interactions were marginally non-significant, *P*-values = 0.088; Fig. 6D, E). The moisture effect remained significant for body condition after Bonferroni corrections, but did not for SVL. Additionally, although the water content of hatchlings was substantial (80.8% of live body mass, SD = 1.2, range = 77.9–83.3%), incubation temperature and moisture did not affect the hydration of hatchlings. Variation in other morphological traits (tail length, limb lengths, head width and head length) was not substantially influenced by incubation temperature or moisture (Table 2). A multivariate analysis of covariance (MANCOVA) indicated no effect of incubation conditions on these morphological variables (temperature: Wilks' $\lambda = 0.92$, $P = 0.392$; moisture: Wilks' $\lambda = 0.03$, $P = 0.556$; interaction: Wilks' $\lambda = 0.96$, $P = 0.750$), but individual ANOVAs suggested that hatchlings from the wet treatment (mean ± SE: 4.04 mm ± 0.02) had slightly wider heads than those from the dry treatment (mean ± SE: 3.93 mm ± 0.03), albeit this small difference was not significant after Bonferroni correction.

Only 13.5% of the eggs that hatched contained non-internalized residual yolk left in the eggshell and this frequency did not differ among treatments (moisture: $\chi^2 = 0.9$, $P = 0.954$; temperature: $\chi^2 = 0.00$, $P = 0.979$; interaction: $\chi^2 = 0.29$, $P = 0.867$). On average, internalized residual yolk made up 1.3% of hatchling live mass (SD = 1.2, range: 0–4.6%) and heavier hatchlings tended to have more residual yolk than lighter individuals (wet yolk mass: $r^2 = 0.12$, $P = 0.001$; dry yolk mass: $r^2 = 0.05$, $P = 0.038$). Moisture levels during egg incubation explained a significant amount of variation in the quantity of wet residual yolk (Table 2) and marginally explained the amount of variation in dry residual yolk mass ($P = 0.070$). Hatchlings from the dry incubation treatment contained more internalized yolk than those from the wet treatment; the effect of incubation moisture on the wet mass of residual yolk was most pronounced when eggs were incubated at 30 °C (Fig. 6F), but the temperature by moisture interaction was not significant ($P = 0.104$; Table 2). Overall, wet residual yolk comprised 68.6% water on average (SD = 21.7) and the proportion of water in internalized residual yolk was not affected by temperature or moisture conditions during incubation (Table 2).

DISCUSSION

The primary objective of this study was to quantify the effects of temperature and moisture conditions on developmental rate, egg water uptake, embryonic yolk metabolism and subsequent effects on fitness-related phenotypes of hatchling lizards. Overall, we found that thermal and hydric conditions had different effects on rates of development, yolk metabolism and offspring morphology. For example, patterns of embryo hydration, yolk metabolism and yolk hydration were indirectly influenced by incubation temperature via thermal effects on embryo stage at different times of incubation. The phenotypic consequences (in terms of body size) of incubation temperature depended on egg size. Additionally, variation in egg water uptake

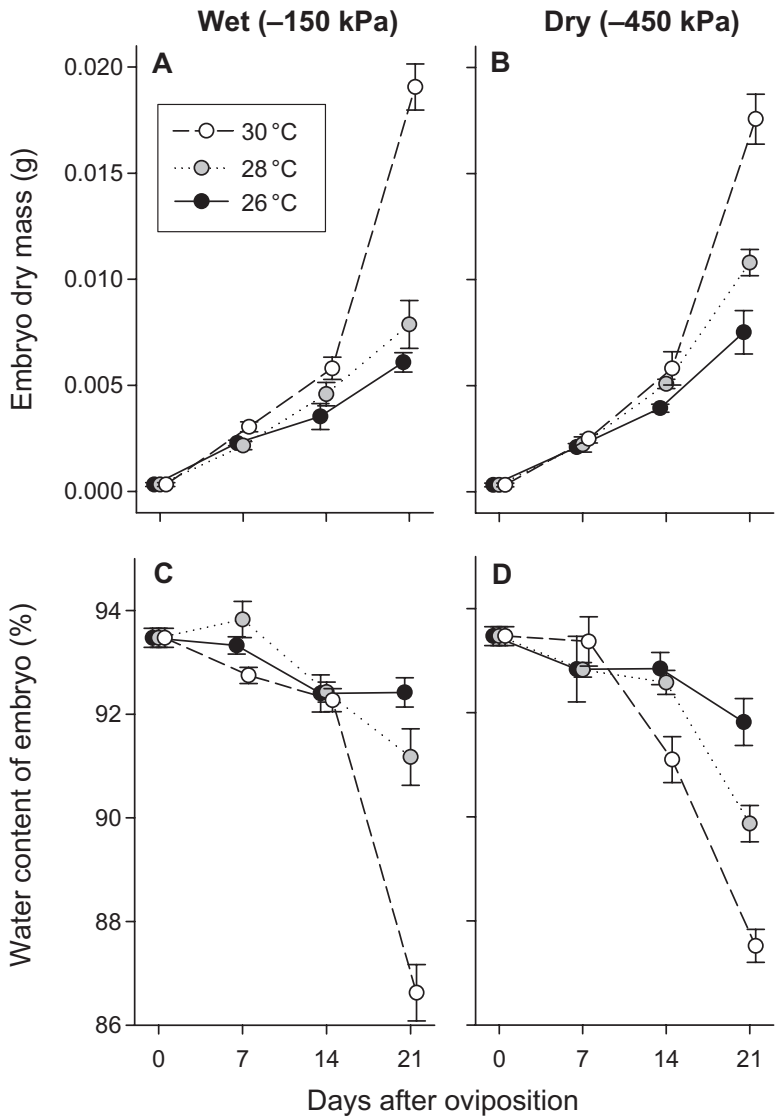

Figure 3. Effect of egg incubation temperature and moisture on dry embryo mass (A, B) and on water content of embryos (C, D) at four different periods during incubation. Left panels represent egg incubation under wet (–150 kPa) conditions and right panels represent egg incubation under dry (–450 kPa) conditions. Data are summarized as means ± 1 standard error. Eggs sampled at oviposition (day 0) were not exposed to any incubation treatment and were not included in the statistical analyses. Statistical results are reported in Table 1.

due to moisture availability influenced both offspring body size and the quantity of internalized residual yolk. This effect of incubation moisture on body size and residual yolk suggests that relatively wet incubation conditions facilitate yolk metabolism (Packard *et al*., 1987; Miller & Packard, 1992).

Thermal and hydric effects on development and offspring phenotypes

As observed in virtually all ectothermic organisms, warm egg incubation temperatures accelerated embryonic development. The temperature effect on developmental rate has important consequences for embryo stage, mass and relative yolk availability at any given time during development (Morris *et al*., 1983; Packard *et al*., 1987). For example, because embryo metabolism increases with developmental stage and increasing temperatures (Miller & Packard, 1992), the relatively high quantities of yolk in eggs from the 26 °C treatment at most sampling periods resulted from slower developmental rate (and, hence, less advanced embryonic stage) at this low temperature. However, despite this thermal effect on the rate of yolk metabolism,

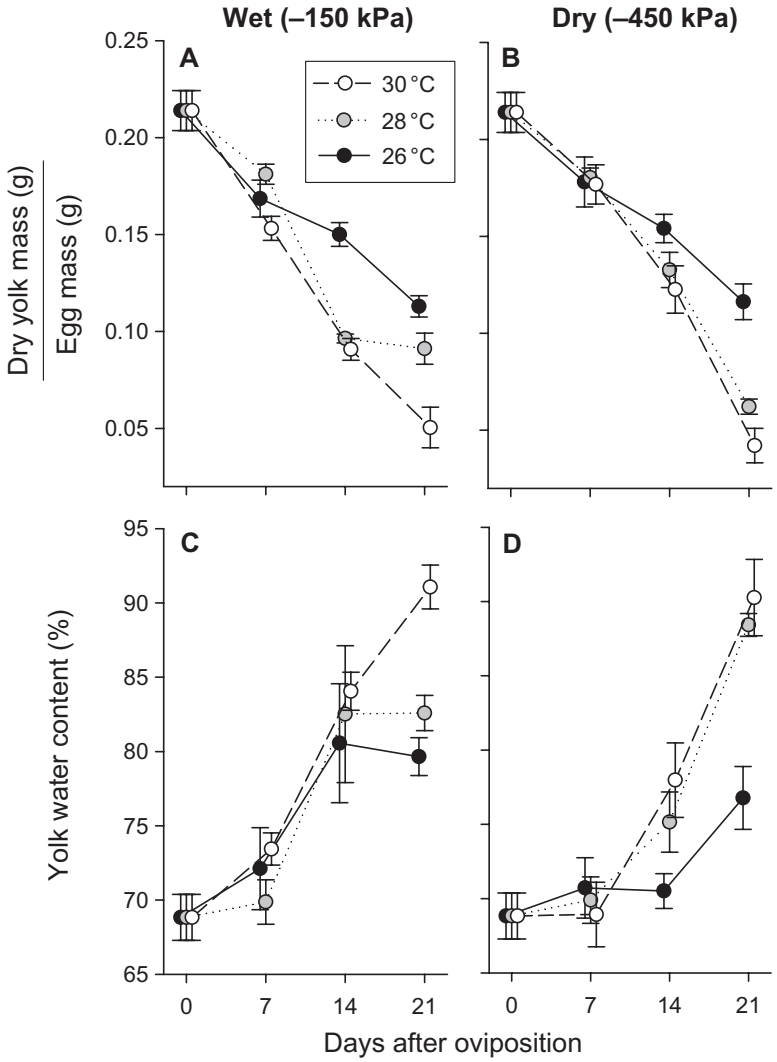

Figure 4. Effect of egg incubation temperature and moisture on dry yolk mass relative to egg mass (A, B) and on proportion of water in yolk (C, D) at four different periods during incubation. Left panels represent egg incubation under wet (–150 kPa) conditions and right panels represent egg incubation under dry (–450 kPa) conditions. Data are summarized as means ± 1 standard error. Eggs sampled at oviposition (day 0) were not exposed to any incubation treatment and were not included in the statistical analyses. Statistical results are reported in Table 1.

offspring in the cool treatment still hatched with quantities of residual yolk (albeit, over a longer incubation duration) similar to those from our warm treatment. Additionally, the relatively well-hydrated embryos under cool incubation conditions (at each day of sampling) did not result in a temperature effect on hydration of hatchlings. These findings demonstrate that final developmental endpoints are similar, but the time to reach the endpoints depends on temperature.

Thermal effects on offspring body size depended on egg size. In most cases, relatively large eggs produced large offspring, but hatchling SVL was not affected by egg size when incubated at the intermediate temperature. The thermal effect on body mass also depended on egg size, but did not mirror the pattern for SVL. Under relatively extreme incubation conditions (hot and dry), small eggs produced heavy hatchlings. This negative relationship between egg mass and hatchling mass is counter-intuitive, but suggests that incubation temperatures might modify yolk conversion into tissue or fat, depending on the quantity of yolk available at oviposition. Although we show that embryo yolk metabolism is embryo-stage-dependent (regardless of temperature), work on other lizards has experimentally shown that yolk quantity in eggs at oviposition can also affect embryonic yolk metabolism (Radder, Shanbhag & Saidapur, 2004) and stressful developmental environments can affect other aspects

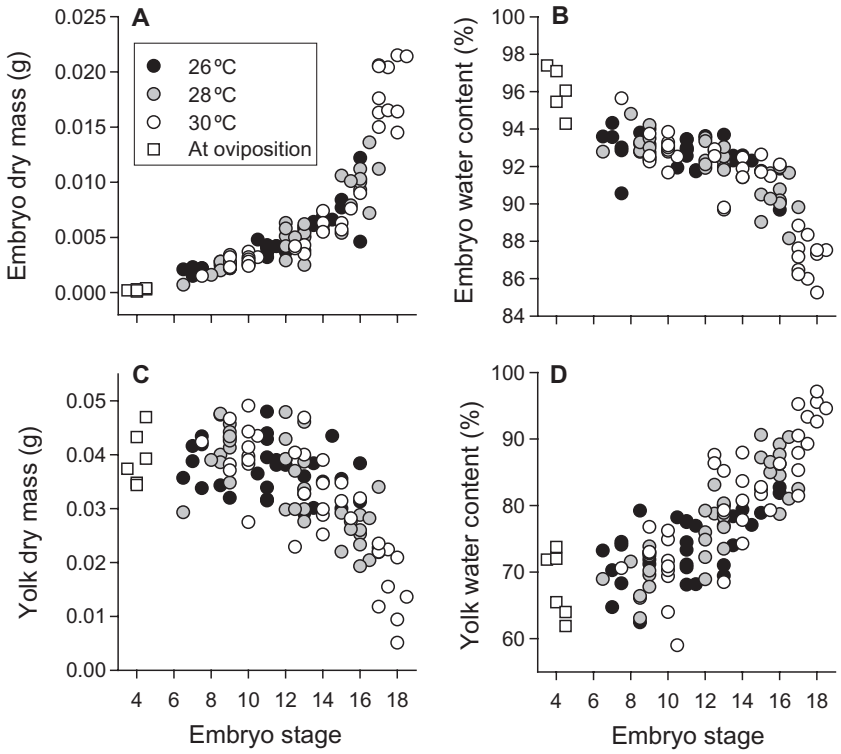

Figure 5. Relationships between embryo stage with (A) embryo dry mass ($r = 0.94$, $P < 0.001$; exponential fit), (B) embryo water content ($r = –0.68$, $P < 0.001$; linear fit), (C) yolk dry mass ($r = –0.66$, $P < 0.001$; linear fit) and (D) yolk water content ($r = 0.76$, $P < 0.001$; linear fit). Embryos at stages greater than 16 were removed from slopes tests so that an equal range of embryo stages was represented among temperature treatments; this linearized the relationships for parametric analyses (ANCOVA) and resultant slopes did not differ among temperature treatments for any analysis (see text). Embryos were staged according to the criteria of Sanger *et al*. (2008b); oviposition is around embryonic stage 4 and full-term embryos hatch at stage 19. Eggs sampled at oviposition (squares; embyro stages ranged from 3.5 to 5) were not assigned a temperature treatment.

of development (e.g. effect of yolk corticosterone on developmental rate, sexual development and post-hatching growth; Weiss, Johnston & Moore, 2007; Warner, Radder & Shine, 2009). Although this finding has important implications for how interactions between maternal investment (egg size) and nest conditions might shape offspring phenotypes, we urge caution with interpretation because this three-way interaction was not significant after Bonferroni correction.

Moisture conditions had relatively little, if any, effect on patterns or rates of development, but had significant effects on hatchling body size. We found no effect of incubation moisture on the water content of embryos or of their unused yolk. These results contrast with those of a similar study on snapping turtles (*Chelydra serpentina*) that show strong effects of substrate water potential on the hydration of embryos and unused yolk during development (Morris *et al*., 1983). These contrasting results may be attributable to our relatively small differences in substrate water potential (–150 vs. –450 kPa compared with –150 vs. –850 used in Morris *et al*., 1983), which resulted in a relatively minor difference in overall water uptake of eggs between moisture treatments (Fig. 2A, B); the only substantial difference in egg mass gain was toward the latter part of incubation under 30 °C (Fig. 2C). These minor differences in water uptake between moisture treatments may explain the lack of a moisture effect on embryo and yolk hydration. Indeed, eggs of *A. sagrei* may be extremely efficient at absorbing moisture from their surrounding environment, even under relatively dry conditions. Similar suggestions have been made in other squamate reptiles because of virtually no moisture effect on embryonic use of energy or resultant offspring phenotypes (Ji & Braña, 1999; Flatt *et al*., 2001; Brown & Shine, 2005; Robbins & Warner, 2010). Additionally, water gained by eggs does not necessarily go into the embryo, but instead can be stored in the allantoic sac (Belinsky *et al*., 2004). Nevertheless, despite no effect of moisture on the water content of embryos and yolk,

Table 2. Effect of egg incubation temperature and moisture conditions on morphology and residual yolk of hatchling brown anoles (*Anolis sagrei*). Non-significant interaction terms between main effects and covariates were removed from final ANCOVA models, but significant terms were retained [only for snout–vent length (SVL) and body mass]. *P*-values in bold face are statistically significant after Bonferroni corrections

Morphological trait	Covariate	Temperature	Moisture	Temperature × moisture	Temperature × covariate	Moisture × covariate	Temperature × moisture × covariate
Snout–vent length (mm)	Egg mass	$F_{2,46} = 6.1$, **$P = 0.005$**	$F_{1,46} = 4.3$, $P = 0.043$	$F_{2,46} = 2.6$, $P = 0.089$	$F_{2,46} = 6.2$, $P =$ **0.004**	–	–
Tail length (mm)	SVL	$F_{2,48} = 0.9$, $P = 0.436$	$F_{1,48} = 0.0$, $P = 0.947$	$F_{2,48} = 2.2$, $P = 0.117$	–	–	–
Body mass (g)							
Live mass (with residual yolk)	Egg mass	$F_{2,43} = 5.1$, **$P = 0.010$**	$F_{1,43} = 12.9$, **$P < 0.001$**	$F_{2,43} = 2.7$, $P = 0.076$	$F_{2,43} = 6.0$, **$P = 0.005$**	$F_{1,43} = 16.7$, $P <$ **0.001**	$F_{2,43} = 3.9$, $P = 0.027$
Dry carcass mass*	Egg mass	$F_{2,43} = 3.9$, $P = 0.029$	$F_{1,43} = 14.7$, **$P < 0.001$**	$F_{2,43} = 3.0$, $P = 0.059$	$F_{2,43} = 4.7$, **$P = 0.015$**	$F_{1,43} = 18.0$, **$P < 0.001$**	$F_{2,43} = 3.9$, $P = 0.028$
Hatchling water content*†	Live body mass	$F_{2,48} = 0.3$, $P = 0.719$	$F_{1,48} = 1.5$, $P = 0.225$	$F_{2,48} = 0.1$, $P = 0.885$	–	–	–
Residual yolk (g)							
Wet residual yolk	Live body mass	$F_{2,48} = 1.1$, $P = 0.350$	$F_{1,48} = 5.9$, **$P = 0.019$**	$F_{2,48} = 2.4$, $P = 0.104$	–	–	–
Dry residual yolk	Dry carcass mass*	$F_{2,48} = 1.6$, $P = 0.205$	$F_{1,48} = 3.5$, $P = 0.070$	$F_{1,48} = 1.5$, $P = 0.246$	–	–	–
Water content of residual yolk‡	Wet residual yolk	$F_{2,48} = 0.3$, $P = 0.741$	$F_{1,48} = 0.3$, $P = 0.589$	$F_{2,48} = 0.3$, $P = 0.711$	–	–	–
Body condition (live mass, g)	SVL	$F_{2,48} = 0.5$, $P = 0.615$	$F_{1,48} = 7.6$, **$P = 0.008$**	$F_{2,48} = 2.6$, $P = 0.088$	–	–	–
Hindlimb length (mm)	SVL	$F_{2,81} = 1.4$, $P = 0.246$	$F_{1,81} = 0.4$, $P = 0.550$	$F_{2,81} = 2.3$, $P = 0.109$	–	–	–
Forelimb length (mm)	SVL	$F_{2,81} = 1.0$, $P = 0.372$	$F_{1,81} = 0.8$, $P = 0.363$	$F_{2,81} = 1.8$, $P = 0.173$	–	–	–
Head width (mm)	SVL	$F_{2,82} = 0.3$, $P = 0.761$	$F_{2,82} = 5.3$, $P = 0.023$	$F_{2,82} = 1.5$, $P = 0.228$	–	–	–
Head length (mm)	SVL	$F_{2,82} = 0.0$, $P = 0.993$	$F_{2,82} = 0.0$, $P = 0.994$	$F_{2,82} = 0.2$, $P = 0.836$	–	–	–

*Dry residual yolk mass was included in the estimate of dry carcass mass.
†Hatchling water content = live mass – dry carcass mass.
‡Water content of residual yolk = wet residual yolk – dry residual yolk.

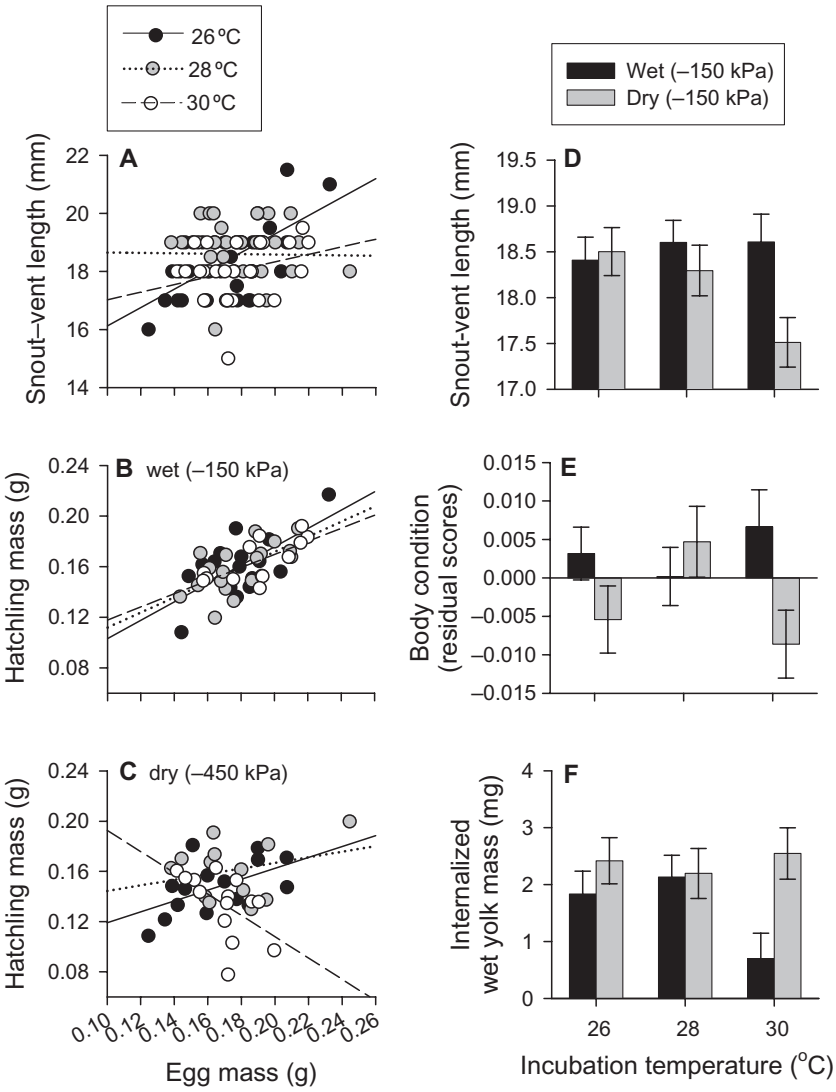

Figure 6. Effect of egg incubation temperature and moisture on offspring morphology and residual yolk in *Anolis sagrei*. Effect of incubation temperature on (A) snout–vent length (SVL) across egg sizes, and incubation temperature effects on hatchling mass across egg sizes under wet (B) and dry (C) incubation conditions. Effect of incubation temperature and moisture on (D) SVL, (E) body condition (residual scores from the regression of body mass on SVL) and (F) internalized wet yolk mass. Data in (D–F) are summarized as least-squares means ± 1 standard error. Statistical results are reported in Table 2.

hatchlings from wet-incubated eggs were significantly heavier than those from dry-incubated eggs. The wet-incubated hatchlings also had lower quantities of internalized residual yolk, but did not differ in overall hydration. These findings are consistent with studies on other reptiles, as wet incubation substrates facilitate yolk metabolism (converted into tissue or fat), resulting in relatively large offspring (attributable to fat or tissue development, rather than hydration) with low quantities of residual yolk (Packard, Packard & Boardman, 1980; Packard *et al*., 1988; Janzen *et al*., 1990; Phillips *et al*., 1990; Christian, Lawrence & Snell, 1991; Miller & Packard, 1992).

Potential fitness consequences of thermal and hydric effects

Thermal and hydric conditions during development can affect offspring phenotypes in ways that can influence fitness (Janzen, 1993; Andrews *et al*., 2000; Warner & Shine, 2008). Thus, quantifying thermal and hydric reaction norms of developing embryos can

provide important insights into optimal egg environments. This information can be used to predict how mothers should adaptively select microhabitats for oviposition (Refsnider & Janzen, 2010).

The effect of egg thermal environments on developmental rate could have fitness consequences for offspring. For instance, eggs that experienced 30 °C during incubation hatched 5–11 days earlier than those at the cooler incubation temperatures. Accelerated development at warm temperatures could potentially enhance offspring fitness via earlier hatching, which has been shown to benefit hatchling survival and future reproductive success in other species (Perrins, 1967; Olsson & Shine, 1997; Warner & Shine, 2007; Wapstra *et al*., 2010). Importantly, however, warm-incubated eggs will not always hatch before cool-incubated eggs because of variation in oviposition date. Given that *A. sagrei* produce *c*. one egg per week across an extended egg-laying season, interactions between nest temperature and oviposition date would dampen any fitness consequences of temperature-related timing of hatching (Warner & Shine, 2009). A second potential benefit of shortened incubation periods (because of temperature) involves a decreased amount of time that developing embryos would be exposed to predators or environmental extremes (Shine, 2002). Given these potential benefits of warm incubation temperatures, we predict that females can enhance their fitness by choosing relatively warm micro-environments (with sufficient moisture to sustain embryogenesis) as oviposition sites.

Although thermal and hydric environments during incubation affected offspring body size, potential fitness benefits are unclear. Most combinations of temperature and moisture conditions had little effect on hatchling body size, but body mass of hatchlings from small eggs was enhanced when incubated under hot and dry conditions (perhaps the most stressful treatment). Thus, if large body size enhances offspring fitness, then females that produce small eggs might benefit from nesting in relatively dry and warm microhabitats; such benefits remain speculative at best. Our results also demonstrate that offspring hatch at relatively large sizes under wet incubation conditions, which may enhance offspring locomotor performance (Miller, Packard & Packard, 1987) or survival (Janzen, 1993). Importantly, however, although large body size has been shown to benefit offspring survival in a diversity of organisms (Janzen, Tucker & Paukstis, 2000; Cleasby *et al*., 2010; Perez & Munch, 2010), associations between body size and survival in hatchling lizards is relatively uncommon (Sinervo *et al*., 1992; Warner & Andrews, 2002; Warner & Shine, 2007; Robbins & Warner, 2010; but see Laurie & Brown, 1990; Marguis, Massot & Le Galliard, 2008). Indeed, offspring from wet-incubated eggs were on average only 2.2% longer (in SVL) and 7.9% heavier than those from dry-incubated eggs. Whether this mass difference is attributable to tissue or fat could impact offspring growth, but substantially greater variation in body size is likely needed to detect size-related variation in survival (e.g. Sinervo *et al*., 1992; Warner & Andrews, 2002).

Internalized residual yolk can serve as an important energy store in many reptiles and birds (Troyer, 1987; Turro *et al*., 1994; Nagle, Burke & Congdon, 1998; Willette, Tucker & Janzen, 2005), but its functional significance in *A. sagrei* is questionable. Hatchlings from wet-incubated eggs contained 34.9% less residual yolk than those from dry-incubated eggs. Although this is a relatively large difference between moisture treatments, residual yolk made up only an average of 1.3% of hatchling body mass. To our knowledge, this is the smallest relative quantity of residual yolk reported in a reptile and it fits well with expectations given the small body size and egg size of *A. sagrei* (Belinsky *et al*., 2004). This small quantity of residual yolk in *A. sagrei* is unlikely to have any serious fitness consequences, as experimentally demonstrated in a moderately sized lizard species (Radder *et al*., 2007). These results suggest that developmental conditions likely have minimal impacts on fitness via an effect on offspring morphology or yolk stores in *A. sagrei*, but studies that evaluate the effects of more extreme conditions are warranted. Indeed, embryos of other lizard species can tolerate conditions beyond those used in the present study, and incubation-induced phenotypic variation may have been more substantial under more extreme conditions (e.g. Andrews *et al*., 2000; Warner & Shine, 2005). Additionally, although natural thermal fluctuations inside *A. sagrei* nests are unknown, future studies that incorporate appropriate thermal diel variation will provide critical insights (e.g. Ashmore & Janzen, 2003; Du & Ji, 2006; Warner & Shine, 2011).

CONCLUSIONS

The genus *Anolis* has served as an important model in ecological and evolutionary research, yet surprisingly little is known about how egg incubation environments impact embryo development and offspring morphology in this group (Andrews & Sexton, 1981; Losos, Schoener & Spiller, 2003; Goodman & Walguarnery, 2007; Goodman, 2008; Warner & Chapman, 2011). We show that hydric and thermal conditions experienced during development can greatly impact rates of development and offspring size, and some phenotypic effects might depend on maternal egg investment (egg size). However, because of the minor differences among treatments, the biological significance these

short-term phenotypic effects are likely small. Because morphological adaptation to local habitats has been critical in the radiation of *Anolis* lizards (Losos, 2009), our findings are important as they partially rule out the developmental environment as a source of substantial morphological variation (at least in *A. sagrei*). For example, although limb morphology is influenced by incubation temperature in other lizard species (Braña & Ji, 2000; but see Warner & Shine, 2005), temperature does not seem to be an important source of variation in *A. sagrei*. Instead, post-hatching environments (e.g. perch diameter) during early growth may be more important in shaping variation in this critical trait (Losos *et al*., 2000; Kolbe & Losos, 2005).

Theory predicts that natural selection will have different impacts on organismal sensitivities to environmental conditions under stable vs. variable environments (reviewed in Angilletta, 2009). Under relatively stable environments (e.g. thermal conditions in the tropics), phenotypic development should be adapted to a narrow range of conditions and conditions outside this narrow range could have serious fitness consequences. Under less stable environments, developmental responses should be less sensitive to environmental variation, resulting in very little environmental effect on phenotypic development. *Anolis sagrei* seems to fit under this latter situation because embryonic conditions likely vary substantially because of an extended oviposition period that spans seasons (Lee *et al*., 1989) and the potential exposure to diverse developmental microhabitats (similar to other *Anolis* species; Andrews, 1982). Accordingly, we show that phenotypic development of *A. sagrei* embryos is relatively robust to variation in environmental conditions (at least the conditions tested here). Such developmental canalization may have evolved as a result of long-term selection on optimal phenotypes of hatchlings (Waddington, 1942; Siegal and Bergman, 2002). Importantly, although we did not detect strong thermal and hydric effects on morphology, incubation conditions could influence fitness-related physiological processes that do not translate into morphological phenotypes. Thus, future studies that elucidate the long-term phenotypic and fitness consequences of variable developmental environments are warranted. Overall, although several traits can aid in colonization of new habitats, the broad thermal and hydric tolerances of embryos demonstrated in this short-term study may have also facilitated the successful establishment of *A. sagrei* into novel environments.

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