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Reproductive modes in lizards: measuring fitness consequences of the duration of uterine retention of eggs

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Summary

1. One of the primary axes of life-history variation involves the proportion of embryonic development for which the offspring is retained within its parent's body; understanding trade-offs associated with prolonging that period thus is a critical challenge for evolutionary ecology.
2. Prior to oviposition, most oviparous squamate reptiles retain developing eggs *in utero* for about one-third of embryogenesis; the strong conservatism in this trait is a major puzzle in reptilian reproduction. To clarify fitness consequences of this prolonged uterine retention, we need to experimentally modify the trait and examine the effects of our manipulation.
3. We used transdermal application of corticosterone to induce gravid scincid lizards (*Bassiana duperreyi*) to lay their eggs 'prematurely', with relatively undeveloped embryos. Corticosterone application induced females to oviposit sooner (mean of 5·41 ± 0·51 days post-treatment) at earlier embryonic developmental stage (27 ± 0·21) than did controls (13·2 ± 1·22 days; embryonic stage 30·4 ± 0·16).
4. Corticosterone levels in the egg yolk were unaffected by maternal treatment, so effects of earlier oviposition should not be confounded by endocrine disruption of embryogenesis. Nonetheless, early oviposition reduced hatchling fitness. Hatching success was lower, incubation periods post-laying were increased, and neonates from eggs laid at earlier embryonic stages were smaller and slower.
5. These results suggest that retention of developing eggs *in utero* by oviparous squamates enhances maternal fitness, and does so via modifications to offspring phenotypes rather than (for example) due to accelerated developmental rates of eggs *in utero* compared to in the nest.
6. More generally, our data support optimality models that interpret interspecific variation in the duration of maternal–offspring contact in terms of the selective forces that result from earlier vs. later termination of that maternal investment.

Key-words: endocrine, oviparity, Squamata, uterine retention, viviparity

Introduction

One of the most fundamental axes of interspecific variation in reproductive biology involves the duration of contact between a reproducing animal and its developing offspring. At one extreme, externally fertilizing species shed their gametes into the outside world and never contact their progeny after fertilization. At the other extreme, the embryo is retained within the female's body or within the family unit until it is ready for independent life (Clutton-Brock 1991).

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Prolonged association between parent and developing offspring has evolved many times within a diverse array of animal lineages, providing an ideal model system with which to investigate the selective forces and constraints that may have shaped these evolutionary events. That potential is greatest for lineages that exhibit multiple phylogenetically independent transitions in the duration of parent–offspring contact among closely-related species, and in this respect the squamate reptiles (lizards and snakes) are of particular interest (Blackburn 2006). Viviparity (oviductal retention of offspring until completion of embryogenesis) has evolved from oviparity (laying of eggs with relatively early-stage embryos) at least 100 times within the squamates (Blackburn 1982, 1985, 2006; Shine 1985).

The high frequency of independent evolutionary transitions in reproductive mode within squamates has stimulated intense research interest in this group, and much has been learnt about issues such as the selective forces driving the evolution of viviparity (reviewed by Shine 1985; Blackburn 2006; Shine & Thompson 2006). Nonetheless, some major puzzles remain. One of the most perplexing is the near-ubiquity of prolonged uterine retention of developing eggs in oviparous squamates (e.g. Shine 1983; Blackburn 1995, 2006; Harlow 2004; Calderón-Espinosa, Andrews & Méndez de la Cruz 2006; Shine & Thompson 2006). In a remarkably diverse array of oviparous lizard and snake species, females delay oviposition until the eggs are about one-third of the way through embryogenesis (Shine 1983; Andrews & Mathies 2000; Harlow 2004; Calderón-Espinosa *et al*. 2006). By this time, the embryos have well-formed bodies and a functional circulatory system (Dufaure & Hubert 1961; stage of *c.* 28–30). Unlike other major lineages of reptiles (turtles, crocodilians, tuatara), very few squamates oviposit with embryos at earlier developmental stages (except for chameleons: Díaz-Paniagua & Cuadrado 2003; Andrews & Donoghue 2004). Ever since this pattern was discovered (Shine 1983), authors have speculated on the reasons for such conservatism (e.g. Blackburn 1995; Qualls *et al*. 1995; Qualls, Andrews & Mathies 1997; Qualls & Andrews 1999; Shine & Thompson 2006).

A low variance in any trait frustrates attempts to test adaptive explanations (e.g. Blackburn & Evans 1986; Rose & Lauder 1996) and thus, the consistency in degree of uterine retention among squamates makes it difficult to clarify the selective forces responsible for this widespread pattern. To do so, ideally we need to manipulate the embryonic stage at oviposition and examine fitness consequences of that experimentally induced variation. That aim has been achieved for one aspect of the question: the issue of why even more prolonged uterine retention of eggs (intermediate between 'normal' oviparity and viviparity) is so rare. Experimental work (e.g. Radder, Shanbhag & Saidapur 1998; Warner & Andrews 2003; Calderón-Espinosa *et al*. 2006) has exploited the tendency of some lizard taxa to retain developing eggs until suitably moist oviposition sites are available; that work has substantially clarified the degree to which such 'extra' retention can influence developmental rates and ontogenetic trajectories of the offspring that hatch from those eggs (e.g. Andrews & Mathies 2000; Warner & Andrews 2003). However, the other side of the coin – potential advantages and disadvantages associated with ovipositing earlier than the 'normal' stage of embryonic development – has remained unstudied. In the course of research on montane scincid lizards, we developed a technique to induce 'premature' oviposition, and hence can address this issue experimentally for the first time.

Methods

STUDY SPECIES AND AREA

Bassiana duperreyi are medium-sized [to 80 mm snout-vent length (SVL)] oviparous scincid lizards that are widely distributed through southeastern Australia. Previous research on this species has focused on populations in the Brindabella Range 40 km west of Canberra in the Australian Capital Territory (1240 m a.s.l.; 148°50′ E, 35°21′ S). These cool high-elevation sites are close to the upper elevational limits for oviparous reproduction by Australian lizards (Shine & Harlow 1996) and thus, nests are concentrated in the few sites that provide sufficient sun penetration for relatively warm nesting conditions (Shine, Barrott & Elphick 2002a). Female *B. duperreyi* aggregate in these sites prior to oviposition, facilitating collection of gravid animals (Shine *et al*. 2002a). We captured lizards ($n = 22$) from these sites during late October (2006), and took them to the University of Sydney. Gravidity was confirmed by abdominal palpation. All gravid females were weighed, measured and individually marked. They were housed in separate cages (each $22 \times 13 \times 7$ cm). Each cage contained moist vermiculite (for oviposition), a shelter site and a water dish. The lizards were fed on live crickets dusted with vitamin and calcium powder three times a week. The room was maintained at 20 °C with a 12 h light : 12 h dark photoperiod. A thermal gradient from 20 °C to 35 °C was provided within each cage by means of an underfloor heating element that was switched on for 8 h per day; cage temperature fell to ambient room temperature (20 °C) overnight (see Shine & Harlow 1996 for details). The lizards thus had ample opportunity for behavioural thermoregulation during daylight hours.

HORMONE TREATMENT APPLIED TO GRAVID FEMALES

After 48 h of acclimatization to laboratory conditions, we randomly allocated gravid females into two groups, and elevated levels of circulating corticosterone (B) in one group of 12 animals using a non-invasive method (modified from Knapp & Moore 1997; Meylan *et al*. 2002; Meylan, Dufty & Clobert 2003). Corticosterone (Sigma, Castle Hill, NSW, Australia, C-2505) diluted in sesame oil (30 μg/0·1 mL of oil) was delivered transdermally to the lizards. Lipids in the lizard skin facilitate entry by liphophilic molecules, elevating plasma B levels (Meylan *et al*. 2002, 2003). The remaining 10 control females received only the vehicle (i.e. 0·1 mL of oil). After this single application, all females were returned to their cages, which were inspected twice daily for eggs.

COLLECTION OF BLOOD AND YOLK SAMPLES, AND B ASSAY

To quantify B levels in maternal plasma and egg yolk, we collected blood serum and yolk samples on the day of oviposition from representatives of both experimental groups. The blood samples were obtained from the post-orbital sinus from randomly selected females ($n = 5$ lizards/treatment group), and the resultant blood serum stored at –80 °C until radioimmunoassay (RIA). Two eggs from each clutch were randomly selected at oviposition for yolk sampling, performed via a sterile syringe with a 24-gauge needle. Approximately 10% (25–30 mg) of yolk was removed from each egg (determined by reweighing eggs after yolk removal; for details see Radder, Ali & Shine 2007). The yolk was transferred into a 2-mL plastic eppendorff tube and homogenized with 500 μL of distilled water, and stored at –80 °C until RIA.

Corticosterone in the serum and egg yolk homogenate was assayed using MP Biomedicals (formerly ICN Biomedicals) RIA kits. Briefly, yolk was thawed and homogenized again in 1 mL of water. Yolk homogenate was extracted with 3 mL diethylether and 0·1 mL distilled water following the protocol of Saino *et al*. (2005)

with modifications as described by Radder *et al*. (2007). Then the water was separated from diethylether by snap freezing and the diethylether was evaporated in a drying centrifuge. The extract was re-suspended in 1·2 mL of steroid diluent, then extracts were vortexed and kept at 37 °C for 30 min. Similarly, blood serum was diluted with steroid diluent (1 : 500). For assay, 500 μL of the yolk homogenate suspension or diluted serum was used. Samples were incubated at 98 °C for 10 min to denature corticosterone binding proteins. A 0·1-mL of anti-corticosterone and ^{3}H corticosterone tracer (*c.* 10 000 cpm) were added to assay tubes. After overnight incubation at 4 °C in a water bath, 0·2 mL of cold charcoal dextran solution was added. After 20 min at 4 °C, assay tubes were centrifuged (2500 r.p.m. for 15 min) and the supernatant was decanted into a scintillation cocktail and counted for 1 min in a β-counter.

All samples were assayed in duplicate and hormone concentrations were compared to a standard curve from 0 to 1 ng/0·5 mL. Specificity and accuracy of the hormone assay were examined following the procedures of Saino *et al*. (2005). From additional samples, we removed endogenous steroids by treatment of yolk homogenate with 1 mL of charcoal solution (10 mg/mL). We then added known amounts of exogenous steroids (0, 0·1 and 0·25 ng/mL of B, Sigma Chemicals) to each sample. After equilibration for 24 h, steroids were extracted and subjected to RIA. The measured steroid levels were similar to expected values (non-detectable, 0·092 ± 0·002 and 0·24 ± 0·002 ng/mL recovery for 0, 0·1 and 0·25 ng/mL, respectively, for yolk homogenate and non-detectable, 0·093 ± 0·005 and 0·24 ± 0·008 ng/mL recovery for 0, 0·1 and 0·25 ng/mL, respectively, for plasma samples; $n = 2$ samples for each concentration) indicating negligible interference (e.g. from antibody–steroid interactions with egg yolk substances). Both yolk homogenates and plasma samples were assayed together in a single assay; intra- and inter-run assays had coefficient of variations of 3·6% and 5·8%, respectively, for both yolk and plasma samples.

EMBRYO STAGING AT OVIPOSITION

We assumed that eggs within a clutch did not vary in stage of development at oviposition. This has been demonstrated for two species of lizards (e.g. Muthukkaruppan *et al*. 1970; Mathies & Andrews 1995) but has not been examined in this particular species. Thus, one of the two eggs that were selected from each clutch for yolk sampling was used to determine embryonic developmental stage at oviposition. The selected egg was dissected in a glass Petri dish, and the stage of embryonic development was determined by methods and embryonic staging criteria developed for reptiles (Dufaure & Hubert 1961; modified for oviparous lizards by Muthukkaruppan *et al*. 1970; Shanbhag, Radder & Saidapur 2001; Radder, Shanbhag & Saidapur 2002).

EGG INCUBATION PROCEDURES AND OFFSPRING MEASUREMENTS

On the day of oviposition, eggs were removed from the cages, weighed, and then placed individually in 64-mL jars containing moist (–200 kPa) vermiculite. The jars were kept in a cycling-temperature incubator with a sinusoidal diurnal thermal cycle (22 ± 7·5 °C). This thermal regime mimics conditions that eggs experience in the field in natural nests (Shine & Elphick 2001), and produces offspring sex ratios close to 50 : 50 (Shine 2002a; Shine *et al*. 2002a). Heteromorphic sex chromosomes (male XY, female XX) determine sex in *B. duperreyi* under these conditions, although these genetic factors can be over-ridden by incubation at lower temperatures (Shine, Elphick & Donnellan 2002b). Incubators were checked twice daily for hatchlings to determine hatching dates and thus, incubation periods. Any hatchling found was removed and measured and weighed, and its sex was determined by squeezing the tailbase to manually evert hemipenes (Harlow 1996). At 1 week of age, we recorded offspring running speeds using a purpose-built raceway, to quantify maximal running speeds. The racetrack (1-m long) contained five infrared photocells, spaced at 25-cm intervals, connected to an electronic stopwatch. The raceway was kept in a room at 25 °C, and lizards were acclimated to this test temperature for 30 min prior to testing (see Shine & Harlow 1996 for details). We measured locomotor performance of hatchlings by chasing them along the racetrack. Each hatchling was raced three times, with at least 10 min rest between successive trials. For a trial, the hatchling was placed at one end of the racetrack and encouraged to run by gently prodding its tail with an artist's paintbrush. We recorded each 25-cm split time, over the entire 1-m distance. For analyses, we used data on speed for the fastest 25 cm split as well as over the full 1-m distance.

DATA ANALYSES

Data were tested for the requirements of parametric testing (normality, variance homogeneity, etc.). We used unpaired *t*-tests to compare control vs. B-treated lizards in terms of maternal body length, maternal mass, clutch size, egg mass, duration of uterine retention of eggs (number of days required for oviposition after treatments), B levels in maternal plasma and eggs, and incubation duration. Our analyses of egg and offspring traits were based on mean values for each clutch to avoid pseudoreplication (because treating siblings as statistically independent would artificially inflate the df). Differences in embryonic stage at oviposition between the two experimental groups were evaluated by Mann–Whitney *U* tests because the dependent variable was categorical. Residuals obtained by regressing ln offspring body mass on ln SVL were used as our index of 'body condition' for analyses. Data on phenotypic traits of the offspring were analysed by ANOVA using experimental groups and sex as factors, and offspring traits or incubation duration as the dependent variables. Although B-treated females tended to produce smaller eggs than did the controls, this difference was not statistically significant (see below) and thus, we did not include egg mass as covariate in the ANOVAs. Significance level was accepted at $P < 0·05$ and all data analyses were carried out using SYSTAT (version 11·0).

Results

Mean body sizes of gravid females, and the mean numbers of eggs they produced, did not differ significantly between the control and B treatment groups (Table 1). Unsurprisingly, B-treated females exhibited higher plasma corticosterone levels than did the control females at oviposition (Table 1). B-treated females began laying eggs 48 h after hormone administration, and oviposited significantly earlier on average (5·4 ± 0·51 days post-treatment; range 2–8 days) than did the control group (mean 13·2 ± 1·22 days, range 8–19 days; unpaired $t = 6·22$, 20 df, $P = 0·0001$; Fig. 1a). Reflecting their earlier oviposition, eggs from B-treated females contained embryos at stages 26–28 at oviposition (mean embryonic stage 27·0 ± 0·21, $n = 12$ eggs used for embryonic staging; one from each clutch), whereas control females laid eggs at stage

Table 1. Summary (mean value ± SEM) of maternal morphological, clutch and egg traits of scincid lizards (*Bassiana duperreyi*) belonging to the corticosterone treatment (B treated; $n = 12$ females) and control groups ($n = 10$ females). Maternal plasma corticosterone levels are based on readings from five females per group; egg data are based on clutch mean values. Total egg yolk B was obtained by multiplying unit hormone concentration (i.e. ng/mg) with egg mass. Data were analysed by unpaired *t*-tests. *P* values in bold font indicate a significant difference in mean values between experimental and control groups

Variable	B treated	Controls	*t*	*P*
Snout-vent length (mm)	66·25 ± 1·72	70·70 ± 1·97	1·70	0·10
Body mass (g)	4·83 ± 0·33	5·19 ± 0·44	0·66	0·51
Clutch size	6·08 ± 0·41	7·0 ± 0·71	1·15	0·26
Egg mass (mg)	361·09 ± 9·60	371·78 ± 17·66	0·55	0·58
Plasma B levels (ng/mL)	246·2 ± 30·93	79·0 ± 18·35	4·64	**0·001**
Egg yolk B levels (ng/mL)	6·23 ± 1·99	5·40 ± 2·1	1·07	0·29
Total egg yolk B (ng)	87·66 ± 7·21	69·52 ± 7·15	–1·78	0·09

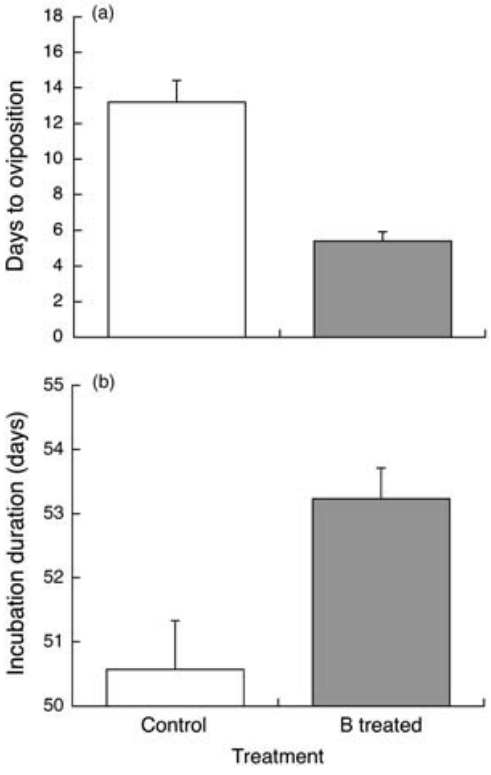

Fig. 1. (a) Duration of oviductal retention of eggs after corticosterone B treatment (days post-treatment; mean ± SEM) for B-treated and control lizards. B-treated females ($n = 12$) oviposited earlier (unpaired $t = 6·22$, $P = 0·0001$) than did the control group ($n = 10$). (b) Incubation duration (days from laying to hatching; mean ± SEM) of the eggs from corticosterone B treated and control lizards. The eggs of B-treated females took longer to hatch (unpaired $t = 2·89$, $P = 0·01$).

30–31 of embryonic development (mean embryonic stage 30·4 ± 0·16, $n = 10$ eggs; one from each clutch). Thus, the mean embryonic stage at oviposition differed between the two experimental groups (Mann–Whitney $U = 120·0$, $P < 0·0001$). Eggs laid by B-treated females weighed slightly but not significantly less than those of control females (Table 1). Eggs from B-treated mothers also exhibited slightly higher mean as well as total yolk corticosterone levels, but again the difference was non-significant (Table 1).

The eggs laid by B-treated females took longer to hatch than did those of control females (using clutch mean values to avoid pseudoreplication, unpaired $t = 2·89$, df = 17, $P = 0·01$; Fig. 1b), with no significant interaction between treatment and offspring sex with respect to incubation duration (ANOVA on raw data: interaction $F_{1,89} = 0·016$, $P = 0·89$). Interestingly, despite a small range in embryonic stages at oviposition, the same patterns were evident within the control group as between the control vs. experimental groups. Within the control group, clutches oviposited at embryonic stage 30 were laid earlier than those with embryos at stage 31 ($F_{1,8} = 19·58$, $P < 0·002$) and had longer subsequent incubation periods ($F_{1,8} = 10·03$, $P < 0·02$). Three out of 12 B-treated female clutches did not produce viable hatchlings. Hatching success for the control group was 91·2% (52 hatched/57 eggs incubated) and 69·5% for the B-treated group (41/59 eggs; Fisher's Exact $P < 0·005$).

Corticosterone-treated females laid their eggs an average of 7·5 ± 0·31 days earlier, but the subsequent incubation periods of those eggs averaged only 3 days longer than control eggs. As a result, the eggs from corticosterone-treated females hatched an average of 2·5 ± 0·48 days earlier overall ($F_{1,17} = 13·56$, $P < 0·002$). We detected no significant interactions between treatment and offspring sex with respect to offspring traits or locomotor performance (in all cases $P > 0·05$). Therefore, analyses were repeated after removing sex, and using clutch means rather than son and daughter means (Table 2). Compared to hatchlings from the eggs of control females, offspring of B-treated females were smaller (SVL: $F_{1,17} = 8·14$, $P < 0·011$) and lighter (body condition: $F_{1,17} = 3·84$, $P < 0·03$) at hatching, and ran more slowly over 25 cm at 1 week of age ($F_{1,17} = 7·85$, $P < 0·012$); the pattern was similar but non-significant for speed over 1 m ($F_{1,17} = 3·07$, $P = 0·10$: see Table 2). The locomotor effect appeared to be a secondary consequence of the body-size effect; when we included SVL as a covariate, the effect of B treatment on running speed relative to body size was non-significant ($F_{1,16} = 1·30$, $P = 0·27$ and $F_{1,16} = 0·001$, $P = 0·98$ for 25 cm and 1 m, respectively). The stage of embryonic development at oviposition was positively correlated with subsequent offspring size ($r = 0·53$, $P < 0·02$ and $r = 0·43$, $P < 0·05$, $n = 19$ clutches for SVL and tail length, respectively) and locomotor performance ($r = 0·59$, $P < 0·01$

Table 2. Summary of morphological traits (at hatching) and locomotor performance (at 1 week of age) of hatchlings from the eggs produced by corticosterone treated (B treated; $n = 9$ clutches as three clutches did not produce viable hatchlings) and control (unmanipulated; $n = 10$ clutches) scincid lizards *Bassiana duperreyi*. Data are expressed as mean ± SEM. P values in bold face indicate significant differences ($P < 0{\cdot}05$) based on ANOVA (with offspring trait or locomotor performance as the dependent variable and with treatment as factors. Degrees of freedom for all = 1, 17). 'Body condition' is based on residual scores from the linear regression of ln body mass on ln snout-vent length. Analyses of offspring traits were performed using clutch mean values to avoid pseudoreplication

Trait	B treated	Control	B effect
Snout-vent length (mm)	24·96 ± 0·17	25·55 ± 0·15	$F = 8{\cdot}14$, $\mathbf{P = 0{\cdot}011}$
Body condition	–2·45 ± 3·28	2·17 ± 4·76	$F = 3{\cdot}84$, $\mathbf{P = 0{\cdot}03}$
Tail length (mm)	31·43 ± 0·46	32·25 ± 0·40	$F = 0{\cdot}73$, $P = 0{\cdot}40$
Running speed (m/s)			
25 cm	0·28 ± 0·02	0·44 ± 0·03	$F = 7{\cdot}85$, $\mathbf{P = 0{\cdot}012}$
1 metre	0·27 ± 0·03	0·34 ± 0·02	$F = 3{\cdot}07$, $P = 0{\cdot}098$

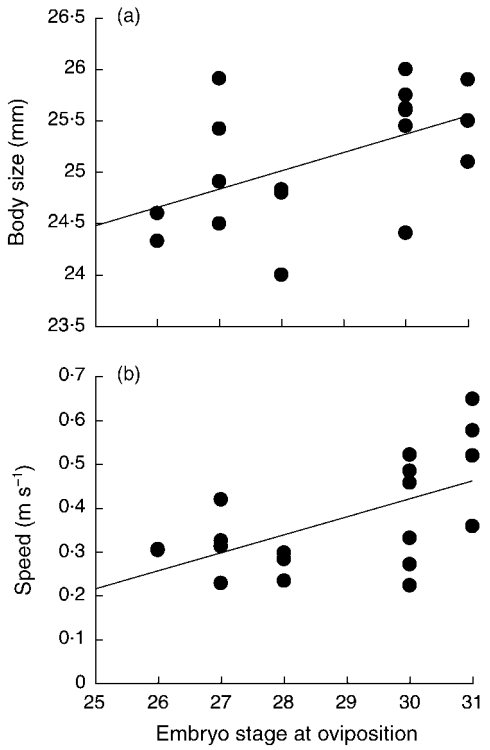

Fig. 2. Relationships between offspring body size (snout-vent length) and embryonic developmental stage at oviposition (a), and between offspring locomotor performance (running speed at 25 °C) and embryonic stage at oviposition (b) in *B. duperreyi*. Eggs that were oviposited at early embryonic stages (Stage < 28; from corticosterone-treated mothers) produced offspring that were both slower and smaller.

and $r = 0{\cdot}54$, $P < 0{\cdot}02$, $n = 19$ clutches for 25 cm and 1 m, respectively: Fig. 2), suggesting a direct link between embryonic stage at oviposition and the phenotypic traits of hatchlings.

Discussion

Our results reveal two intriguing patterns: (i) elevated corticosterone levels in gravid lizards induced 'premature' oviposition, and (ii) earlier embryonic stages at oviposition resulted in lower offspring fitness (reduced hatching success, and offspring that were smaller, thinner-bodied and slower). Below, we focus on these two results.

How and why did corticosterone application induce 'premature' oviposition by *B. duperreyi*? In birds, exogenously elevated B levels delay the onset of egg laying (Salvante & Williams 2003), and previous authors have speculated that maternally-experienced stress might delay rather than accelerate oviposition in reptiles also (Shine & Guillette 1988). However, several studies on oviparous vertebrates, especially passerine birds, have reported a natural elevation in B levels coincident with egg production, ovulation or oviposition (see Salvante & Williams 2003). Although some reptile species exhibit higher baseline levels of B during the breeding season than non-breeding season (e.g. *Uta stansburiana, Geochelone nigra, Chelonia mydas, Thamnophis sirtalis parietalis*), others (e.g. *Anolis sagrei, Pogona barbata, Alligator mississippiensis, Gopherus polyphemus*) do not increase maternal basal plasma B levels during late pregnancy or breeding (e.g. Girling & Cree 1995; reviewed by Moore & Jessop 2003). Elevated B levels (in mid-pregnancy) cause premature abortion in a viviparous gecko, *Hoplodactylus maculatus* (Cree *et al.* 2003), and early hatching (by accelerating embryonic development) in the oviparous lizard *Urosaurus ornatus* when near-term embryos (85% of incubation) were treated with B (Weiss, Johnston & Moore 2007). Generalizations about the role of corticosteroid levels in reptile reproduction are likely to prove elusive, because of substantial interspecific as well as intraspecific divergence in responses (Moore & Jessop 2003).

Functionally, 'premature' oviposition by corticosterone-treated female lizards may act to protect the eggs. Eggs that are retained in the oviduct in the presence of elevated maternal B levels may take up corticosterone (Johnston & Moore 2006; but see Painter, Jennings & Moore 2002), negatively affecting offspring traits including growth and immune function (Morici, Elsey & Lance 1997). Therefore, 'premature' oviposition may shield the developing offspring from such effects – albeit, at the cost of other negative impacts as demonstrated in the current study.

The most critical issue in terms of interpreting our own results is whether the negative impacts of the experimental treatment on offspring viability were due to 'premature' oviposition or to some experimental artefact. Our analyses of egg yolk show that the most obvious such potential problem (corticosterone transfer to the egg) did not occur to any measurable degree; and encouragingly, correlations between embryonic stage at oviposition and traits such as date of oviposition and duration of subsequent incubation were evident even within the control group alone. These results strengthen the inference that the effects we saw on offspring viability were direct consequences of embryonic stages at oviposition, rather than artefacts of the experimental methods that we used to generate variation in this trait. Also encouraging is the observation that the maternal blood corticosterone levels produced by our exogenous administration (Table 1) fell within the range of B levels induced by 'natural' stressors in a sympatric scincid species (Langkilde & Shine 2006), further suggesting that our experimental manipulations did not introduce major artefacts.

If early oviposition indeed caused the reduction to offspring viability, by what processes did this occur? Our results fit well with extensive earlier work on *B. duperreyi*, in showing that offspring phenotypes are very sensitive to the conditions under which the egg develops (e.g. Shine, Elphick & Harlow 1997; Elphick & Shine 1998; Shine 1999, 2002a). In particular, the earliest post-oviposition stages are more sensitive to physical conditions than are later stages (Shine 2002a), and experiments manipulating maternal body temperatures prior to laying have confirmed that phenotypic sensitivity extends back to these pre-oviposition stages of embryogenesis (Shine 2006). In snakes, even brief periods of exposure to specific hydric conditions at the time of oviposition can influence neonatal phenotypes measured when the offspring emerge months later (Shine & Brown 2002; Brown & Shine 2006). In *B. duperreyi*, subtle shifts in incubation conditions (the number of other eggs in contact with the focal egg) can modify rates of water uptake and thus, offspring size – and remarkably, lower water uptake can generate larger not smaller offspring (Radder & Shine 2007). Thus, the sensitivity of offspring phenotypes to minor differences in incubation conditions, and the stage dependency of such reaction norms, offers a plausible basis for the effects we have seen in the current study.

Incubation temperatures were identical for control vs. treatment-group eggs in our experiment, so that rates of water exchange (which depend upon the condition of the embryo, eggshell and other egg components) are the most likely causal influences on offspring viability. This inference accords well with arguments by Shine & Thompson (2006), who attribute the scarcity of early-embryo oviposition in squamates to the advantages of delaying oviposition until the embryo and its egg have developed hydroregulatory capacity. In our study, 'premature' oviposition may have altered water uptake via changes to eggshell thickness or egg turgidity; future work could usefully explore such possibilities. The most likely alternative mechanism involves developmental rates rather than routes of embryogenesis: maternal retention might enhance fitness because retained eggs develop faster (either because they are kept warmer by maternal thermoregulation, or because of some physiological contribution by the uterus). Under this hypothesis, we would expect 'prematurely' laid eggs to hatch later (in absolute terms) than control-group eggs. We found the reverse situation: corticosterone-treated females laid their eggs much earlier, but the subsequent incubation periods of those eggs averaged only slightly longer than control eggs and thus, hatched earlier overall. Thus, 'premature' oviposition resulted in earlier not later hatching in absolute terms.

The great diversity of squamate reproductive tactics makes it difficult to extrapolate our results to other taxa. Although oviposition at embryonic stages 26–32 encompasses the vast majority of records for oviparous lizards (Andrews & Mathies 2000; Harlow 2004), there is significant within- and among-population variation within that range (Huey 1977; Shine 1983; Braña, Bea & Arrayago 1991; Andrews & Mathies 2000; Andrews 2004; Calderón-Espinosa *et al*. 2006). For lizards, the minimum viable embryonic stage at oviposition appears to be about stage 26 (Muthukkaruppan *et al*. 1970; Shine 1983; Harlow 2004). Attempts to incubate eggs containing embryos below this stage (by surgical removal from the oviducts) generally fail (Muthukkaruppan *et al*. 1970; R. Radder pers. obs.). Thus, eggs may need to remain inside the oviduct until the embryos pass this critical early stage of development – perhaps until they are able to control water balance within the egg (Shine & Thompson 2006). Selection within each population then presumably fine-tunes the embryonic stage at oviposition to match fitness optima (e.g. Huey 1977). Very prolonged retention (as required for the transition to viviparity) may be opposed by physiological constraints such as oxygen supply to the oviductal embryo (Andrews 2002) as well as by increasing costs to the female (Shine 1980). These two endpoints thus define a range (approximately embryonic stages 26–32) wherein oviposition can occur without requiring major physiological adjustments. Specific selective forces and local environmental conditions can move embryonic stages at oviposition within that range. For example, multiple-clutching species tend to lay their eggs at earlier embryonic stages than do single-clutching species (Andrews & Mathies 2000), and lack of suitably moist incubation sites can delay oviposition (e.g. Radder *et al*. 1998; Warner & Andrews 2003; reviewed by Andrews 2004). Our data hint that maternal stress also can induce earlier oviposition. To move outside the range of embryonic stages 26–32 may require major selection differentials, such as are imposed by cold climates (favouring retention all the way through to viviparity: Huey 1977; Shine 1985).

Although many uncertainties remain, recent work is beginning to fill in some of the major gaps in our understanding of evolutionary aspects of reptilian reproductive modes (e.g. see Thompson & Blackburn 2006). We now have plausible hypotheses about the selective forces responsible not only for many of the major transitions, but also for the striking phylogenetic conservatism of many traits (such as the degree of embryonic development at oviposition). The challenging

task of robustly testing those hypotheses is still in its infancy, but progress is encouraging (Andrews 2002; Shine 2002b). Techniques to experimentally manipulate critical parameters (such as the degree of uterine retention of eggs) are clearly feasible (Andrews 2002; present study) and should provide increasingly powerful methods to clarify the fitness consequences of reproductive diversity within squamate reptiles.

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