

## RESEARCH ARTICLE

# Facultative mobilization of eggshell calcium promotes embryonic growth in an oviparous snake

James R. Stewart<sup>1,\*</sup>, Rebecca A. Pyles<sup>1</sup>, Kaitlyn A. Mathis<sup>1</sup> and Tom W. Ecyay<sup>2</sup>

## ABSTRACT

The mineralized eggshell of Reptilia was a major innovation in the evolution of the amniotic egg. Inorganic components strengthen the eggshell and are a potential source of nutrients to developing embryos. Embryos of oviparous reptiles do extract calcium from eggshells but vary interspecifically in exploitation of this resource. The pattern of embryonic calcium nutrition of the corn snake, *Pantherophis guttatus*, is similar to a diversity of squamate species: embryos obtain most calcium from yolk, yet also mobilize calcium from the eggshell. We tested the hypothesis that embryonic development is not dependent on eggshell calcium by manipulating calcium availability. We peeled away the outer calcareous layer of the eggshell of recently oviposited eggs; control eggs were left intact. Eggs were sampled periodically and calcium content of egg compartments (embryo, yolk, eggshell) was measured. We also analyzed skeletal development and size of hatchlings. There was no difference in survivorship or length of incubation between treatments. However, hatchlings from intact eggs contained more calcium and were larger in mass and length than siblings from peeled eggs. There were no observable differences in ossification but hatchlings from intact eggs had larger skeletal elements (skull, vertebrae). Our results indicate that mobilization of eggshell calcium is not a requirement for embryonic development of *P. guttatus* and that embryos augment yolk calcium by extracting calcium from the eggshell. This pattern of embryonic calcium nutrition would favor embryos with a greater capacity to mobilize calcium from the eggshell by promoting growth and thereby potentially enhancing hatchling fitness.

**KEY WORDS:** Oviparity, Eggshell, Skeletogenesis, Embryonic nutrition

## INTRODUCTION

Calcium-rich yolk, which supports the development of free-living hatchlings, was a key innovation in the evolution of amniotes (Packard and Seymour, 1997). The addition of a calcareous eggshell surrounding the large mass of yolk in the ancestors of modern reptiles (Packard, 1994; Stewart, 1997) provided both a buffer with the external environment and a secondary source of nutritional calcium for embryonic development. Extraction of calcium from the eggshell in combination with mobilization of calcium from yolk is widespread among reptilian embryos, but varies among species

(Packard and Packard, 1984; Packard, 1994; Stewart and Ecyay, 2010). Patterns of embryonic mobilization of calcium are influenced by phylogeny (Packard and Packard, 1984; Packard, 1994). Generally, embryos of crocodylians and birds rely most heavily on calcium from the eggshell, whereas embryonic snakes are least dependent on eggshell calcium (Packard, 1994). Embryonic turtles and lizards are somewhat intermediate in use of eggshell calcium (Stewart and Ecyay, 2010).

Early estimates of the calcium content of the yolk of several species of squamate reptiles (lizards and snakes) led to the hypothesis that yolk calcium is sufficient to sustain embryonic development (Simkiss, 1967; Jenkins and Simkiss, 1968). Subsequent research demonstrating that squamate embryos do extract calcium from the eggshell (Packard and Packard, 1984; Packard et al., 1984, 1985), which can represent more than half of calcium content of hatchlings in some species (Stewart and Ecyay, 2010), appears to contradict this hypothesis. However, we know little about calcium requirements of squamate embryos because dependence on eggshell calcium for successful development has not been tested directly on squamates. There is considerable interspecific variation in the embryonic pattern of calcium mobilization. If yolk is the primary source of calcium and embryonic mobilization of eggshell calcium varies intraspecifically as well, calcium mobilization from eggshells may not be a requisite for successful embryonic development. The existence of such a pattern would not be surprising if the evolution of embryonic acquisition of calcium from eggshells occurred in species with calcium-rich yolks (Packard and Seymour, 1997).

Embryos of the red corn snake, *Pantherophis guttatus*, represent a calcium mobilization pattern near one end of the continuum of variation in reliance on calcium from the eggshell; less than 30% of hatchling calcium is derived from the eggshell, with the remainder acquired from yolk (Stewart et al., 2004). We tested the hypothesis that calcium mobilized from yolk provides sufficient nutrition to produce hatchlings (Simkiss, 1967; Jenkins and Simkiss, 1968) by experimentally manipulating eggshell calcium content by manual removal of the outer calcareous eggshell layers shortly after oviposition.


## MATERIALS AND METHODS

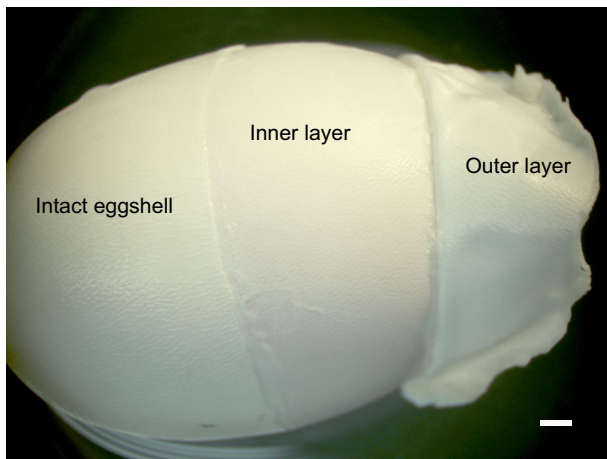
### Embryonic growth and calcium mobilization

Recently oviposited eggs were obtained from ten female *Pantherophis guttatus* Linnaeus 1766 housed in the animal care facility at East Tennessee State University, USA over two reproductive seasons (2011, 5 clutches; 2012, 5 clutches; mean=18 eggs per clutch). Egg wet mass was recorded and the outer layer of the eggshell was removed manually from approximately 50% of the eggs in each clutch within 3 days of oviposition. The eggshell is composed of fibrous protein organized in two distinct layers that can be separated (Fig. 1). Eggshells were left intact for the remaining eggs. One peeled egg from each clutch was sampled to estimate calcium distribution in oviposited eggs

<sup>1</sup>Department of Biological Sciences, East Tennessee State University, Johnson City, Tennessee 37614, USA. <sup>2</sup>Department of Biomedical Sciences, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614, USA.

\*Author for correspondence (stewarjr@etsu.edu)

 J.R.S., 0000-0001-9032-1163



**Fig. 1. *Pantherophis guttatus* egg with the outer fibrous layer of the eggshell partially peeled.** The inner fibrous layer remains uncompromised. Scale bar: 2 mm.

prior to incubating the remaining eggs in a low-temperature incubator at 26°C (Precision Model 818; Union, NJ, USA). Embryonic stage (Zehr, 1962) at sampling ranged from 21 to 28 (median=27, mode=27/28). Eggs were incubated in plastic containers with a medium consisting of 2 parts water:1 part vermiculite (w/w). Under these incubation conditions, eggs hatched in 71–78 days. Peeled and intact eggs were aligned alternately in incubation containers. Incubated eggs were sampled at one of three time points (19 days, range 17–22; 14 days, range 12–16; 9 days, range 8–10 prior to hatching) with the fourth and final sample as hatchlings. Egg or hatchling wet mass was recorded at each time point. For each sample, we removed the outer layer of the eggshell of an intact egg from each clutch and collected the outer shell, inner shell, yolk and embryo; peeled eggs sampled at the same time yielded an inner shell, yolk and embryo. Hatchlings were killed with an overdose of ethyl carbamate. Any residual (internal) yolk was removed and analyzed separately. All components of eggs were weighed for wet mass and frozen for later analysis. Dry mass for each of these components was estimated by lyophilization (Labconco Freeze Dryer Model 77500; Kansas City, MO, USA) to constant mass. Lyophilized samples were ashed in a muffle furnace (Fisher Isotemp; Hampton, NH, USA) at 600°C for 12 h, prior to digestion in hot nitric acid for 12 h. Nitric acid was evaporated to near dryness and digestates were stored in 2.5% hydrochloric acid. Lanthanum chloride was added (10% of sample volume) to reduce ionic interference prior to analyzing sample calcium content with flame atomic absorption spectroscopy (Shimadzu model AA-6300; Kyoto, Kyoto Prefecture, Japan), calibrated against samples of known calcium concentration.

Differences in total calcium in oviposited eggs were tested by mixed-model analysis of variance (ANOVA) with egg compartment (yolk, outer shell, inner shell), year, interaction between year and compartment as fixed factors and clutch as a random factor. Differences in total calcium in oviposited eggs and in hatchlings were tested by mixed-model ANOVA with treatment (oviposited eggs, hatchlings from eggs with intact eggshells, hatchlings from eggs with the outer eggshell removed), year, interaction between year and treatment as fixed factors and clutch as a random factor. Differences between samples of incubated eggs in wet mass and in total calcium of egg compartments (embryos or hatchlings, yolk) were tested by mixed-model ANOVA with treatment (intact, peeled), days to hatch, year and the interaction between treatment and days to

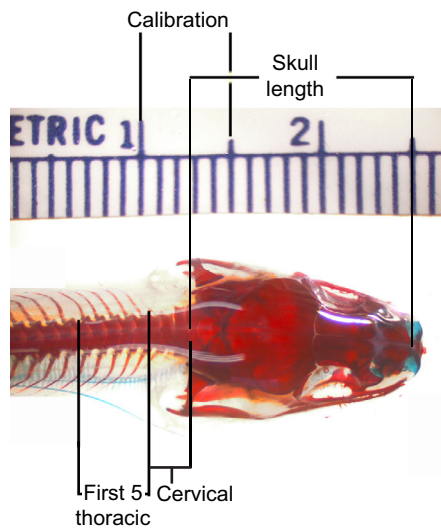
hatch as fixed factors. Initial egg wet mass served as a covariate and clutch (i.e. maternal source) as a random factor. Differences in relative calcium mass were tested using the same fixed factors and clutch as a random factor, but with tissue dry mass (embryos or hatchlings, yolk) as a covariate. Differences in total egg wet mass at each sampling time point were tested by mixed-model ANOVA with treatment, days prior to hatching, year and interaction between treatment and days to hatch as fixed factors, with initial egg wet mass as a covariate and clutch as a random factor. Differences in mass and length of hatchlings and of hatchling internal yolk were tested by mixed-model ANOVA with initial egg mass as a covariate, clutch as a random factor and treatment (intact, peeled) and year as fixed factors. We tested for homogeneity of variances using Bartlett's test prior to ANOVA. Multiple comparisons among least-squares means were tested using the Scheffe procedure. Statistical software was SAS 9.2 (Cary, NC, USA).

### Hatchling size and skeletal development

Fifteen clutches of oviposited eggs (mean=3 eggs per clutch) were sampled from 14 female *P. guttatus* over two seasons (2010, 2 clutches; 2011, 13 clutches). Females were housed in either the animal care facility at East Tennessee State University ( $n=7$ ) or the Trinity College Ophidian Research Facility ( $n=7$ ). Egg wet mass was recorded prior to incubation, and eggs were sampled, treated (peeled or intact eggshell) and incubated to hatching as for the embryonic calcium samples. Embryonic stage at initial sampling ranged from 25 to 31 (median and mode=28). Hatchlings were killed with an overdose of ethyl carbamate, weighed intact (carcass+internal yolk), measured for length (snout–vent, tail), and processed using a clearing and staining protocol (Hanken and Wassersug, 1981). Skull length and the lengths and number of vertebrae (by region) were recorded from cleared-and-stained specimens. For accurate vertebral counts, photographs were taken of each specimen using a Leica (Wetzler, Germany) DFC 420 camera attached to a Wild (Heerbrugg, Germany) M3Z Type-S dissecting scope. Dissecting pins placed between intercostal spaces served as landmarks among the multiple photos of each specimen. Vertebral counts for cervical, thoracic and caudal regions were obtained directly from grayscale printouts of these photos; counts were summed to provide total number of vertebrae. Photographs used for size measurements were obtained separately (same equipment). Each size photograph included a ruler in the field of view to provide measurement scale (Fig. 2). Measurements were made of skull length, and lengths of the three cervical vertebrae, the first five and last five thoracic vertebrae, and the first five and last five caudal vertebrae. The regional samples of first five and last five were intended to test for any cranial-to-caudal differences, reflecting the development and ossification pattern of vertebrae in snakes. Measurements were obtained by conversion of pixel counts; a pixel-to-millimeter ratio was secured using the ruler in each photo, a pixel count for the measurement needed was obtained (Adobe Premier Photoshop 12; Fig. 2) and then the ratio was used to convert pixel counts to actual size (mm) of vertebral segments sampled.

Count and measurement data were analyzed using a general linear model procedure (IBM SPSS; Armonk, NY, USA). Differences in count and size data were tested by mixed model ANOVA (general linear model, SPSS) with clutch (maternal source) as a random effect, initial egg wet mass as a covariate and with treatment as a fixed effect. We tested for homogeneity of variances using Bartlett's test prior to ANOVA.

All procedures involving animals were approved by the East Tennessee State University Committee on Animal Care and Use (protocol #P100201).



**Fig. 2.** Image capture showing a photograph of a cleared and stained (Alizarin Red S, Alcian Blue) *P. guttatus* hatchling from the peeled treatment group. Shown are measurement tools (Photoshop) for size measurements of skull and representative vertebrae.

## RESULTS

### Embryonic growth and calcium mobilization

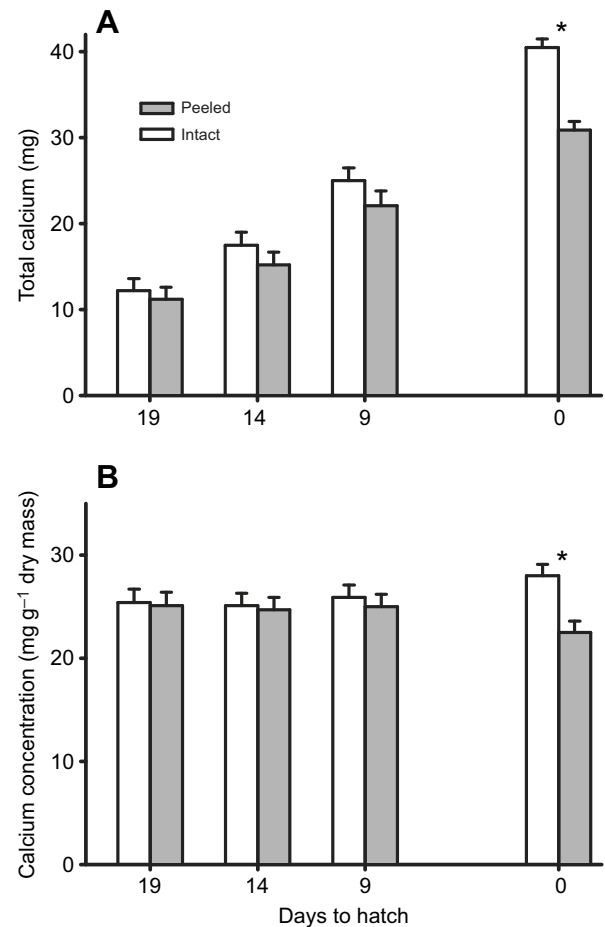
Total calcium in recently oviposited eggs (yolk+eggshell) was  $54.7 \pm 8.5$  mg (mean  $\pm$  s.e.m.). Yolk contained the greatest amount of calcium (64%), with most of the remaining calcium deposited in the outer layer of the shell (Table 1). Total calcium in hatchlings (hatchling+residual yolk) from peeled eggs did not differ from calcium in yolk at oviposition, but total calcium in hatchlings from intact eggs exceeded that in both the initial yolk and in hatchlings from peeled eggs (Table 1). Calcium content of embryos sampled 19, 14 or 9 days prior to hatching did not differ significantly between treatments, but hatchlings from peeled eggs contained 24% less calcium than hatchlings from intact eggs (Fig. 3A, Table S1). Calcium mass relative to dry mass of embryos did not differ between treatments at 19, 14 or 9 days prior to hatching, but hatchlings from peeled eggs had significantly less calcium relative to dry mass than hatchlings from intact eggs (Fig. 3B, Table S2). Total calcium in yolk decreased gradually during incubation and then dropped dramatically within 9 days of hatching in both treatment groups ( $F_{3,79}=245$ ,  $P<0.0001$ , Table 2, Table S3). However, total yolk calcium did not differ between treatments

**Table 1.** Least-squares means ( $\pm$ s.e.m.) of distribution of calcium in recently oviposited eggs and hatchlings (carcass+residual yolk) of *Pantherophis guttatus*

|                    | Calcium in egg compartments (mg) |                  |                 |
|--------------------|----------------------------------|------------------|-----------------|
|                    | Egg contents                     | Outer shell      | Inner shell     |
| Oviposited egg     | $35.1 \pm 1.3^{a,*}$             | $17.9 \pm 1.3^*$ | $1.8 \pm 1.3^*$ |
| Hatchling (peeled) | $34.2 \pm 2.2^a$                 | –                | –               |
| Hatchling (intact) | $45.3 \pm 2.2^b$                 | –                | –               |

Prior to incubation, eggs were treated either by removal of the outer layer of the eggshell (peeled) or the eggshell was left intact.  $N=10$  clutches.

\* $P<0.0001$ , differences between egg compartments of oviposited eggs (row 1). Differences between recently oviposited yolk and hatchlings are indicated by different letters ( $P<0.0001$ ) (column 1).



**Fig. 3.** Calcium in embryos and hatchlings of *P. guttatus* at four developmental time points. (A) Total calcium. (B) Calcium concentration (calcium mass adjusted for dry mass). Time '0' denotes hatchlings. Peeled, eggs with the outer calcareous layer of the eggshell removed; intact, control eggs. Data are least-squares means ( $N=10$  clutches both treatments)  $\pm$  1 s.e.m. \* $P<0.0001$ .

( $F_{1,81}=2.6$ ,  $P=0.11$ , Table 2). There was no difference in yolk calcium mass relative to yolk dry mass between treatments (intact, mean= $13.4 \pm 0.7$ ; peeled, mean= $13.1 \pm 0.7$ ;  $F_{1,77}=0.73$ ,  $P=0.40$ ) or among sampling times ( $F_{3,79}=1.8$ ,  $P=0.16$ , Table S4). There was a significant reduction in calcium from outer eggshells of intact eggs between 14 and 9 days before hatching, but calcium content of inner eggshells of both treatments remained stable throughout incubation (Table 2).

When adjusted for initial wet mass, there was a significant increase in egg wet mass between 19, 14 and 9 days prior to hatching ( $F_{2,37}=18.9$ ,  $P<0.0001$ ), and peeled eggs had significantly greater mass than intact eggs ( $F_{1,39}=19.6$ ,  $P<0.0001$ , Table S5). Hatchlings from intact eggs were significantly larger (i.e. heavier and longer) than those from peeled eggs (Table 3, Tables S6, S7), but there was no difference between treatments in either wet mass or dry mass of embryos at any sampling time prior to hatching (20, 15, 10 days; Fig. 4A,B, Tables S7, S8). Yolk mass, wet and dry, decreased gradually from 19 to 9 days before hatching, then dropped dramatically in parallel in both treatment groups (Fig. 4C,D, Tables S9, S10). Embryonic mortality did not differ between treatment groups (intact, 10 of 104 eggs died; peeled, 10 of 92 eggs died), and there was no difference in length of incubation (Table 3).

**Table 2. Least-squares means ( $\pm$ s.e.m.) of total calcium (mg) in yolks and eggshells of *P. guttatus***

| Days to hatch | Intact eggs                 |                             |                | Peeled eggs                 |                |
|---------------|-----------------------------|-----------------------------|----------------|-----------------------------|----------------|
|               | Yolk                        | Outer eggshell              | Inner eggshell | Yolk                        | Inner eggshell |
| 19            | 27.0 $\pm$ 1.2 <sup>a</sup> | 14.4 $\pm$ 1.5 <sup>a</sup> | 0.99 $\pm$ 0.3 | 24.9 $\pm$ 1.2 <sup>a</sup> | 0.67 $\pm$ 0.2 |
| 14            | 21.6 $\pm$ 1.2 <sup>a</sup> | 13.1 $\pm$ 1.5 <sup>a</sup> | 0.82 $\pm$ 0.3 | 20.9 $\pm$ 1.2 <sup>b</sup> | 0.75 $\pm$ 0.2 |
| 9             | 15.9 $\pm$ 1.3 <sup>b</sup> | 10.1 $\pm$ 1.6 <sup>b</sup> | 1.39 $\pm$ 0.3 | 14.3 $\pm$ 1.4 <sup>c</sup> | 0.98 $\pm$ 0.2 |
| 0             | 4.4 $\pm$ 0.9 <sup>c</sup>  | –                           | –              | 4.3 $\pm$ 0.9 <sup>d</sup>  | –              |

Prior to incubation, eggs were treated either by removal of the outer layer of the eggshell (peeled) or the eggshell was left intact.  $N=10$  clutches.

Differences between rows within columns are indicated by different letters (outer eggshell,  $P<0.05$ ; yolk of intact eggs,  $P<0.0004$ ; yolk of peeled eggs  $P<0.005$ ).

### Hatchling size and skeletal development

In this sample (14 clutches), the effect of treatment (peeled eggshell) on hatchling size duplicated that found in the experiment analyzing calcium content. Hatchlings from peeled eggs were smaller in mass and snout–vent length than hatchlings from intact eggs (Table 4, Tables S11, S12). Initial egg mass and maternal source also had significant effects on hatchling mass and snout–vent length (not tail length), but no interactions were significant. Pattern of ossification, as revealed by Alizarin Red S staining, did not differ between treatments (Fig. S1). Individual bones of the braincase, palatamaxillary complex, suspensorium and mandible, as well as vertebral components, stained intensely in both treatment groups. There were no significant differences in number of total vertebrae or numbers of vertebrae by region in hatchlings between treatments (Table 5). However, hatchling skull length and lengths of thoracic and the first five caudal vertebrae differed significantly between treatments (Table 4, Fig. 5, Tables S13, S14); skull lengths also differed among females but there was no interaction between maternal source and treatment. Hatchlings from eggs with intact eggshells had significantly longer skulls and vertebrae. Differences in lengths of vertebrae were distributed across the vertebral column, with the exception of the cervical region and the last five caudal vertebrae (Table 4, Fig. 5).

### DISCUSSION

Embryos of oviparous reptiles are provided with two potential sources of calcium: as a constituent of yolk, synthesized during vitellogenesis, and as a component of the eggshell, secreted by the uterus (Packard, 1994). The allocation of calcium to these two egg compartments and the pattern of embryonic acquisition of calcium vary (Packard, 1994; Stewart and Eca, 2010). Factors that influence evolution of the pattern of calcium provisioning to eggs are unknown, but mineral deposition is thought to provide structural reinforcement to the proteinaceous eggshell (Nys et al., 2004). Thus, eggshells with greater calcium content would be more resistant to physical and biological disruption (Packard and Packard, 1980). Heavily calcified eggshells also reduce water exchange with the environment (Packard and Packard, 1980) and are a potential

**Table 3. Size and length of incubation of hatchling *P. guttatus* from eggs analyzed for calcium mobilization**

| Treatment | Hatchling mass (g) | Hatchling length (mm) | Retained yolk mass (g) | Length of incubation (days) |
|-----------|--------------------|-----------------------|------------------------|-----------------------------|
| Intact    | 6.2 $\pm$ 0.14*    | 280 $\pm$ 3.4**       | 0.8 $\pm$ 0.09         | 76 $\pm$ 0.6                |
| Peeled    | 5.5 $\pm$ 0.14*    | 264 $\pm$ 3.4**       | 0.9 $\pm$ 0.09         | 76 $\pm$ 0.6                |

Prior to incubation, eggs were treated either by removal of the outer layer of the eggshell (peeled) or the eggshell was left intact.  $N=10$  clutches. Values are least-squares means ( $\pm$ s.e.m.) adjusted for initial egg mass and year (2011, 2012).

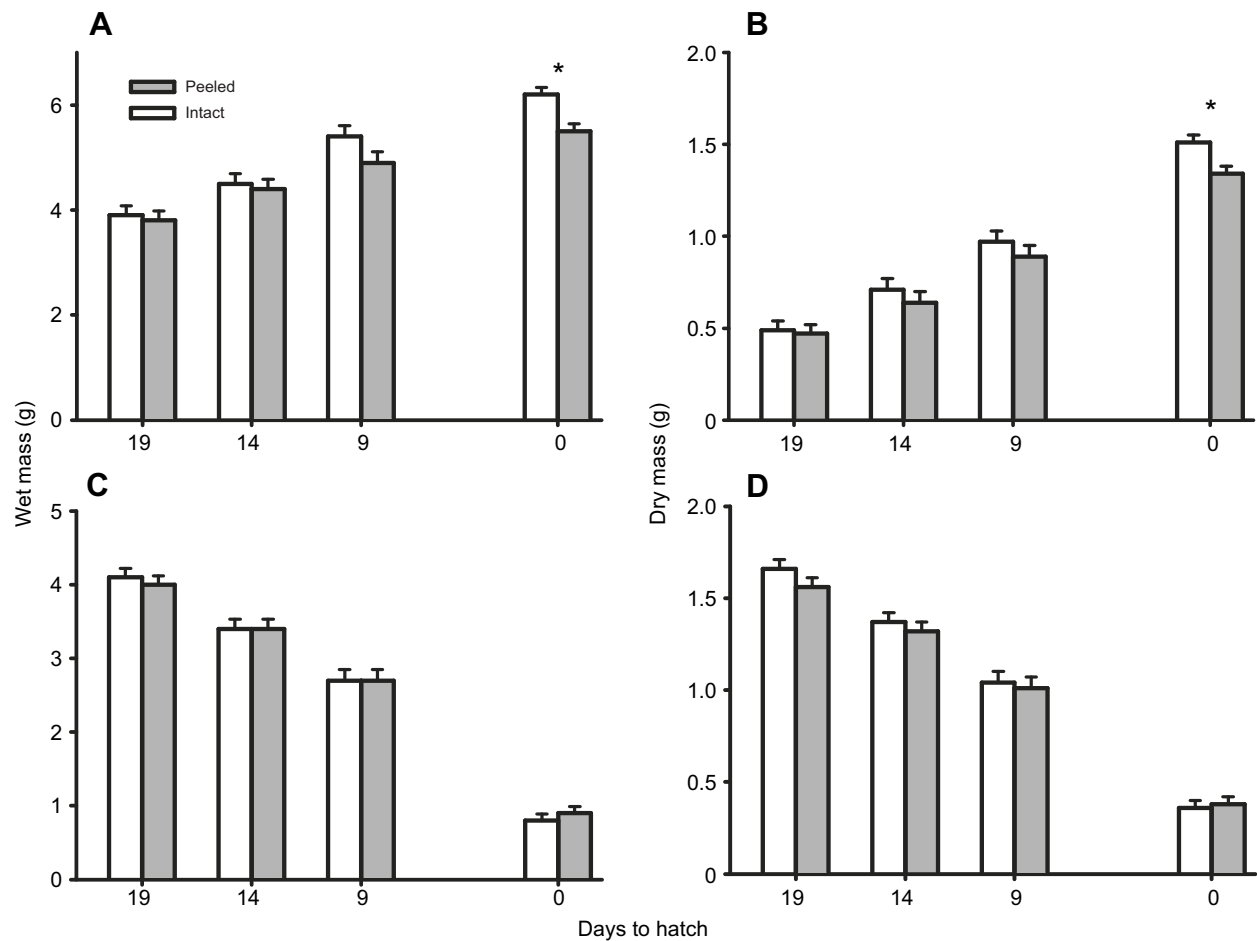
\* $P<0.005$ ; \*\* $P<0.0001$ .

response to arid nest conditions (Packard et al., 1982). Whatever the selective advantage, turtles and archosaurs (Archelosauria) have evolved calcium-rich eggshells and embryos are heavily dependent on these eggshells as a source of calcium (Packard and Packard, 1984). In contrast, the calcareous layer of the eggshell of most squamate reptiles is a thin, loosely organized deposit on the outside of a thick fibrous protein matrix (Packard and DeMarco, 1991). In addition to variable amounts of calcium in the eggshell, squamates also exhibit considerable variation in pattern of embryonic calcium nutrition (Stewart and Eca, 2010). Embryos of some species obtain most of their calcium from the eggshell, as do turtles, crocodylians and birds, while those of other species mobilize most of their calcium from yolk. Unlike turtles, crocodylians and birds, viviparity is common among squamates, and thus the potential role of embryonic calcium nutrition in the evolution of mode of parity is of interest (Packard et al., 1977; Stewart and Eca, 2010; Stewart, 2013). Squamate reptiles thus offer an opportunity to test hypotheses for the role of calcium in the evolution of life history patterns.

Embryonic calcium nutrition of *P. guttatus* exemplifies a pattern in which yolk provides sufficient calcium for successful embryonic development. This finding documents a pattern that was predicted to be a general characteristic of squamates (Simkiss, 1967; Jenkins and Simkiss, 1968). A similar, hypothetical pattern is an intermediate condition in two scenarios for the evolution of reptile life history patterns: embryonic acquisition of eggshell calcium is an adaptation arising in species with calcium-rich yolks (Packard and Seymour, 1997) and evolution of viviparity would be favored in species that are not dependent on calcium from the eggshell (Packard et al., 1977). In addition, acquisition of eggshell calcium by embryos of *P. guttatus* is facultative and contributes significantly to hatchling size, providing a mechanism for selection to promote embryonic calcium mobilization from the eggshell.

### Embryonic growth and calcium mobilization

Quantitative analysis of layers of the eggshell of *P. guttatus* confirms microscopic analysis that most of the calcium is associated with the outer layers of fibrous protein (Kohring, 1996; Table 1). Allocation of calcium to the egg is greater in yolk compared with the eggshell, and yolk is the primary source of calcium for embryonic development. Calcium is mobilized from yolk gradually throughout development, but embryonic uptake increases during the latest embryonic stages when embryonic growth is also elevated (Stewart et al., 2004). As a result of this pattern of growth, embryonic calcium content is equivalent to total calcium in yolk of oviposited eggs after approximately 90% of the incubation interval (Stewart et al., 2004). Extraction of calcium from the eggshell contributes importantly to growth during late stages of incubation. Consequently, removal of the outer layer of the eggshell did not influence the pattern of growth or calcium acquisition by the embryo until the last 10 days of incubation. Hatchlings from eggs with intact



**Fig. 4. Mass of embryos, hatchlings and yolk of *P. guttatus* at four developmental time points.** (A) Wet mass of embryos and hatchlings. \* $P=0.005$ . (B) Dry mass of embryos and hatchlings. \* $P=0.04$ . (C) Wet mass of yolk. (D) Dry mass of yolk. Time '0' denotes hatchlings. Peeled, eggs with the outer calcareous layer of the eggshell removed; intact, control eggs. Data are least-squares means ( $N=10$  clutches both treatments)  $\pm 1$  s.e.m.

eggshells contained more calcium and were larger than those from eggs in which most of the calcium was removed from the eggshell. In addition, the concentration of calcium in hatchlings from eggs with intact shells was higher. There were no differences in the incubation period or in embryonic mortality, and there were no apparent physical or behavioral differences between hatchlings from the two treatments. Embryos achieved hatching and neonate stage in the absence of eggshell calcium but, if eggshell calcium was available, embryos continued to mobilize calcium and grow. Interestingly, the additional increase in embryonic growth did not prolong the incubation period.

Comparison of skeletal development of hatchlings from the two treatments supported the interpretation from patterns of embryonic growth and uptake of calcium. There were no apparent differences between treatments in skeletal anomalies or in pattern of ossification based on Alizarin Red S staining (Fig. S1). However, hatchlings

from eggs with intact eggshells had larger skulls and larger vertebrae. Skeletal elements appeared fully formed in hatchlings from eggs in which eggshell calcium had been removed, but were significantly larger in hatchlings from eggs with intact eggshells.

As predicted for squamate embryos with calcium-rich yolks (Simkiss, 1967; Jenkins and Simkiss, 1968), embryos of *P. guttatus* do obtain sufficient calcium from yolk reserves for skeletal development to hatching. Eggshell calcium is a supplement to yolk calcium for embryos in that eggshell calcium is not required for survival to hatching. However, extraction of calcium from the eggshell is an important component of the pattern of embryonic calcium nutrition of *P. guttatus*. The ability of embryos to extract calcium from the eggshell has the potential to enhance fitness, if hatchling survivorship is influenced by size as has been shown for some snake species (Jayne and Bennett, 1990; Brown and Shine, 2005).

**Table 4. Overall size and axial skeletal length (means  $\pm$  s.e.m.) of hatchling *P. guttatus* from eggs analyzed for skeletal development**

| Treatment | Mass (g)         | Snout–vent (mm)   | Tail (mm)      | Skull (mm)        | Cervical (mm)   | First 5 thoracic (mm) | Last 5 thoracic (mm) | First 5 caudal (mm) | Last 5 caudal (mm) |
|-----------|------------------|-------------------|----------------|-------------------|-----------------|-----------------------|----------------------|---------------------|--------------------|
| Intact    | 7.3 $\pm$ 0.15** | 244.4 $\pm$ 1.8** | 48.3 $\pm$ 0.6 | 12.29 $\pm$ 0.08* | 2.46 $\pm$ 0.04 | 3.99 $\pm$ 0.06*      | 3.97 $\pm$ 0.06*     | 3.79 $\pm$ 0.04*    | 1.85 $\pm$ 0.13    |
| Peeled    | 6.6 $\pm$ 0.17** | 232.9 $\pm$ 2.0** | 46.5 $\pm$ 0.7 | 12.02 $\pm$ 0.09* | 2.41 $\pm$ 0.04 | 3.77 $\pm$ 0.07*      | 3.75 $\pm$ 0.07*     | 3.59 $\pm$ 0.04*    | 1.95 $\pm$ 0.15    |

Prior to incubation, eggs were treated either by removal of the outer layer of the eggshell (peeled) or the eggshell was left intact.  $N=14$  clutches.

\* $P<0.05$  between treatments (within columns).

\*\* $P\leq 0.01$  between treatments (within columns).

**Table 5. Vertebral number (means $\pm$ s.e.m.) in hatchling *P. guttatus***

| Treatment | Cervical | Thoracic        | Caudal         | Total           |
|-----------|----------|-----------------|----------------|-----------------|
| Intact    | 3        | 224.1 $\pm$ 2.2 | 71.5 $\pm$ 1.8 | 298.6 $\pm$ 2.5 |
| Peeled    | 3        | 223.6 $\pm$ 2.4 | 68.9 $\pm$ 2.1 | 295.4 $\pm$ 2.9 |

Prior to incubation, eggs were treated either by removal of the outer layer of the eggshell (peeled) or the eggshell was left intact.  $N=14$  clutches.

### Utilization of yolk

Embryos of *P. guttatus* from both treatments (intact versus peeled) mobilized nearly all of the calcium available in yolk, which is common for embryonic squamate reptiles (Packard et al., 1984, 1985, 1992; Packard and Packard, 1988). The absence of a difference in calcium mobilization from yolk between treatments suggests that the mechanism for transport of yolk calcium is regulated independently from extraction of calcium from the eggshell. Likewise, residual yolk calcium of turtle and lizard embryos is almost completely depleted independent of differences in embryonic growth and calcium mobilization from eggshell (Packard and Packard, 1986, 1989; Packard et al., 1992).

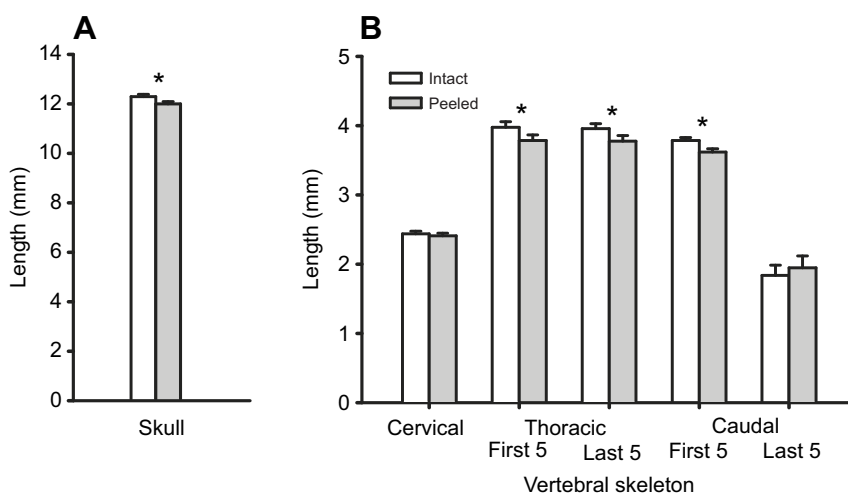
However, the similarity in total mass of residual yolk in corn snake hatchlings from peeled and intact eggs is puzzling. Yolk contains all of the organic nutrients for embryonic metabolism and our expectation was that yolk utilization should be correlated with embryonic growth, as is the case in turtles and lizards (Packard and Packard, 1986, 1989; Packard et al., 1992). One possible explanation is that our experimental conditions increased maintenance costs at the expense of growth, as a result of either reduced calcium availability or another factor arising from manipulation of the eggs. In addition to differences in calcium availability, our experimental conditions produced differences in water content of eggs, as reflected in egg mass. Peeled eggs absorbed and retained more water than intact eggs. Water is known to influence embryonic growth of turtles, lizards and snakes, but higher water flux results in larger hatchlings with less residual yolk (Gutzke and Packard, 1987; Packard and Packard, 1986; Packard et al., 1992).

Although we have no data to address the apparent differences in metabolism between *P. guttatus* embryos in the two treatment groups, the uptake of calcium was uncoupled from uptake of water, and this experimental model could be appropriated to test separately the effects of water uptake and calcium uptake on embryonic metabolism and growth.

### Squamate embryonic calcium nutrition

Patterns of embryonic calcium acquisition among squamate reptiles can theoretically be represented by a continuum with mobilization from yolk at one extreme and mobilization from the eggshell at the other extreme. Although few species have been studied, a sample of six snake and eleven lizard species reveals that the percentage of hatchling total calcium acquired from yolk ranges widely (19–86%; Stewart and Ecaj, 2010). Taxon sampling is biased, i.e. few lineages are represented, but we can infer that embryos of most oviparous squamates obtain calcium from both yolk and eggshell, but in different proportions. Pattern of embryonic calcium mobilization of corn snakes is yolk dependent, i.e. toward the yolk end of the yolk–eggshell continuum. Indeed, female corn snakes provide all the calcium necessary for embryonic development during vitellogenesis, yet also draw on calcium reserves to provision eggshells in response to a variety of potential selective factors. Embryonic development is dependent on mobilization of calcium from yolk, but embryos have the ability to extract calcium from the eggshell. Comparative timing of calcium mobilization from yolk and eggshells of *P. guttatus* is similar to other squamate species (Packard et al., 1984, 1985; Stewart et al., 2004, 2009a,b), and the mechanism of calcium mobilization from eggshells may also be conserved across lineages (Ecaj et al., 2004; Stewart et al., 2011). Yolk calcium concentration (calcium mass/dry mass) varies among squamates (Stewart and Ecaj, 2010) and embryos mobilize nearly all of the calcium available in yolk (Packard et al., 1984, 1985; Stewart et al., 2004, 2009a,b). If calcium uptake is proportional to embryonic growth, as has been shown for a diversity of squamate species (Packard et al., 1984, 1985; Stewart et al., 2004, 2009a,b), embryonic dependence on calcium from eggshells may be driven in part by maternal allocation of calcium to yolk. The evolution of different patterns of embryonic calcium mobilization among squamates may not require substantial adjustments of embryonic physiology and may be facilitated by embryonic responses to variation in the pattern of maternal allocation. This hypothesis predicts an inverse correlation between yolk calcium density and embryonic mobilization of calcium from eggshells both within and between species.

This study shows that facultative exploitation of eggshell calcium contributes significantly to size of hatchlings. This pattern of embryonic calcium mobilization has been modeled as a stage in the early evolution of the reptilian egg (Packard and Seymour, 1997). We provide the first verification that this pattern occurs, but its



**Fig. 5. Length of elements of the axial skeleton of hatchling *P. guttatus*.** (A) Skull. (B) Vertebrae. Peeled, eggs with the outer calcareous layer of the eggshell removed; intact, control eggs. Data are least-squares means ( $N=14$  clutches both treatments)  $\pm$ 1 s.e.m. \* $P<0.05$  between treatments.

presence in corn snakes also raises historical questions. The data that are available (reviewed in Stewart and Ecay, 2010) suggest that snake embryos mobilize proportionally more calcium from yolk than do lizard embryos. Phylogenetically, snakes are a deeply imbedded lineage within lizards (Pyron et al., 2013). Have snakes retained an ancestral pattern or have they evolved dependence on calcium in yolk from an ancestral lizard lineage in which embryos obtained most calcium from the eggshell? Broader taxonomic sampling will provide a test for this question and the outcome will shape our understanding of the evolution of the reptilian egg.

#### Acknowledgements

We thank Brian Evanshen for assistance in lyophilizing the specimens, and Ray Mohseni for advice and assistance with atomic absorption spectroscopy. Dan Blackburn kindly provided some of the eggs used in the study.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: J.R.S., R.A.P., T.W.E.; Methodology: J.R.S., R.A.P., K.A.M., T.W.E.; Formal analysis: J.R.S., R.A.P., K.A.M., T.W.E.; Resources: R.A.P., T.W.E.; Data curation: J.R.S., R.A.P., K.A.M., T.W.E.; Writing - original draft: J.R.S., R.A.P., K.A.M.; Writing - review & editing: J.R.S., R.A.P., K.A.M., T.W.E.; Project administration: J.R.S., R.A.P., T.W.E.; Funding acquisition: R.A.P.

#### Funding

This work was funded in part by the National Science Foundation (DBI-REU 1062645).

#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.193565.supplemental>

#### References

- Brown, G. P. and Shine, R.** (2005). Links between female phenotype, life-history and reproductive success in free-ranging snakes (*Tropidonophis mairii*, Colubridae). *Ecology* **86**, 2763-2770.
- Ecay, T. W., Stewart, J. R. and Blackburn, D. G.** (2004). Expression of calbindin-D<sub>28k</sub> by yolk sac and chorioallantoic membranes of the Corn Snake, *Elaphe guttata*. *J. Exp. Zool.* **302B**, 517-525.
- Gutzke, W. H. N. and Packard, G. C.** (1987). Influence of the hydric and thermal environments on eggs and hatchlings of bull snakes *Pituophis melanoleucus*. *Physiol. Zool.* **60**, 9-17.
- Hanken, J. and Wassersug, R.** (1981). The visible skeleton: a new double-stain technique reveals the nature of the "hard" tissues. *Funct. Photog.* **44**, 22-26.
- Jayne, B. C. and Bennett, A. F.** (1990). Selection on locomotor performance capacity in a natural population of garter snakes. *Evolution* **44**, 1204-1229.
- Jenkins, N. K. and Simkiss, K.** (1968). The calcium and phosphate metabolism of reproducing reptiles with particular reference to the Adder (*Vipera berus*). *Comp. Biochem. Physiol.* **26**, 865-876.
- Kohring, R.** (1996). Structure and biomineralization of eggshell of *Elaphe guttata* (Linne 1766) (Serpentes: Colubridae). *Berliner Geowiss. Abh.* **E18**, 191-200.
- Nys, Y., Gautron, J., Garcia-Ruiz, J. M. and Hincke, M. T.** (2004). Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *C. R. Palevol.* **3**, 549-562.
- Packard, G. C. and Packard, M. J.** (1980). Evolution of the cleidoic egg among reptilian antecedents of birds. *Amer. Zool.* **20**, 351-362.
- Packard, G. C., Tracy, C. R. and Roth, J. J.** (1977). The physiological ecology of reptilian eggs and embryos, and the evolution of viviparity within the class Reptilia. *Biol. Rev.* **52**, 71-105.
- Packard, M. J.** (1994). Patterns of mobilization and deposition of calcium in embryos of oviparous, amniotic vertebrates. *Israel J. Zool.* **40**, 481-492.
- Packard, M. J. and DeMarco, V. G.** (1991). Eggshell structure and formation in eggs of oviparous reptiles. In *Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles* (ed. D. C. Deeming and M. W. J. Ferguson), pp. 53-69. Cambridge, England: Cambridge University Press.
- Packard, M. J. and Packard, G. C.** (1984). Comparative aspects of calcium metabolism in embryonic reptiles and birds. In *Respiration and Metabolism of Embryonic Vertebrates* (ed. R. S. Seymour), pp. 155-179. Dordrecht, Netherlands: W Junk Publ.
- Packard, M. J. and Packard, G. C.** (1986). Effect of water balance on growth and calcium mobilization of embryonic painted turtles (*Chrysemys picta*). *Physiol. Ecol.* **59**, 398-405.
- Packard, M. J. and Packard, G. C.** (1988). Sources of calcium and phosphorus during embryogenesis in Bullsnakes (*Pituophis melanoleucus*). *J. Exp. Zool.* **246**, 132-138.
- Packard, M. J. and Packard, G. C.** (1989). Environmental modulation of calcium and phosphorus metabolism in embryonic Snapping Turtles (*Chelydra serpentina*). *J. Comp. Physiol. B.* **159**, 501-508.
- Packard, M. J. and Seymour, R. S.** (1997). Evolution of the amniote egg. In *Amniote Origins* (ed. S. S. Sumida and K. L. M. Martin), pp. 265-290. San Diego, California: Academic Press.
- Packard, M. J., Packard, G. C. and Boardman, T. J.** (1982). Structure of eggshells and water relations of reptilian eggs. *Herpetologica* **38**, 136-155.
- Packard, M. J., Packard, G. C. and Gutzke, W. H. N.** (1984). Calcium metabolism in embryos of the oviparous snake *Coluber constrictor*. *J. Exp. Biol.* **110**, 99-112.
- Packard, M. J., Packard, G. C., Miller, J. D., Jones, M. E. and Gutzke, W. H. N.** (1985). Calcium mobilization, water balance, and growth in embryos of the agamid lizard *Amphibolurus barbatus*. *J. Exp. Zool.* **235**, 349-357.
- Packard, M. J., Phillips, J. A. and Packard, G. C.** (1992). Sources of mineral for green iguanas (*Iguana iguana*) developing in eggs exposed to different hydric environments. *Copeia* **1992**, 851-858.
- Pyron, R. A., Burbrink, F. T. and Wiens, J. J.** (2013). A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* **13**, 93.
- Simkiss, K.** (1967). *Calcium in Reproductive Physiology*. London, England: Chapman and Hall Ltd.
- Stewart, J. R.** (1997). Morphology and evolution of the egg of oviparous amniotes. In *Amniote Origins* (ed. S. S. Sumida and K. L. M. Martin), pp. 291-326. San Diego, California: Academic Press.
- Stewart, J. R.** (2013). Fetal nutrition in lecithotrophic squamate reptiles: toward a comprehensive model for evolution of viviparity and placentation. *J. Morphol.* **274**, 824-843.
- Stewart, J. R. and Ecay, T. W.** (2010). Patterns of maternal provision and embryonic mobilization of calcium in oviparous and viviparous squamate reptiles. *Herp. Cons. Biol.* **5**, 341-359.
- Stewart, J. R., Ecay, T. W. and Blackburn, D. G.** (2004). Sources and timing of calcium mobilization during embryonic development of the Corn Snake, *Pantherophis guttatus*. *Comp. Biochem. Physiol. A* **139**, 335-341.
- Stewart, J. R., Ecay, T. W. and Heulin, B.** (2009a). Calcium provision to oviparous and viviparous embryos of the reproductively bimodal lizard, *Zootoca vivipara*. *J. Exp. Biol.* **212**, 2520-2524.
- Stewart, J. R., Ecay, T. W., Garland, C. P., Fregoso, S. P., Price, E. K., Herbert, J. F. and Thompson, M. B.** (2009b). Maternal provision and embryonic uptake of calcium in an oviparous and a placental viviparous Australian lizard (Lacertilia: Scincidae). *Comp. Biochem. Physiol. A* **153**, 202-208.
- Stewart, J. R., Ecay, T. W., Heulin, B., Fregoso, S. P. and Linville, B. J.** (2011). Developmental expression of calcium transport proteins in extraembryonic membranes of oviparous and viviparous *Zootoca vivipara* (Lacertilia, Lacertidae). *J. Exp. Biol.* **214**, 2999-3004.
- Zehr, D. R.** (1962). Stages in the normal development of the common garter snake, *Thamnophis sirtalis sirtalis*. *Copeia* **1962**, 322-329.