Received: 5 November 2017 | Revised: 8 March 2018 | Accepted: 13 March 2018

DOI: 10.1002/jez.b.22800

RESEARCH ARTICLE

WILEY JEZ-B MOLECULAR AND DEVELOPMENTAL EVOLUTION

Uterine and eggshell modifications associated with the evolution of viviparity in South American water snakes (*Helicops* spp.)

Henrique B. Braz[1,2,3] | **Selma M. Almeida-Santos**[2,3] | **Christopher R. Murphy**[4] | **Michael B. Thompson**[1]

[1]School of Life and Environmental Sciences, University of Sydney, Sydney, Australia

[2]Laboratório de Ecologia e Evolução, Instituto Butantan, São Paulo, Brazil

[3]Departamento de Anatomia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil

[4]Discipline of Anatomy and Histology, School of Medical Science and Bosch Institute, University of Sydney, Sydney, Australia

Correspondence
Henrique B. Braz, Laboratório de Ecologia e Evolução, Instituto Butantan, São Paulo, Brazil.
Email: h.braz@hotmail.com

Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 235248/2014-2; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: 2009/54478-3; Australian Research Council, Grant/Award Number: DP120100649

All authors have read the paper and agreed to have their names listed as authors.

Abstract

The evolution of viviparity requires eggshell thinning to bring together the maternal uterus and extraembryonic membranes to form placentae for physiological exchanges. Eggshell thinning likely involves reduced activity of the uterine glands that secrete it. We tested these hypotheses by comparing the uterine and eggshell structure and histochemistry among oviparous and viviparous water snakes (*Helicops*) using phylogenetic methods. Eggshell thinning occurred convergently in all three origins of viviparity in *Helicops* and was accomplished by the loss of the mineral layer and thinning of the shell membrane. Uterine glands secrete the shell membrane in both oviparous and viviparous *Helicops*. These glands increase during vitellogenesis regardless of the reproductive mode, but they always reach smaller sizes in viviparous forms. As there is no phylogenetic signal in eggshell thickness and gland dimensions, we conclude that interspecific differences are related to reproductive mode and not phylogeny. Therefore, our results support the hypothesis that eggshell thinning is associated with the evolution of viviparity and that such thinning result from a reduction in gland size in viviparous taxa. Interestingly, the shell membrane thickness of viviparous females of the reproductively bimodal *Helicops angulatus* is intermediate between their oviparous and viviparous congeners. Thus, although eggshell thinning is required by the evolution of viviparity, a nearly complete loss of this structure is not. However, uterine gland dimensions are similar across viviparous *Helicops*. Fewer glands or their functional repurposing may explain the thinner shell membrane in viviparous species of *Helicops* in comparison to viviparous females of the bimodal *H. angulatus*.

KEYWORDS

comparative methods, eggshell formation, oviduct histochemistry, oviparity, shell glands, uterine morphology

1 | INTRODUCTION

Oviparity (the deposition of shelled eggs) is the ancestral and predominant reproductive mode in squamate reptiles. Viviparity (parturition of young) occurs in nearly 20% of the squamate species (Blackburn, 1985), but it has evolved from oviparity at least 115 times independently via prolonging intrauterine egg retention (Blackburn, 2015; Shine & Thompson, 2006). Despite these numerous origins, the evolution of viviparity is a complex change that requires a series of morphological and physiological modifications for pregnancy to occur successfully (Murphy & Thompson, 2011; Thompson & Speake, 2006). Such modifications include prolonging of intrauterine embryonic development, reduction of the eggshell thickness, and formation of placentae for physiological exchanges between mother and embryo (Murphy & Thompson, 2011; Thompson, Adams, Herbert, Biazik, & Murphy, 2004).

Eggshell reduction is one of the fundamental modifications that must occur during the evolutionary transition from oviparity to viviparity. The term eggshell encompasses all layers deposited on the egg after ovulation, and in oviparous squamates, it consists of the following three layers: the inner boundary, the shell membrane, and the mineral layer (Packard & DeMarco, 1991; Packard, Packard, & Boardman, 1982). The inner boundary is the thin, innermost layer of the eggshell. The shell membrane comprises a relatively thick layer of proteinaceous fibers overlying the inner boundary. The mineral layer overlies the shell membrane, and in most squamates, it consists of calcium carbonate as

calcite (Packard & DeMarco, 1991). Most viviparous species may also have an eggshell surrounding the egg, but it lacks the mineral layer and the shell membrane is considerably reduced compared with oviparous species (Blackburn, 1998; Weekes, 1935). In some viviparous squamates, a shell membrane exists early in development but deteriorates through embryogenesis (Jerez & Ramírez-Pinilla, 2003; Murphy, Brandley, Murphy, & Thompson, 2012; Stewart & Thompson, 2009). Eggshell reduction in viviparous species is considered an obligatory correlate during the transition from oviparity to viviparity because this structure acts as a physical barrier to diffusion of gases between the embryo and its external environment (Deeming & Thompson, 1991; Thompson et al., 2004, but see Mathies & Andrews, 2000). Moreover, oxygen requirements of the embryo increase throughout development, especially during late growth (Robert & Thompson, 2000; Van Dyke and Beaupre, 2011; Vleck & Hoyt, 1991). Thus, the prolonged retention of shelled eggs within the uterus would impose serious restrictions for maternal–fetal exchanges, and the increases in intrauterine egg retention may require a correlated decrease in eggshell thickness (Guillette, 1993; Packard, Tracy, & Roth, 1977; Shine & Thompson, 2006). Alternatively, eggshell reduction is suggested to occur after complete intrauterine embryonic development has evolved (Tinkle & Gibbons, 1977). Independently of the timing of such reduction, a thinner eggshell brings together the uterine epithelium and extraembryonic membranes to form placentae for physiological exchanges (Griffith, Blackburn, Brandley, Van Dyke JU, & Thompson, 2015; Guillette, 1993; Thompson et al., 2004).

The eggshell components are secreted in the oviduct after ovulation. Despite the potential role of the uterine luminal epithelium in secreting some components of the eggshell (e.g., the inner boundary; Heulin et al., 2005; Hoffman, 1970; Stewart et al., 2010), the thicker eggshell component (i.e., the shell membrane) is secreted by uterine glands (Corso, Delitala, & Carcupino, 2000; Heulin et al., 2005; Palmer, DeMarco, & Guillette, 1993; Stewart et al., 2010). Therefore, the proximate mechanism by which the eggshell is reduced in thickness during the evolution of viviparity likely involves reducing the activity of these uterine glands (Guillette, 1992, 1993), which may be achieved by reduction in gland size (Heulin et al., 2005). Previous researchers have stated that uterine glands are sparse or less developed in viviparous squamates, although no quantitative evidence is provided (e.g., Angelini & Ghiara, 1984; Blackburn, 1998; Boyd, 1943; Girling et al., 1998; Guillette, 1992; Picariello, Ciarcia, & Angelini, 1989). However, these comparisons are made among distantly related taxa, which may be misleading because some features exhibited by viviparous taxa may actually reflect subsequent adaptations to the evolution of viviparity (Albergotti & Guillette, 2011; Guillette, 1993). Thus, comparisons among distantly related species may say little about the steps required for the evolutionary transition from oviparity to viviparity. A more robust approach focuses on closely related taxa exhibiting variation in reproductive modes (e.g., Adams, Biazik, Stewart, Murphy, & Thompson, 2007; Qualls, 1996). In these situations, any difference is much more readily related to the evolution of viviparity and less confounded by changes occurred after it has evolved. Two studies have quantitatively evaluated the association between uterine gland size and eggshell thickness in closely related taxa, but they produced conflicting results. Eggshell reduction has occurred in viviparous females of the reproductively bimodal lizards *Zootoca vivipara* and *Saiphos equalis*, but such reduction is correlated with a decrease in size of the uterine glands only in *Z. vivipara* (Heulin et al., 2005; Stewart et al., 2010). Therefore, additional studies with closely related taxa varying in reproductive modes are needed to investigate uterine gland reduction and its association with eggshell reduction and the evolution of viviparity.

The South American water snakes of the genus *Helicops* provide an excellent model system to test for the association between eggshell reduction and uterine gland reduction during the evolution of viviparity in a phylogenetic structure. Reproductive mode varies within the genus. Of the 17 species currently recognized (Uetz & Hošek, 2017), at least two are oviparous and nine are viviparous (Braz, Scartozzoni, & Almeida-Santos, 2016; Costa et al., 2016). In addition, one species (*Helicops angulatus*) exhibits geographic variation in reproductive mode. This species is oviparous from northern to mid-eastern and north-eastern South America, but viviparous populations occur in north-western to mid-western South America (Braz et al., 2016). Importantly, viviparity has evolved independently at least three times in *Helicops*, thus providing replication for both comparisons of closely related taxa varying in reproductive mode and reconstructions of the morphological modifications associated with the evolution of viviparity (Braz et al., 2016).

Here, we used the water snakes of the genus *Helicops* to test the hypotheses that eggshell reduction is associated with the evolution of viviparity and such reduction results from the decreased size of the uterine glands. For that, we used light microscopy to describe and quantify uterine glands, uterine epithelium, and eggshell components in oviparous and viviparous *Helicops*. We also employed several histochemical techniques to characterize the eggshell composition and identify the uterine structures responsible for secreting each eggshell component. Then we compare uterine structures and eggshell thickness across oviparous and viviparous *Helicops* and verify the correlation between uterine gland dimensions and shell membrane thickness.

2 | MATERIAL AND METHODS

2.1 | Study species and sample collection

We studied the following five species of *Helicops*: two oviparous (*H. gomesi* and *H. hagmanni*), two viviparous (*H. carinicaudus* and *H. infrataeniatus*), and both oviparous and viviparous individuals of the reproductively bimodal *H. angulatus* (Braz et al., 2016). These species represent a fraction of the diversity of *Helicops*. However, all the oviparous *Helicops* known to date were included, and importantly, the viviparous forms studied are representative of all three distinct origins of viviparity identified in the genus (Braz et al., 2016). We also included the oviparous *Hydrops martii* as the sister group of *Helicops* for morphometric comparisons only (see below). Uterine and egg samples were collected from specimens preserved in museums (Table 1; see Supporting Information Table S1 for a full list), which proved to be a useful approach since some of the species studied are rare and difficult to locate in nature (e.g., *H. gomesi*, *H. hagmanni*). The use of museum

TABLE 1 Sample sizes per reproductive stage for *Helicops* spp. and *Hydrops martii*

Species	Reproductive mode	Primary vitellogenesis	Secondary vitellogenesis	Gravid/Pregnant
Helicops gomesi	Oviparous	3	3	1
Helicops hagmanni	Oviparous	7	7	1
Helicops angulatus	Oviparous	8	10	10
Helicops angulatus	Viviparous	7	5	5
Helicops carinicaudus	Viviparous	7	6	4
Helicops infrataeniatus	Viviparous	8	5	4
Hydrops martii	Oviparous	6	6	1

specimens has been invaluable in generating knowledge on various aspects of animal biology, including squamate viviparity (Blackburn & Flemming, 2010).

Females were dissected through a mid-ventral incision, and the sizes of ovarian follicles were recorded. We assigned nonpregnant females to one of two stages of vitellogenesis based on follicular size (Aldridge, 1979). Females in primary vitellogenesis had ovarian follicles bellow 6.0 mm, and females in secondary vitellogenesis had enlarged ovarian follicles ranging from 11.0 to 21.7 mm (Supporting Information Table S2). Follicular size for females in secondary vitellogenesis were in general consistent with the size of preovulatory follicles for each studied species (Aguiar & Di-Bernardo, 2005; Scartozzoni, 2009; H. B. Braz, pers. obs.). Preliminary analyses showed that the mean follicular size differed between the two stages of vitellogenesis (being larger in secondary than in primary vitellogenesis) but not among species (two-way analysis of variance (ANOVA), reproductive stage: $F_{1,74} = 1347.0$, $P < 0.0001$; species: $F_{6,74} = 1.1$, $P = 0.38$; interaction: $F_{6,74} = 1.8$, $P = 0.11$). Thus, our assignment of females to either primary or secondary vitellogenesis based on follicular size produced two distinctive categories of preovulatory reproductive stages (Supporting Information Table S2). Fragments of the uterus of nongravid females and incubation chambers (uterus containing eggs) from gravid/pregnant females were collected and stored in 70% ethanol solution until processing for light microscopy. As a standard procedure, we collected only samples from the right uterus. For each gravid/pregnant female, we dissected one egg under a stereomicroscope and determined the embryonic developmental stage using the staging system for the snake *Thamnophis sirtalis*, which divides the embryonic development into a series of 37 stages (Zehr, 1962).

2.2 | Histological and histochemical procedures

Before processing for paraffin embedding, the incubation chambers were immersed in Bouin's fluid for 24 h to make the tissues harder and more readily trimmed. Incubation chambers were then washed in successive changes of 70% ethanol solution until the yellow color of Bouin's fluid was almost fully removed. Each incubation chamber was then transversally cut using a sharp blade and returned to 70% ethanol solution. All samples were dehydrated through a 70–100% series of ethanol concentrations, cleared in xylene, and embedded in paraffin (Kiernan, 2008). Sections were cut at 6–9 μm using a rotary microtome (Thermo-Microm HM 325), mounted on gelatin-coated or super-frosted slides (Menzel-Gläser, Thermo Scientific), deparaffinized with Histolene® (Fronine Lab Supplies, Riverstone, NSW, Australia), and rehydrated in descending ethanol concentrations to tap water. Sections were stained with hematoxylin and eosin for examination of general morphology. Several histochemical techniques were performed to characterize eggshell composition and identify the uterine structures responsible for secreting each eggshell component. The periodic acid-Schiff (PAS) reaction was performed for identification of neutral carbohydrates, and Alcian blue 8GX (pH 2.5) was used for carboxyl and sulphate-ester groups of acid mucosubstances (Kiernan, 2008). To detect proteins, sections were stained with Coomassie brilliant blue R250 (Kiernan, 2008) diluted at 0.04 mg/mL. Histological sections were dehydrated in increasing graded ethanol, cleared with Histolene® and coverslipped with DPX. Sections were photographed with an Olympus DP73 digital camera (Olympus Corporation, Tokyo, Japan) mounted on an Olympus BX53 light microscope (Olympus Corporation, Tokyo, Japan), and the software cellSens Standard, version 1.11 was used for image capture. Photomicrographs were minimally processed for sharpness and color balance using the same software. Images were cropped and labeled with Microsoft PowerPoint 2010.

2.3 | Morphometry

Uterine gland dimensions and the luminal epithelium height were estimated for each female. We also estimated the thickness of the shell membrane and the outer layer of the eggshell for each gravid/pregnant female. Measurements were taken on digital photos (magnification of 10 times) of two different regions of the histological sections of each female using the software ImageJ, version 1.51d (Abràmoff, Magalhães, & Ram, 2004). Dimensions of uterine glands were estimated by taking two measures (height and width; Stewart et al., 2010) of all glands visualized in the digital photos. The epithelial height was estimated by measuring at least 10 epithelial cells (five per region). The eggshell thickness was estimated by taking at least 10 measures (five per region) from two different sections of the same egg. Values for all measured variables were then averaged to obtain a mean value per female.

2.4 | Analyses

Statistical analyses were performed using R (R Development Core Team, 2016) with significance assumed at $P < 0.05$. We used Welch's *t*-test and one-way ANOVA to verify intraspecific differences in the

uterine gland dimensions and epithelial height across reproductive stages.

We employed phylogenetic comparative methods to analyze our data and account for the shared history among species (Felsenstein, 1985; Harvey & Pagel, 1991). Phylogenetic relationships for *Helicops* were extracted from the most comprehensive phylogeny available for snakes (Figueroa, McKelvy, Grismer, Bell, & Lailvaux, 2016). This phylogeny includes only five out of 17 known species of *Helicops*, but despite being incomplete, it includes all species studied here. In this phylogeny, a clade formed by *Hydrops triangularis* and *Pseudoeryx plicatilis* was recovered as the sister group of *Helicops* (Figueroa et al., 2016). In our analyses, we used *Hy. martii* as the sister group of *Helicops* because it was the only species of *Hydrops* we had access to gravid females for histological examinations. We consider this modification acceptable since species of *Hydrops* form a cohesive group sharing several unique cranial and hemipenian features (Di Pietro, Alcalde, & Williams, 2014; Zaher, 1999). For the reproductively bimodal *H. angulatus*, we assumed oviparous and viviparous populations represent two closely related evolutionary lineages (Braz et al., 2016). By doing these modifications in the tree, we lost information on branch lengths. Thus, we tested four different types of arbitrary branch lengths: all = 1 (constant), Grafen (1989), Pagel (1992), and Nee (Purvis, 1995). We computed branch lengths using PDAP:PDTREE module, version 1.16 (Midford et al., 2011) in Mesquite software, version 3.2 (Maddison & Maddison, 2017). Results were the same for all types of branch lengths. We then report results based on the method of Grafen (1989).

We tested for phylogenetic signal in the continuous variables (uterine gland dimensions, epithelial thickness, and shell membrane thickness) estimating Blomberg's *K* (Blomberg, Garland, & Ives, 2003) and Pagel's (1999) lambda (λ) and their significance using the function 'phylosig' in the Phytools package (Revell, 2012). We also estimated the strength of phylogenetic signal of reproductive mode by computing the Fritz and Purvis's *D* statistic for binary traits (Fritz & Purvis, 2010) using the 'phylo.d' function in Caper package (Orme et al., 2015). Significant departures from both phylogenetic randomness and Brownian threshold models were tested comparing the observed *D*-value with the value found using 1,000 simulations for each model (Fritz & Purvis, 2010).

We conducted phylogenetic ANOVAs (Garland, Dickerman, Janis, & Jones, 1993) with post hoc comparisons to test for interspecific differences in uterine gland height, uterine gland width, epithelial height, and shell membrane thickness using the package Phytools (Revell, 2012). The analyses for gland dimensions and epithelial height were conducted separately for primary and secondary vitellogenesis categories. Because of the small sample size for gravid females, we did not include the oviparous *H. gomesi*, *H. hagmanni*, and *Hy. martii* in the comparisons of the shell membrane thickness. In this case, the phylogenetic tree was pruned to contain only the taxa included in the analysis. For the phylogenetic ANOVAs, we first carried out 1,000 simulations of the evolution of each dependent variable (log-transformed) by Brownian motion on the phylogeny (Garland et al., 1993). Then, we generated 1,000 simulations at the individual level similarly as implemented by Arias et al. (2016). For that, we used the 'rnorm' function to sample the same number of observations per species as in the empirical dataset based on the simulated mean value per species and with the coefficient of variation computed from the empirical data (Arias et al., 2016). We performed ANOVA on the simulated datasets and obtained a null distribution of *F*-values for each variable. Then, we carried out conventional ANOVAs in the empirical data and compared the observed *F*-values with those from the null distribution (Arias et al., 2016; Garland et al., 1993). The *P*-value for the phylogenetic ANOVA was calculated as the proportion of the *F*-values from the simulated data that were higher than the observed *F* statistic (Garland et al., 1993). Post hoc comparisons of the means were conducted similarly. We performed pairwise *t* statistics on each simulated dataset and calculated the proportion of simulated *t*-values that were higher than the observed *t* statistic while controlling for multiple tests with the Holm–Bonferroni method.

Finally, we tested whether shell membrane thickness is correlated with uterine gland dimensions (in secondary vitellogenic females) using phylogenetic generalized least squares (PGLS) regressions (Grafen, 1989). For that, we used the 'pgls' function in Caper to fit a model that uses maximum likelihood to simultaneously estimate the regression parameters and phylogenetic signal (Pagel's λ) of the residual error and therefore accounting for the phylogenetic signal in the correlation between variables (Revell, 2010; Symonds & Blomberg, 2014). The PGLS_{λ} is nearly equivalent to ordinary least squares regression if there is no phylogenetic signal in the model residuals (i.e., $\lambda = 0$), but can equal phylogenetic independent contrasts if a strong phylogenetic signal is present (i.e., $\lambda = 1$) (Revell, 2010; Symonds & Blomberg, 2014).

3 | RESULTS

The uteri of all oviparous and viviparous species studied are structurally similar. Although morphological and histochemical changes occur throughout the reproductive cycle, the observed interspecific differences are attributable to reproductive modes. Thus, for brevity, the general uterine morphology of oviparous and viviparous species is described together. Moreover, we present below representative photomicrographs of the uterus from different species but arranged to compare oviparous and viviparous forms. Photomicrographs of the uteri for each species of *Helicops* studied and all staining techniques used are provided in the Supporting Information Material (Supporting Information Figures S1–S6).

3.1 | General morphology of the uterus

The uterus of *Helicops* has three layers: the muscularis externa, the lamina propria, and the luminal epithelium (Figure 1). The muscularis externa is composed of inner circular and outer longitudinal smooth muscle (Figure 1). The lamina propria contains glands, blood vessels, and fibroblasts intercalated within irregular connective tissue (Figure 1). The glandular cells are organized in a circular or ovoid arrangement around a central lumen. The nuclei of glandular cells are basally located in the cells (Figure 1). The luminal epithelium is formed by a single layer of ciliated and nonciliated cells, which are cuboidal to low columnar in nongravid females and low cuboidal to squamous in gravid/pregnant females (Figure 1).

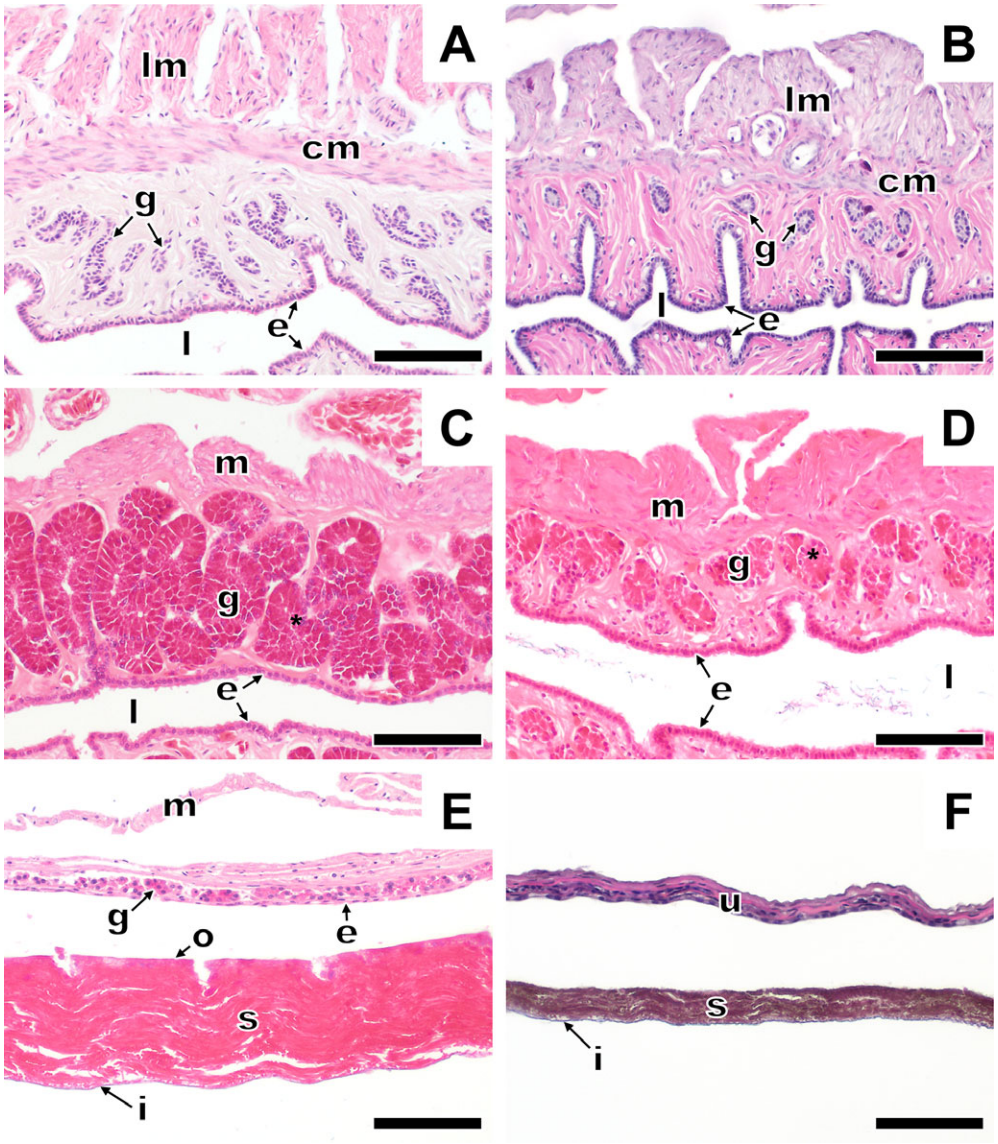

FIGURE 1 Representative histology (hematoxylin–eosin) of the uterus of oviparous and viviparous *Helicops* in three reproductive stages. (A) Oviparous (*H. hagmanni*), primary vitellogenesis. (B) Viviparous (*H. angulatus*, viviparous populations), primary vitellogenesis. (C) Oviparous (*H. hagmanni*), secondary vitellogenesis. (D) Viviparous (*H. angulatus*, viviparous populations), secondary vitellogenesis. (E) Oviparous (*H. hagmanni*), gravidity. (F) Viviparous (*H. angulatus*, viviparous populations), pregnancy. cm, circular muscle; e = uterine luminal epithelium; g = uterine glands; i = inner boundary of the eggshell; l = uterine lumen; lm = longitudinal muscle; m, muscle; o = outer surface of the eggshell; s = shell membrane; u = uterus. Asterisk indicates secretory material in uterine glands. Scale bar = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Cyclical variation in uterine morphology and histochemistry

The morphology of the uterus of *Helicops* varies across reproductive stages regardless of reproductive modes. During primary vitellogenesis, the uteri show cuboidal to low columnar epithelia (with ciliated and nonciliated cells) and poorly developed glands distantly spaced from each other (Figure 1A and B; Supporting Information Figures S1–S6). These glands contain little or no secretory materials (Figure 1A and B; Supporting Information Figures S1–S6). In secondary vitellogenesis, the uterine luminal epithelium of oviparous and viviparous females undergoes significant hypertrophy (Supporting Information Table S3) and shows ciliated and nonciliated epithelial cells varying between cuboidal to columnar (Figure 1C and D; Supporting Information Figures S1–S6). The uterine glands also become hypertrophied and filled with secretory material in all species irrespective of the reproductive mode (Figure 1C and D; Supporting Information Table S3), but they are more densely packed in oviparous than in viviparous forms (Figure 1C and D; Supporting Information Figures S1–S6). Unfortunately, we had no access to any gravid uterus of the oviparous *H. gomesi* (and the outgroup *Hy. martii*). For all other species, the uterus is quite distended during gravidity/pregnancy. The muscularis externa is extremely thin (Figure 1E and F; Supporting Information Figures S2–S6). The luminal epithelium is significantly reduced (Supporting Information Table S3) with cells varying between low cuboidal to squamous in oviparous and viviparous females

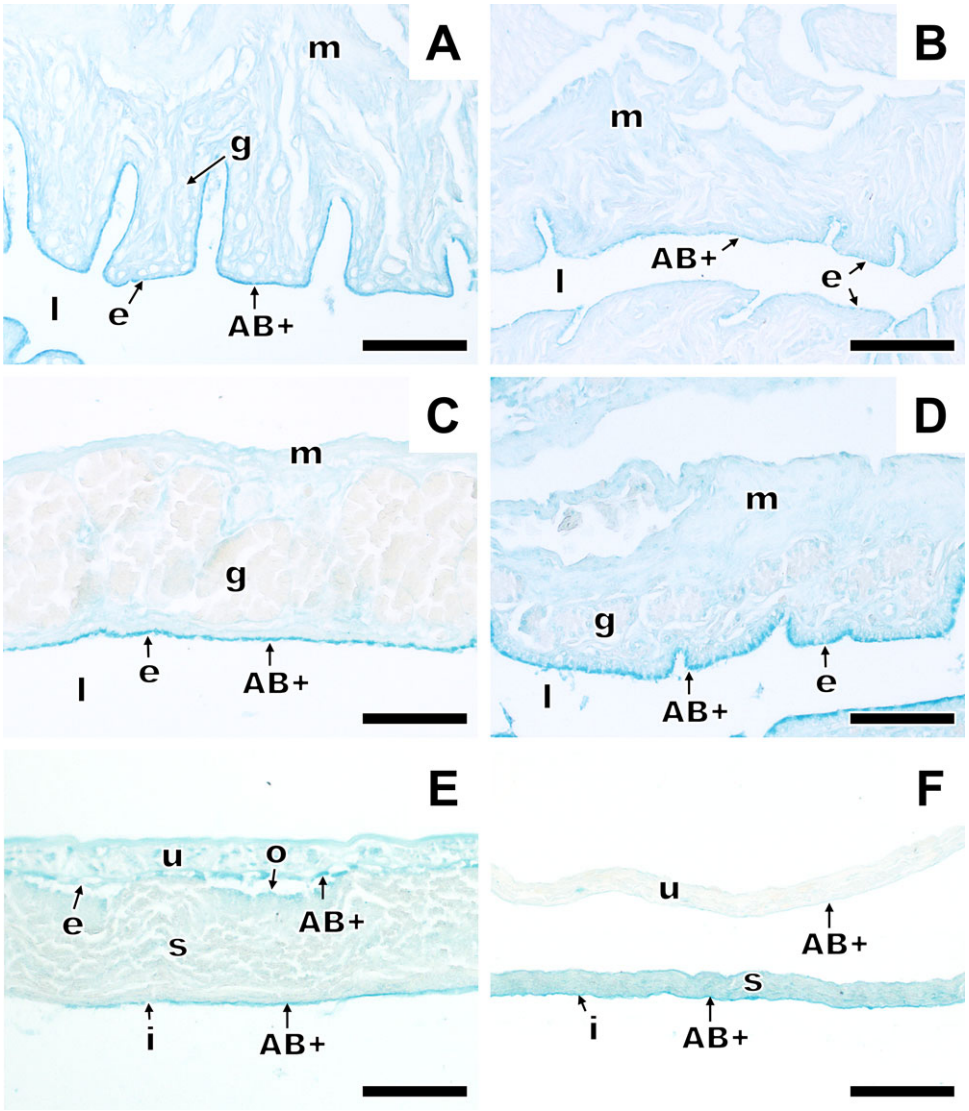

FIGURE 2 Representative histology (Alcian blue, AB) of the uterus of oviparous and viviparous *Helicops* in three reproductive stages. (A) Oviparous (*H. angulatus*, oviparous populations), primary vitellogenesis. (B) Viviparous (*H. infrataeniatus*), primary vitellogenesis. (C) Oviparous (*H. angulatus*, oviparous populations), secondary vitellogenesis. (D) Viviparous (*H. carinicaudus*), secondary vitellogenesis. (E) Oviparous (*H. angulatus*, oviparous populations), gravidity. (F) Viviparous (*H. angulatus*, viviparous populations), pregnancy. e = uterine luminal epithelium; g = uterine glands; i = inner boundary of the eggshell; l = uterine lumen; m = muscle; o = outer surface of the eggshell; s = shell membrane; u = uterus. Scale bar = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 1E and F; Supporting Information Figures S2–S6). The uterine glands are still visible in all gravid oviparous females of *H. hagmanni* and *H. angulatus*, but they are smaller than glands in secondary vitellogenesis (Figure 1E; Supporting Information Figures S2 and S3; Supporting Information Table S3). In contrast, no uterine glands are noticeable in pregnant viviparous forms (Figure 1F; Supporting Information Figures S4–S6).

The apical surface of the uterine epithelium reacts positively to both Alcian blue for acidic mucosubstances (Figure 2) and PAS for neutral carbohydrates (Figure 3) but does not react to Coomassie blue for proteins (Figure 4) in any reproductive stages of both oviparous and viviparous taxa (Supporting Information Figures S1–S6). However, we observed a decrease in the secretion of acidic mucosubstances and neutral carbohydrates in some gravid/pregnant females with some portions of the epithelium not reacting to Alcian blue and PAS. The uterine glands do not react to Alcian blue for acidic mucosubstances (Figure 2) or PAS for neutral carbohydrates (Figure 3) in any reproductive stage in both reproductive modes (Supporting Information Figures S1–S6). The uterine glands do not react to Coomassie blue for proteins in oviparous and viviparous females in primary vitellogenesis (Figure 4A and B) but do react positively in oviparous and viviparous females in secondary vitellogenesis (Figure 4C and D; Supporting Information Figures S1–S6). The intensity of reaction to Coomassie blue is stronger in the oviparous *H. hagmanni* and *H. angulatus* than in viviparous forms (Figure 4C and D; Supporting Information Figures S2–S6). The uterine glands do not react to Coomassie blue in gravid/pregnant females (Figure 4E and F; Supporting Information Figures S2–S6).

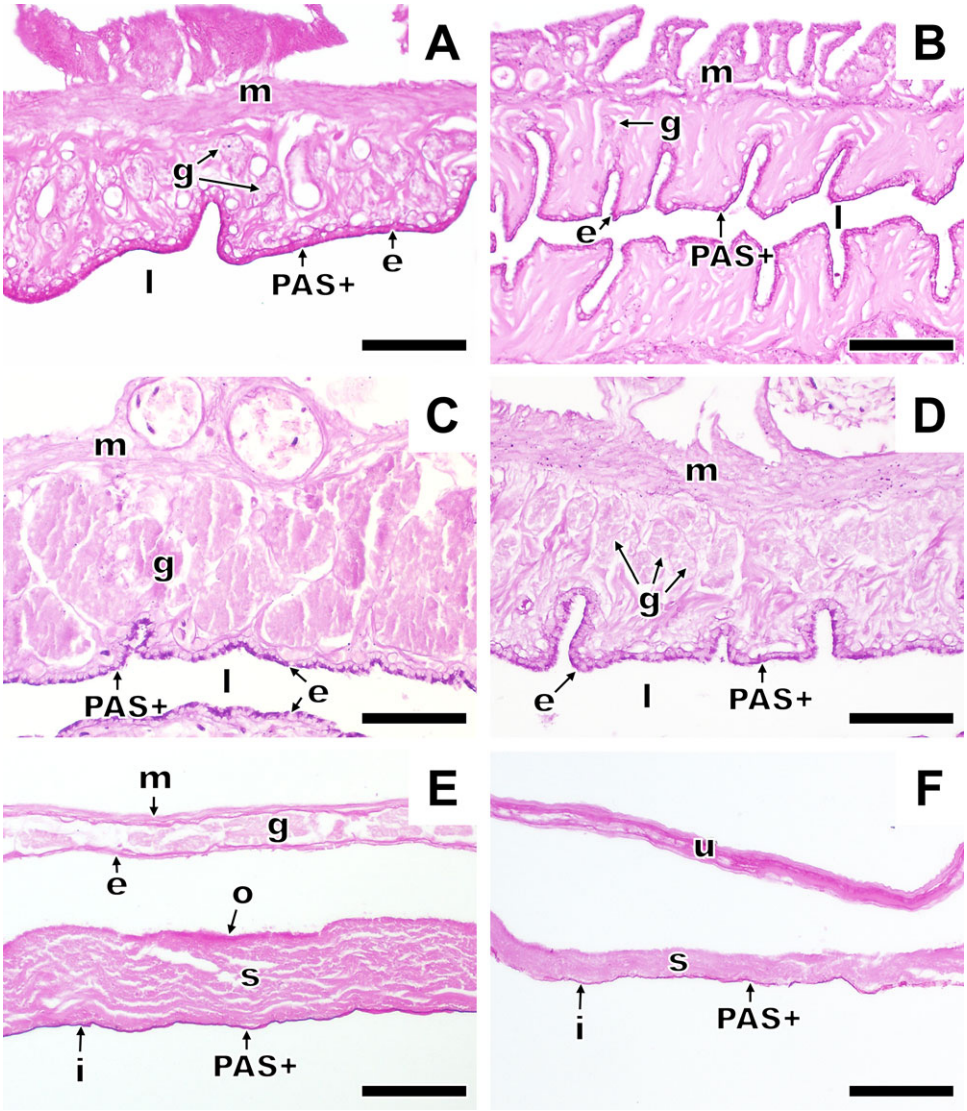

FIGURE 3 Representative histology (PAS) of the uterus of oviparous and viviparous *Helicops* in three reproductive stages. (A) Oviparous (*H. angulatus*, oviparous populations), primary vitellogenesis. (B) Viviparous (*H. angulatus*, viviparous populations), primary vitellogenesis. (C) Oviparous (*H. hagmanni*), secondary vitellogenesis. (D) Viviparous (*H. carinicaudus*), secondary vitellogenesis. (E) Oviparous (*H. hagmanni*), gravidity. (F) Viviparous (*H. angulatus*, viviparous populations), pregnancy. e = uterine luminal epithelium; g = uterine glands; i = inner boundary of the eggshell; l = uterine lumen; m = muscle; o = outer surface of the eggshell; s = shell membrane; u = uterus. Scale bar = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Eggshell structure and histochemistry

An eggshell encloses the eggs of all oviparous and viviparous females examined, but the structure of the eggshell differs between reproductive modes. The eggshells of the oviparous *H. gomesi* and *H. hagmanni* (and the sister species *Hy. martii*; data not shown) consist of an inner boundary overlain by a thick layer of intertwined fibers (shell membrane) and a distinct amorphous layer resting on the outer surface of the shell membrane (Figure 1E; Supporting Information Figures S1 and S2). This amorphous layer does not enclose fibers and is thinner than the shell membrane (Figure 1E; Supporting Information Figures S1 and S2). In contrast, the eggshells of the viviparous *H. carinicaudus* and *H. infrataeniatus* consist of a faintly discernible layer of fibers overlying the inner boundary. In some cases, only the inner boundary is noticeable under light microscopy (Supporting Information Figures S5 and S6). An outer amorphous layer was not observed (Supporting Information Figures S5 and S6). The eggshells of the oviparous individuals of the bimodal *H. angulatus* exhibit the same structure as that of the oviparous species (Supporting Information Figures S1–S3), except for two females whose eggs do not exhibit the outer amorphous layer. The eggs of these two females did not have visible developing embryos and egg shelling was presumably incomplete. The eggshells of the viviparous individuals of *H. angulatus* also exhibit an inner boundary, overlain by a layer of intermingled fibers, but with a noticeably reduced thickness and no outer layer (Figure 1F).

The inner boundary of the eggshell of both oviparous and viviparous *Helicops* reacts positively to Alcian blue for acidic mucosubstances (Figure 2E and F; Supporting Information Figures S1–S6) and PAS for neutral carbohydrates (Figure 3E and F; Supporting Information Figures S1–S6) but does not react to Coomassie blue for proteins

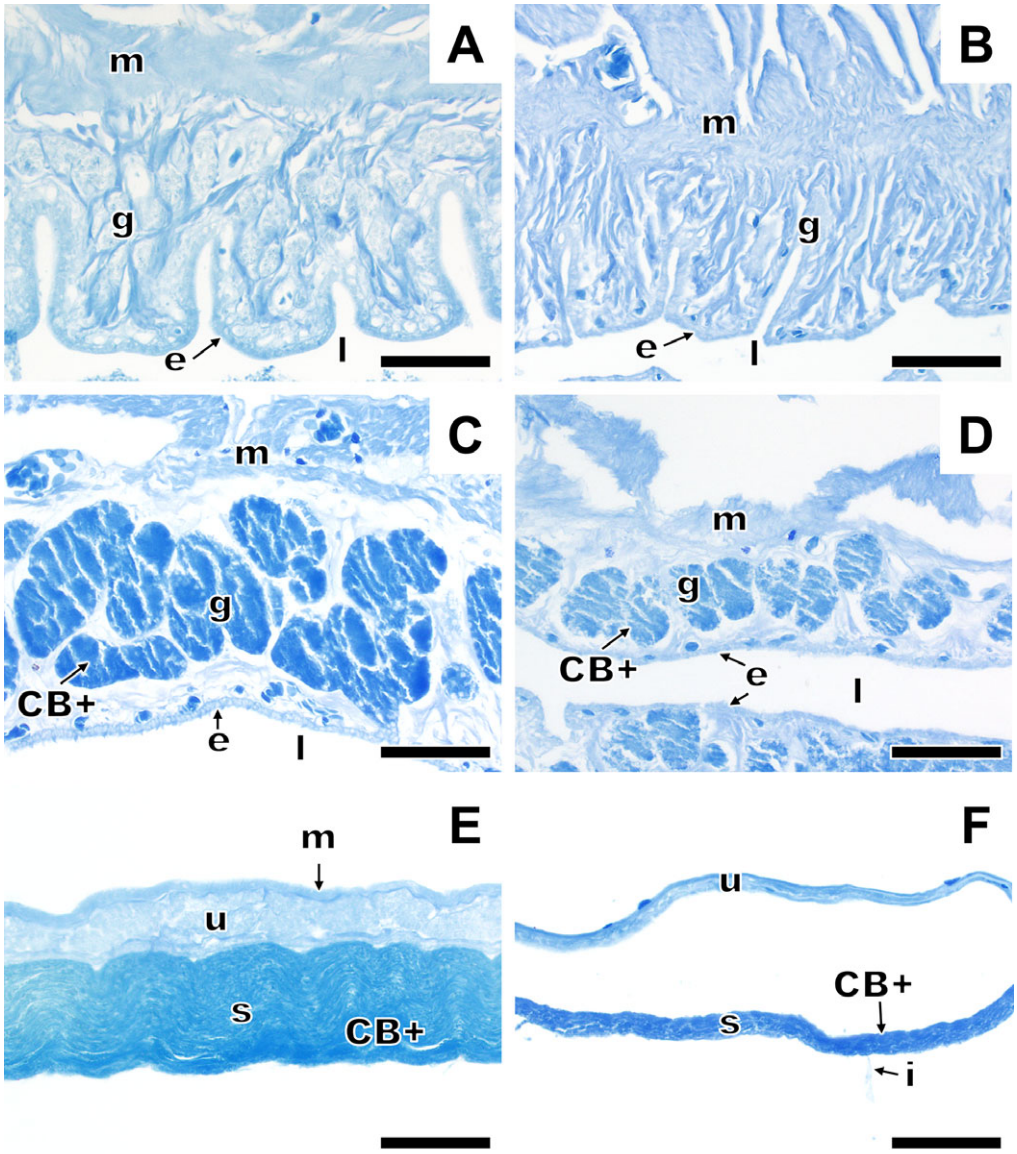

FIGURE 4 Representative histology (Coomassie brilliant blue, CB) of the uterus of oviparous and viviparous *Helicops* in three reproductive stages. (A) Oviparous (*H. angulatus*, oviparous populations), primary vitellogenesis. (B) Viviparous (*H. carinicaudus*), primary vitellogenesis. (C) Oviparous (*H. hagmanni*), secondary vitellogenesis. (D) Viviparous (*H. angulatus*, viviparous populations), secondary vitellogenesis. (E) Oviparous (*H. angulatus*, oviparous populations), gravidity. (F) Viviparous (*H. angulatus*, viviparous populations), pregnancy. e = uterine luminal epithelium; g = uterine glands; i = inner boundary of the eggshell; l = uterine lumen; m = muscle; s = shell membrane; u = uterus. Scale bar = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 4E and F; Supporting Information Figures S1–S6). The fibers of the shell membrane of both oviparous and viviparous *Helicops* react positively to Coomassie blue for proteins (Figure 4E and F; Supporting Information Figures S1–S6), moderately to PAS for neutral carbohydrates (Figure 3E and F; Supporting Information Figures S1–S6), but do not stain with Alcian blue for acidic mucosubstances (Figure 2E and F; Supporting Information Figures S1–S6). The outer surface of the shell membrane of oviparous forms stains moderately with Alcian blue and PAS (Figures 2E and 3E; Supporting Information Figures S1–S3). There is also moderate staining with Alcian blue on the outer surface of the shell membrane from a viviparous female *H. angulatus* (Figure 2F), but no amorphous layer is discernible in hematoxylin–eosin (Figure 1F).

3.4 | Phylogenetic signal and comparative morphometric analysis

There is no significant phylogenetic signal in any of the traits evaluated (Table 2), indicating that closely related species do not have similar reproductive modes, uterine gland size, epithelial height, or eggshell thickness.

We found no interspecific difference in uterine gland height ($F_{6,39}$ = 0.77; phylogenetic ANOVA, P = 0.87; conventional ANOVA, P = 0.60), gland width ($F_{6,39}$ = 0.64; phylogenetic ANOVA, P = 0.78; conventional ANOVA, P = 0.69), and epithelial height ($F_{6,39}$ = 1.16, phylogenetic ANOVA, P = 0.55; conventional ANOVA, P = 0.35) of females in primary vitellogenesis (Figure 5A and B, and Figure 6A). In secondary

TABLE 2 Test for phylogenetic signal in reproductive mode, dimensions of uterine glands, epithelial height, and shell membrane thickness

Trait	Fritz and Purvis's *D*			Blomberg's *K*		Pagel's lambda (λ)	
	D	$P_{H0: D=1}$	$P_{H0: D=0}$	*K*	*P*-value	λ	*P*-value
Reproductive mode	3.536	0.942	0.033	–	–	–	–
Uterine gland height	–	–	–	0.393	0.940	0.000	1.000
Uterine gland width	–	–	–	0.563	0.597	0.000	1.000
Epithelial height	–	–	–	0.654	0.475	0.000	1.000
Shell membrane thickness	–	–	–	0.622	0.571	0.000	1.000

Tests for uterine gland dimensions and epithelial height are only for secondary vitellogenic stage. Note that the absence of phylogenetic signal is supported by the significant difference from 0 but not from 1 in the *D* statistic (Fritz & Purvis, 2010) and the nonsignificant *P*-values for Blomberg's *K* (Blomberg et al., 2003) and Pagel's (1999) lambda (λ). See Material and Methods for details.

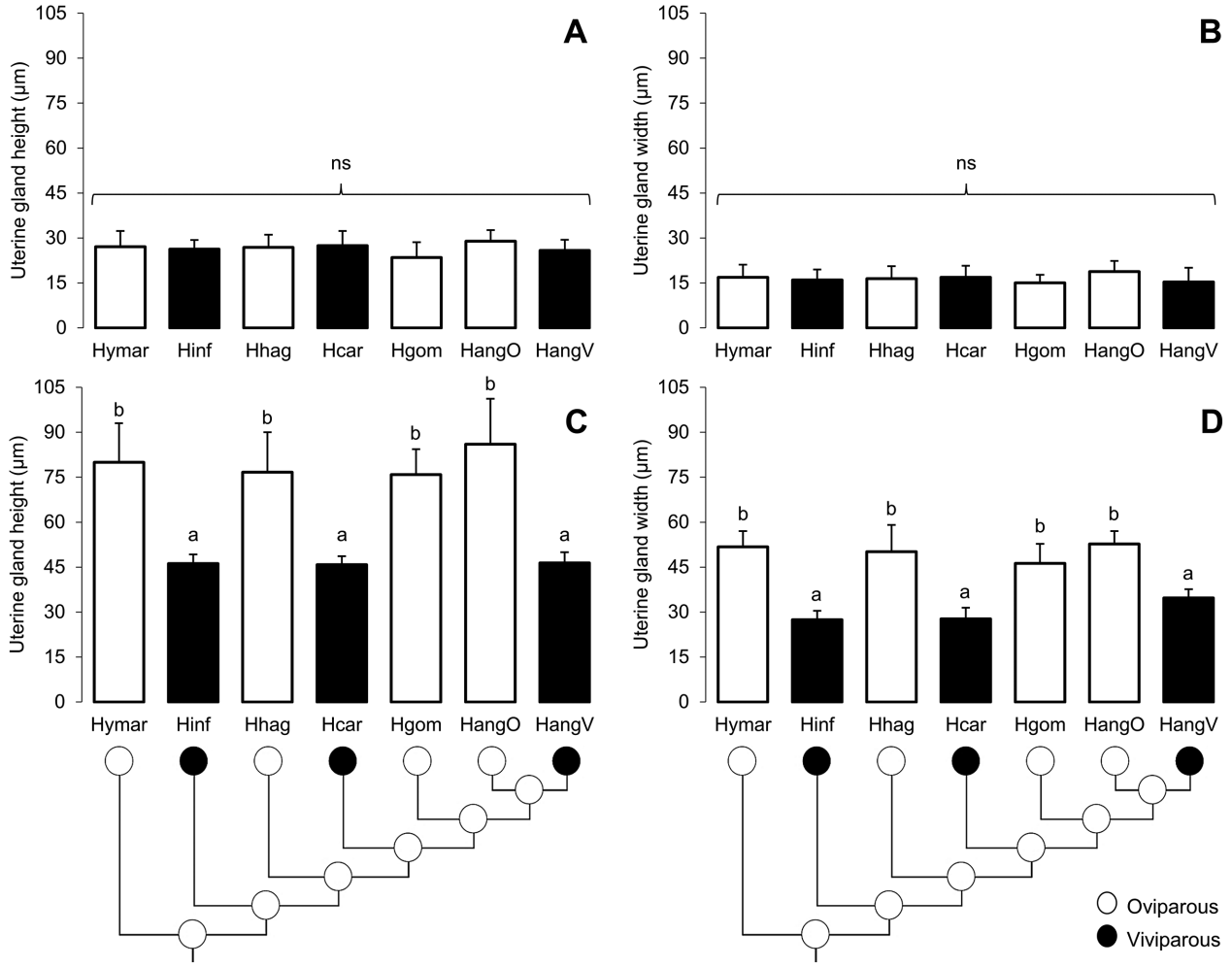

FIGURE 5 Variation in uterine gland dimensions during preovulatory stages in *Helicops* spp. and *Hydrops martii*. (A and B) Primary vitellogenesis. (C and D) Secondary vitellogenesis. Hymar, *Hydrops martii*; Hinf, *Helicops infrataeniatus*; Hhag, *H. hagmanni*; Hcar, *H. carinicaudus*; Hgom, *H. gomesi*; HangO, oviparous *H. angulatus*; HangV, viviparous *H. angulatus*. Reproductive modes (oviparity and viviparity) are mapped onto a phylogeny modified from Figueroa et al. (2016). Different lowercase letters indicate significant differences (post hoc tests for phylogenetic and conventional ANOVA, < 0.001 for all pairwise comparisons, a < b). ns, nonsignificant difference

vitellogenesis, however, the gland height ($F_{6,35} = 27.37$, $P < 0.0001$ for both conventional and phylogenetic ANOVA) and gland width ($F_{6,35} = 36.91$; $P < 0.0001$ for both conventional and phylogenetic ANOVA) differ significantly among species. The uterine glands are larger in oviparous than in viviparous forms (post hoc tests, $P < 0.05$), but similar among species with the same reproductive mode (Figure 5C and D). The epithelial height of females in secondary vitellogenesis differs only between the viviparous *H. carinicaudus* and the oviparous forms of the reproductively bimodal *H. angulatus* using conventional analysis ($F_{6,35} = 2.65$, $P = 0.032$), being lower in the latter. However, the epithelial height of secondary vitellogenic females is similar across species after accounting for phylogeny ($P = 0.065$; Figure 6B).

The shell membrane thickness in the examined individuals of the oviparous *H. gomesi*, *H. hagmanni*, and *Hy. martii* (not included in the

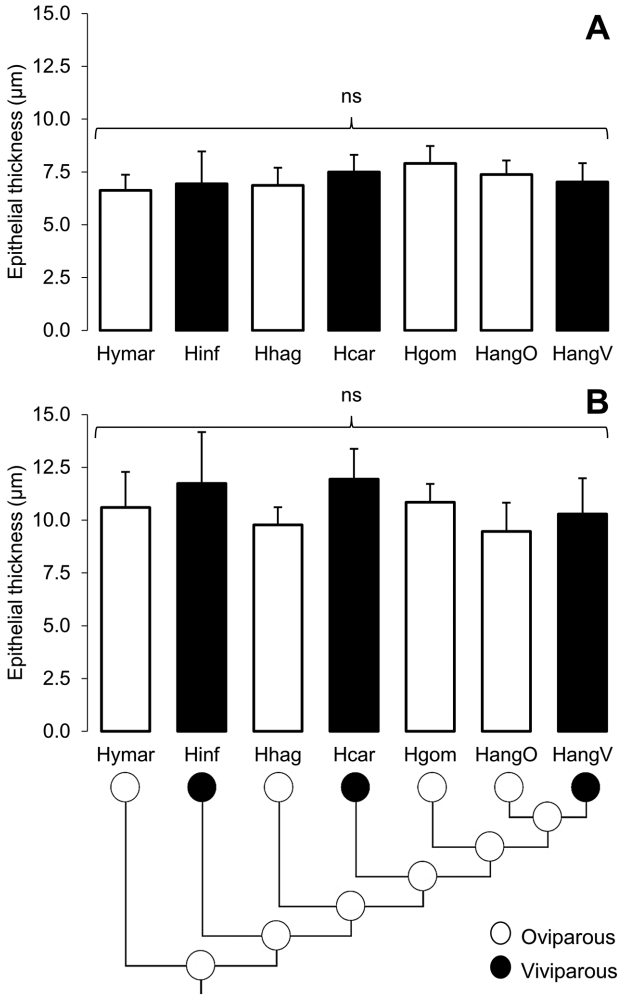

FIGURE 6 Variation in epithelial cell height during preovulatory stages in *Helicops* spp. and *Hydrops martii*. (A) Primary vitellogenesis. (B) Secondary vitellogenesis. Hymar, *Hydrops martii*; Hinf, *Helicops infrataeniatus*; Hhag, *H. hagmanni*; Hcar, *H. carinicaudus*; Hgom, *H. gomesi*; HangO, oviparous *H. angulatus*; HangV, viviparous *H. angulatus*. Reproductive modes (oviparity and viviparity) are mapped onto a phylogeny modified from Figueroa et al. (2016). ns, nonsignificant difference

statistical analyses) is similar-sized to that of the oviparous *H. angulatus* (Table 3). The thickness of the shell membrane differs across species ($F_{3,19} = 125.09$, $P < 0.0001$ for both conventional and phylogenetic ANOVA), being thicker in the oviparous *H. angulatus* than in viviparous *Helicops* (post hoc tests, $P < 0.01$; Table 3). We also found significant interspecific differences in shell membrane thickness among viviparous *Helicops*. The shell membrane in the viviparous females of the bimodal *H. angulatus* is thicker than in the viviparous *H. carinicaudus* and *H. infrataeniatus* (post hoc tests, $P < 0.05$; Table 3), but there is no significant difference in the shell membrane thickness between the two viviparous *Helicops* (Table 3).

The thickness of the shell membrane is positively correlated with both the uterine gland height ($PGLS_\lambda$: $N = 7$ terminal taxa, slope = 4.70, intercept = −6.91, $\lambda = 0$, $R^2 = 0.898$, $P = 0.0012$) and uterine gland width ($PGLS_\lambda$: $N = 7$ terminal taxa, slope = 4.96, intercept = −6.41, $\lambda = 0$, $R^2 = 0.985$, $P < 0.0001$).

4 | DISCUSSION

4.1 | Morphology and cyclical variation of the uterus

The uterine structure of oviparous and viviparous *Helicops* is similar to that of other squamates. Regardless of the reproductive mode, the squamate uterus is subdivided into three layers: the muscularis externa, the lamina propria, and the luminal epithelium (Blackburn, 1998; Girling, 2002; Siegel, Miralles, Chabarria, & Aldridge, 2011). The muscularis externa is a bilayer of smooth muscle with circular (inner) and longitudinal (outer) layers (Blackburn, 1998; Girling, 2002). The lamina propria is a thick medial layer containing blood vessels, glands, connective tissue, fibroblasts, and mast cells (Blackburn, 1998; Girling, 2002). The luminal epithelium is formed by a single layer of ciliated and nonciliated (secretory) cells, which are usually cuboidal or columnar in nongravid uteri and low cuboidal and squamous in gravid uteri (Blackburn, 1998; Girling, 2002; Siegel et al., 2011). Therefore, the uterine structure of *Helicops* reflects the highly conserved pattern of squamates (Blackburn, 1998).

Another commonality observed between *Helicops* and other squamates is the marked cyclical changes in uterine morphology and histochemistry. As in *Helicops*, the uterine epithelium of several squamates stains with PAS for neutral carbohydrates and Alcian blue for acidic mucosubstances throughout the reproductive cycle (Botte, 1973; Girling, Cree, & Guillette, 1998; Perkins & Palmer, 1996; Rojas, Barros, & Almeida-Santos, 2015). Moreover, the epithelial height increases substantially during secondary vitellogenesis (Girling, Cree, & Guillette, 1997; Guillette, Fox, & Palmer, 1989; Picariello et al., 1989, but see Heulin et al., 2005). These observations imply that the uterine epithelial cells are secretory throughout the reproductive cycle, but secretory activity potentially increases during secondary vitellogenesis. We did not detect increases in the intensity of the histochemical reactions in the uterine epithelium during secondary vitellogenesis. However, increased secretion of at least acidic mucosubstances occurs in the oviparous snake *Philodryas patagoniensis* (Rojas et al., 2015). As in *Helicops*, the uterine glands stain for proteins and increase in size during secondary vitellogenesis in several oviparous and viviparous squamates (Botte, 1973; Corso et al., 2000; Guillette et al., 1989; Heulin et al., 2005; Stewart et al., 2010). This indicates that the synthesis of proteins by the uterine glands is cyclic in *Helicops*, peaking in secondary vitellogenesis, as in other squamates (Heulin et al., 2005; Stewart et al., 2010).

4.2 | Eggshell structure and formation

Both oviparous and viviparous *Helicops* have an eggshell around their eggs, but the eggshell structure differs between reproductive modes. Macroscopically, the eggshell is thick, opaque, and parchment-like in oviparous *Helicops*, but it is thin and transparent in viviparous congeners (Braz et al., 2016). Histologically, the eggshell of oviparous *Helicops* exhibits the same three layers (a thin inner boundary, a thick shell membrane, and an outer amorphous layer) as the flexible-shelled eggs of other oviparous squamates (Packard & DeMarco, 1991; Packard et al., 1982). Similarly, the eggshell of viviparous *Helicops*

exhibits only the inner boundary and a thin or vestigial shell membrane as in other viviparous squamates (Blackburn, 1998; Guillette, 1992; Stewart, 1985; Stewart & Brasch, 2003). The outer amorphous layer observed in the eggshells of oviparous *Helicops* is presumably the mineral layer. This structure may be highly sculpted in some lizards but is relatively amorphous in many snakes such as *Python regius* and *Hydrodynastes gigas* (Packard & Hirsch, 1986). Scanning electron microscopy is required to provide a better visualization of the outer layer of the eggshell of oviparous *Helicops*.

Secretions from the uterine epithelium likely contribute to the inner boundary of the eggshell of oviparous and viviparous *Helicops* because both structures stain for neutral carbohydrates and acidic mucosubstances. A similar staining pattern occurs in oviparous and viviparous females of reproductively bimodal lizards (Heulin et al., 2005; Stewart et al., 2010). However, it is noteworthy that the epithelium of the infundibulum (the oviductal region anterior to the uterus; Blackburn, 1998) of several squamates also stains for neutral carbohydrates and acidic mucosubstances (de Resende and Nascimento, 2015; Girling et al., 1998; Guillette et al., 1989; Siegel & Sever, 2008). Thus, neutral carbohydrates and acidic mucosubstances from the infundibular epithelium may also be incorporated to the inner boundary as the eggs enter the infundibulum at ovulation (Guillette et al., 1989; Stewart et al., 2010), as observed, for example, in the lizard *Sceloporus woodi* (Palmer et al., 1993). However, secretions from the infundibular epithelial cells are suggested to have other functions including lubrication to facilitate egg transport (Botte, 1973; Weekes, 1927). Additionally, the posterior infundibulum is a site of sperm storage and fertilization in many squamates (Fox, 1963; Rojas et al., 2015; Sever & Hamlett, 2002). Although the inner boundary is thin, its full deposition around the ovum in the infundibulum could act as a physical barrier impairing fertilization. More specific staining techniques are needed to detect whether the inner boundary is secreted in the infundibulum, the uterus, or both.

Our results strongly demonstrate that the uterine glands secrete the fibers of the shell membrane of oviparous and viviparous *Helicops*. This conclusion is based on the observation that (1) uterine glands are the only uterine structure that reacts to Coomassie blue for proteins in preovulatory females; (2) these glands are depleted (or absent) and no longer stain for proteins once eggs reach the uterus; and (3) the shell membrane surrounding oviductal eggs stains for proteins. These observations indicate that the secretory material from the uterine glands is converted into shell membrane. This finding corroborates previous studies in oviparous (Botte, 1973; Guillette et al., 1989; Palmer et al., 1993), viviparous (Corso et al., 2000; Hoffman, 1970), and reproductively bimodal squamates (Heulin et al., 2005; Stewart et al., 2010). Moderate staining with PAS and Alcian blue also occurs in the shell membrane of other oviparous and viviparous squamates, suggesting that acidic mucosubstances and carbohydrates from the luminal epithelium are present throughout the shell membrane (Corso et al., 2000; Hoffman, 1970; Stewart et al., 2010). Our findings partially agree with those results as the shell membrane of oviparous and viviparous *Helicops* reacts only moderately to PAS and negatively to Alcian blue.

The external surface of the outer amorphous layer stains with Alcian blue for acidic mucosubstances in the oviparous lizards *Crotaphytus collaris* and *Plestiodon obsoletus* (Guillette et al., 1989), PAS for neutral carbohydrates in the viviparous skink *Chalcides ocellatus* (Corso et al., 2000), and both dyes in oviparous and viviparous females of the bimodal lizard *Z. vivipara* (Heulin et al., 2005). The surface of the outer layer of shell membrane also stains with PAS and Alcian blue in oviparous *Helicops* and the viviparous *H. angulatus*. The uterine epithelium is likely responsible for secreting the outer organic layer in *Helicops* because the epithelial cells continue reacting to Alcian blue and PAS in gravid/pregnant females (although to a lesser degree), which indicates ongoing secretory activity.

In squamates, most of the shell membrane is deposited immediately after eggs reach the uterus (Heulin et al., 2005; Ortiz & Morales, 1974; Palmer et al., 1993; Stewart et al., 2010). For example, shell membrane deposition is nearly complete within 24 hr from ovulation in the oviparous lizard *S. woodi* (Palmer et al., 1993). Similarly, in the lizard *Z. vivipara*, the shell membrane thickness of eggs with embryos at segmentation is essentially the same as eggs at more advanced stages (Heulin et al., 2005). We examined only museum specimens, therefore, we could not determine the exact length of time eggs were in the uterus. However, the shell membrane thickness of oviductal eggs with unnoticeable embryos (and presumably at early stages) of oviparous *H. angulatus* was similar to that of eggs with embryos at Zehr stages 21–24 and thus close to oviposition. In viviparous *H. angulatus*, the shell membrane thickness was slightly thicker in the egg without a noticeable embryo than in eggs with partially developed embryos (stages 21–28). Moreover, the uterine glands were either smaller (in oviparous females) or not noticeable (in viviparous females) and no longer reacting for proteins in all gravid/pregnant *Helicops*. Collectively, these observations suggest that shell membrane deposition also occurs rapidly after ovulation in oviparous and viviparous *Helicops*.

4.3 | Eggshell reduction and the evolution of squamate viviparity

Closely related species are expected to exhibit phylogenetic signal in phenotypic traits because of their common evolutionary history. The lack of phylogenetic signal in reproductive mode, uterine gland dimensions, uterine epithelial height, and shell membrane thickness suggests that phylogeny does not predict the observed variation in these traits. The power of any comparative method is strongly affected by the number of terminal taxa included in the analyses and the quality of the phylogeny (Blomberg et al., 2003; Freckleton, Harvey, & Pagel, 2002). As our comparative analyses included only seven terminal taxa, phylogeny is far from inclusive, and *K*-values for some traits were relatively high, some caution is advisable in interpreting the lack of statistical support for phylogenetic signal. Despite these caveats, we are confident to suggest the lack of phylogenetic signal in the traits evaluated because the viviparous lineages studied are likely unrelated (Braz et al., 2016). Moreover, conventional ANOVAs produced essentially the same results and trait values are clearly overdispersed across the phylogeny used here (Figures 5 and 6). This implies that the traits

examined evolved independently of the phylogeny and are a direct response to the evolution of viviparity.

Our results corroborate the hypothesis that eggshell thinning is associated with the evolution of viviparity. Eggshell reduction occurred convergently in the three origins of viviparity in *Helicops* and was accomplished by the loss of the mineral layer and thinning of the shell membrane. Interestingly, the shell membrane thickness of viviparous females of the bimodal *H. angulatus* is intermediate between its oviparous and viviparous congeners. The shell membrane of viviparous *H. angulatus* is six times thinner than that of its oviparous congeners, whereas the shell membrane of the viviparous *H. infrataeniatus* and *H. carinicaudus* is vestigial and much thinner (20–25 times) than that of their oviparous congeners. This difference indicates that, although eggshell reduction is a requirement for the evolution of viviparity, a nearly complete loss of the shell membrane (as in the viviparous *Helicops*) is not. Information on the shell membrane of other squamates corroborates this idea. The evolution of viviparity in the bimodal lizards *S. equalis*, *L. bougainvilliii*, and *Z. vivipara* was also accompanied by a reduction of the shell membrane (Heulin et al., 2005; Qualls, 1996; Stewart et al., 2010). Despite this reduction, the shell membrane of viviparous females is still prominent and ranges from two to nine times thinner than in their oviparous conspecifics (Heulin et al., 2005; Qualls, 1996; Stewart et al., 2010) and, therefore, proportionally comparable to that of viviparous females of *H. angulatus*. Unfortunately, we cannot verify whether the shell membrane thickness in viviparous females of these bimodal lizards is also intermediate between their oviparous and viviparous congeners. *Saiphos equalis* and *Z. vivipara* are monotypic genera and there is no information on the shell membrane of *L. microtus*, the other viviparous species of *Lerista* (Shine, 1985). However, several viviparous squamates exhibit only a vestigial shell membrane throughout pregnancy comparable to that of the viviparous *H. carinicaudus* and *H. infrataeniatus* (e.g., Blackburn, Anderson, Lo, Marquez, & Callard, 2017; Hoffman, 1970; Stewart, 1985; Stewart & Brasch, 2003). In many cases, the shell membranes are barely seen or degenerate at late stages of development (e.g., Blackburn & Flemming, 2012; Jerez & Ramírez-Pinilla, 2003; Murphy et al., 2012). Thus, eggshell reduction is a requirement for viviparity, but the nearly complete loss and eventually disruption of the shell membrane are subsequent steps which occurred after viviparity has evolved (i.e., specialization). Additionally, these observations indirectly support the hypothesis that eggshell reduction occurs concomitantly (gradually) with the increases in intrauterine egg retention (see also Heulin, Ghielmi, Vogrin, Surget-Groba, & Guillaume, 2002; Mathies & Andrews, 1995; Qualls, 1996; Shine & Thompson, 2006) because the nearly complete loss of the shell membrane is accomplished only in viviparous species, whereas it is still present in viviparous females of the bimodal *H. angulatus* (in which viviparity probably has evolved more recently).

Eggshell reduction is critical for the evolution of viviparity because it allows a close apposition of maternal and fetal tissues for enhancing physiological exchanges (Thompson et al., 2004). A direct consequence of the difference in shell membrane thickness among viviparous *Helicops* is that the thicker shell membrane in viviparous *H. angulatus* increases the distance between maternal and fetal tissues. Therefore, it is reasonable to expect that a prominent shell membrane present throughout gestation may somehow impair efficient maternal–fetal exchanges (Stewart, Heulin, & Surget-Groba, 2004). This limitation could even explain why eggshell is vestigial in viviparous species. However, other features as increased vascularity or differential structure of the shell membrane might facilitate maternal–fetal exchanges (Mathies & Andrews, 2000; Stewart et al., 2010). Nevertheless, even if a prominent shell membrane limits exchanges, it does not necessarily jeopardize successful pregnancy (Linville et al., 2010).

Our data also support the hypothesis that eggshell thinning results from the reduced size of uterine glands in viviparous compared to oviparous taxa. Because seasonal fluctuations in uterine morphology are correlated with follicular growth (Botte, 1973; Girling et al., 1997; Heulin et al., 2005; Picariello et al., 1989), any interspecific divergence in gland size could also be the result of comparing specimens at different follicular stages (Guillette & Jones, 1985). We avoided this problem by comparing uterine traits among species at equivalent vitellogenic stages (as confirmed by the similar follicular size in primary and secondary vitellogenesis). Therefore, the differences in gland dimensions are indeed related to reproductive modes and not to seasonal fluctuations. Uterine gland recruitment occurs early in primary vitellogenesis by infoldings of the uterine luminal epithelium (Ortiz & Morales, 1974). Thus, the similarity in glandular dimensions of oviparous and viviparous *Helicops* during primary vitellogenesis was expected. However, uterine glands always grow larger in oviparous than in viviparous *Helicops* (including the sister species *Hy. martii*) by secondary vitellogenesis. Thus, reduced growth of uterine glands occurred convergently in all three origins of viviparity in *Helicops*. Smaller glands reflect less stored material (and consequently less material secreted), as indicated by the positive correlation between gland dimensions and eggshell thickness. Therefore, the shell membrane is thinner in viviparous than in oviparous *Helicops* because the uterine glands that secrete it are smaller in viviparous forms just before ovulation. This conclusion is consistent with a previous study on the reproductively bimodal lizard *Z. vivipara* (Heulin et al., 2005).

Interestingly, we found that although the shell membrane is thicker in viviparous females of the reproductively bimodal *H. angulatus* than in its viviparous congeners, the uterine glands that secrete this structure have similar dimensions across the viviparous forms of *Helicops*. In the reproductively bimodal lizard *S. equalis*, eggshell reduction is not correlated with a decrease in uterine gland size (Stewart et al., 2010). These authors suggested that differences in eggshell thickness in this species might be explained by lower number of glands in viviparous females (Stewart et al., 2010; see also Guillette, 1993), but they could not test this hypothesis. Fewer glands could also explain the thinner shell membrane in *H. carinicaudus* and *H. infrataeniatus*. Unfortunately, our sampling protocol does not allow us to accurately estimate gland abundance. Nevertheless, our observations suggest that gland abundance is similar across oviparous and viviparous *Helicops*. Alternatively, uterine glands and their secretory material could have been repurposed for other functions rather than formation of the shell membrane. Despite being small, uterine glands are still present and hold secretory potential in other viviparous squamates (e.g., Corso et al., 2000; Hoffman, 1970; Siegel & Sever, 2008). In mammals, uterine glands synthesize and

TABLE 3 Thickness of the shell membrane and outer layer in *Helicops* spp. and *Hydrops martii*

Species	Reproductive mode	*N*	Embryo stage	Shell membrane thickness (μm)	Outer layer (μm)
Helicops gomesi[*]	Oviparous	1	25	96.3	9.2
Helicops hagmanni[*]	Oviparous	1	30	117.2	3.6
Helicops angulatus	Oviparous	8	Non-visible	115.1 ± 21.1	9.8 ± 5.1
		2	21 and 24	108.3 and 102.5	9.1 and 10.5
		10	All stages	113.1 ± 19.1^{c}	9.8 ± 3.6
Helicops angulatus	Viviparous	1	Non-visible	31.0	Absent
		3	21, 26, and 28	16.9 ± 7.3	Absent
		1	37	18.2	Absent
		5	All stages	19.5 ± 8.3^{b}	Absent
Helicops carinicaudus	Viviparous	4	26, 31, 33.5, and 36	5.7 ± 2.3^{a}	Absent
Helicops infrataeniatus	Viviparous	4	25.5, 28, 31, and 31.5	4.7 ± 1.9^{a}	Absent
Hydrops martii[*]	Oviparous	1	19	106.7	5.1

Means are followed by standard deviation. Means with different superscripts differ significantly (*Post hoc* pairwise *t*-tests, $P < 0.05$ for all pairwise comparisons, $a < b < c$).

[*]Species not included in the statistical analyses due to the small sample size

secrete a variety of proteins and related substances essential for development and survival of the conceptus (Gray et al., 2001).

Although eggshell reduction results from smaller glands in viviparous forms, the mechanism responsible for uterine gland reduction remains largely unknown. Since oviductal hypertrophy coincides with elevated circulating estrogen concentrations during vitellogenesis (reviewed in Girling, 2002), reduction of estrogen concentrations plausibly acts on uterine gland reduction (Guillette, 1993). However, concentrations of circulating estradiol during vitellogenesis do not vary between oviparous and viviparous females of the reproductively bimodal lizard *Z. vivipara* (Heulin, Garnier, Surget-Groba, & Deunff, 2008). Alternatively, smaller uterine glands may result from modifications of estrogen receptors (Guillette, 1993; Heulin et al., 2008). Estrogen receptor concentrations vary throughout reproductive cycle in oviparous lizards (Paolucci, Di Fiore, & Ciarcia, 1992; Young, Godwin, Grammer, Gahr, & Crews, 1995), but no study has specifically compared estrogen receptors between species differing in reproductive modes. Such comparisons would be enlightening to understand the mechanism responsible for uterine gland reduction. Additionally, transcriptome of the uteri of oviparous and viviparous squamates can detect differentially expressed genes throughout reproductive cycle (Brandley, Young, Warren, Thompson, & Wagner, 2012; Griffith, Brandley, Belov, & Thompson, 2016). At least estrogen receptor 1 gene is significantly downregulated during pregnancy in the viviparous lizard *Chalcides ocellatus* (Brandley et al., 2012). Similar studies comparing gene expression profile between oviparous and viviparous conspecifics at preovulatory stages could detect differential estrogen receptor gene expression and thus to test the hypothesis that smaller glands result from modifications of estrogen receptors.

In summary, the uterine glands secrete the proteinaceous fibers of the eggshell (the shell membrane) of oviparous and viviparous *Helicops*. The luminal epithelium secretes the outer surface of the eggshell in oviparous *Helicops* and potentially the inner boundary. Despite similarities in the histochemical properties of the uterus and eggshell of oviparous and viviparous *Helicops*, the shell membrane thickness is always thinner in viviparous than in oviparous forms, thus supporting the hypothesis that eggshell thinning is associated with the evolution of squamate viviparity. In turn, shell membrane thinning in viviparous forms is correlated with reduction in the uterine gland dimensions (but not the luminal epithelium) during secondary vitellogenesis in every single origin of viviparity in *Helicops*, supporting the hypothesis that eggshell thinning is a direct result of less developed glands in viviparous compared with oviparous taxa.

ACKNOWLEDGMENTS

We thank Marcos Carvalho, Hipócrates Chalkidis, Giselle Cotta, Francisco Franco, Giuseppe Puorto, Gláucia Funk-Pontes, Paulo Manzani, Marcio Martins, Julio Moura-Leite, Maria Ermelinda Oliveira, Paulo Passos, Ana Prudente, Moisés Souza, Richard Vogt, and Noeli Zanella for allowing access to museum specimens under their care. We also thank Rodrigo Scartozzoni for kindly providing some egg samples, Valdir Germano, J. Moura-Leite, Maria C. Santos-Costa, P. Manzani, and P. Passos for assistance in the museums. We are thankful to Sam Dowland, Darryl Cameron, Jacquie Herbert, Jessica Dudley, Laura Lindsay, and Karina Kasperoviczus for valuable help in the laboratory, and Mónica Arias for kindly providing the R code for performing phylogenetic ANOVAs and guidance on how to implement it. Early drafts of the manuscript were improved by comments from Camilla Whittington, James Van Dyke, Melanie Laird, and J. Herbert. This work was partially supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and fully supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brasil (CNPq) through doctoral (FAPESP: 2009/54478-3) and post-doctoral (CNPq: 235248/2014-2) fellowships provided to H.B.B. This work was also supported by funding from the Murphy laboratory.

CONFLICTS OF INTEREST

None.

ORCID

Henrique B. Braz http://orcid.org/0000-0003-4829-6827

LITERATURE CITED

Abràmoff, M. D., Magalhães, P. J., & Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics International*, *11*, 36–42.

Adams, S. M., Biazik, J., Stewart, R. L., Murphy, C. R., & Thompson, M. B. (2007). Fundamentals of viviparity: Comparison of seasonal changes in the uterine epithelium of oviparous and viviparous *Lerista bougainvillii* (Squamata: Scincidae). *Journal of Morphology*, *268*, 624–635.

Aguiar, L. F. S., & Di-Bernardo, M. (2005). Reproduction of the water snake *Helicops infrataeniatus* (Colubridae) in southern Brazil. *Amphibia-Reptilia*, *26*, 527–533.

Albergotti, L. C., & Guillette, L. J. (2011). Viviparity in reptiles. In In D. O. Norris & K. H. Lopez (Eds.), *Hormones and reproduction of vertebrates—Volume 3: Reptiles* (Vol. 3, pp. 247–275). San Diego, CA: Academic Press.

Aldridge, R. D. (1979). Female reproductive cycles of the snakes *Arizona elegans* and *Crotalus viridis*. *Herpetologica*, *35*, 256–261.

Angelini, F., & Ghiara, G. (1984). Reproductive modes and strategies in vertebrate evolution. *Italian Journal of Zoology*, *51*, 121–203.

Arias, M., Meichanetzoglou, A., Elias, M., Rosser, N., De-Silva, D. L., Nay, B., & Llaurens, V. (2016). Variation in cyanogenic compounds concentration within a *Heliconius* butterfly community: Does mimicry explain everything? *BMC Evolutionary Biology [Electronic Resource]*, *16*, 272.

Blackburn, D. G. (1985). Evolutionary origins of viviparity in the Reptilia. II. Serpentes, Amphisbaenia, and Ichthyosauria. *Amphibia-Reptilia*, *6*, 259–291.

Blackburn, D. G. (1998). Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. *Journal of Experimental Zoology. Part A Ecological Genetics and Physiology*, *282*, 560–617.

Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *Journal of Morphology*, *276*, 961–990.

Blackburn, D. G., Anderson, K. E., Lo, A. R., Marquez, E. C., & Callard, I. P. (2017). Placentation in watersnakes II: Placental ultrastructure in *Nerodia erythrogaster* (Colubridae: Natricinae). *Journal of Morphology*, *278*, 675–688.

Blackburn, D. G., & Flemming, A. F. (2010). Reproductive specializations in a viviparous African skink: Implications for evolution and biological conservation. *Herpetological Conservation and Biology*, *5*, 263–270.

Blackburn, D. G., & Flemming, A. F. (2012). Invasive implantation and intimate placental associations in a placentotrophic African lizard, *Trachylepis ivensi* (Scincidae). *Journal of Morphology*, *273*, 137–159.

Blomberg, S. P., Garland, T., & Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. *Evolution; International Journal of Organic Evolution*, *57*, 717–745.

Botte, V. (1973). Morphology and histochemistry of the oviduct in the lizard, *Lacerta sicula*. The annual cycle. *Bolletino Di Zoologia*, *40*, 305–314.

Boyd, M. M. M. (1943). The oviduct, foetal membranes, and placentation in *Hoplodactylus maculatus* Gray. *Proceedings of the Zoology Society of London A*, *112*, 65–104.

Brandley, M. C., Young, R. L., Warren, D. L., Thompson, M. B., & Wagner, G. P. (2012). Uterine gene expression in the live-bearing lizard, *Chalcides ocellatus*, reveals convergence of squamate reptile and mammalian pregnancy mechanisms. *Genome Biology and Evolution*, *4*, 394–411.

Braz, H. B., Scartozzoni, R. R., & Almeida-Santos, S. M. (2016). Reproductive modes of the South American water snakes: A study system for the evolution of viviparity in squamate reptiles. *Zoologischer Anzeiger*, *263*, 33–44.

Corso, G., Delitala, G. M., & Carcupino, M. (2000). Uterine morphology during the annual cycle in *Chalcides ocellatus tiligugu* (Gmelin) (Squamata: Scincidae). *Journal of Morphology*, *243*, 153–165.

Costa, H. C., Santana, D. J., Leal, F., Koroiva, R., & Garcia, P. C. A. (2016). A new species of *Helicops* (Serpentes: Dipsadidae: Hydropsini) from south-eastern Brazil. *Herpetologica*, *72*, 157–166.

de Resende FC, Nascimento L. B. (2015). The female reproductive cycle of the Neotropical snake *Atractus pantostictus* (Fernandes and Puorto, 1993) from south-eastern Brazil. *Anatomia, Histologia, Embryologia*, *44*, 225–235.

Deeming, D. C., & Thompson, M. B. (1991). Gas exchange across reptilian eggshells. In In D. C. Deeming & M. W. J. Ferguson (Eds.), *Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles* (pp. 277–284). Cambridge: Cambridge University Press.

Di Pietro, D. O., Alcalde, L., & Williams, J. D. (2014). New cranial characters in the tribe Hydropsini (Serpentes: Dipsadidae: Xenodontinae). *Acta Herpetologica*, *9*, 1–14.

Felsenstein, J. (1985). Phylogenies and the comparative method. *American Naturalist*, *125*, 1–15.

Figueroa, A., McKelvy, A. D., Grismer, L. L., Bell, C. D., & Lailvaux, S. P. (2016). A species-Level phylogeny of extant snakes with description of a new colubrid subfamily and genus. *Plos One*, *11*, e0161070.

Fox, W. (1963). Special tubules for sperm storage in female lizards. *Nature*, *198*, 500–501.

Freckleton, R. P., Harvey, P. H., & Pagel, M. (2002). Phylogenetic analysis and comparative data: A test and review of evidence. *American Naturalist*, *160*, 712–726.

Fritz, S. A., & Purvis, A. (2010). Selectivity in mammalian extinction risk and threat types: A new measure of phylogenetic signal strength in binary traits. *Conservation Biology*, *24*, 1042–1051.

Garland, T., Dickerman, A. W., Janis, C. M., & Jones, J. A. (1993). Phylogenetic analysis of covariance by computer simulation. *Systematic Biology*, *42*, 265–292.

Girling, J. E. (2002). The reptilian oviduct: A review of structure and function and directions for future research. *Journal of Experimental Zoology*, *293*, 141–170.

Girling, J. E., Cree, A., & Guillette, L. J. (1997). Oviductal structure in a viviparous New Zealand gecko, *Hoplodactylus maculatus*. *Journal of Morphology*, *234*, 51–68.

Girling, J. E., Cree, A., & Guillette, L. J. (1998). Oviducal structure in four species of gekkonid lizard differing in parity mode and eggshell structure. *Reproduction, Fertility, and Development*, *10*, 139.

Grafen, A. (1989). The phylogenetic regression. *Proceedings of the Royal Society of London B*, *326*, 119–156.

Gray, C. A., Bartol, F. F., Tarleton, B. J., Wiley, A. A., Johnson, G. A., Bazer, F. W., & Spencer, T. E. (2001). Developmental biology of uterine glands. *Biology of Reproduction*, *65*, 1311–1323.

Griffith, O. W., Blackburn, D. G., Brandley, M. C., Van Dyke JU, Whittington C. M., & Thompson, M. B. (2015). Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: A case study examining the evolution of reproductive mode in squamate reptiles. *Journal of Experimental Zoology Part B Molecular and Developmental Evolution*, *324*, 493–503.

Griffith, O. W., Brandley, M. C., Belov, K., & Thompson, M. B. (2016). Reptile pregnancy is underpinned by complex changes in uterine gene expression: A comparative analysis of the uterine transcriptome in viviparous and oviparous lizards. *Genome Biology and Evolution*, *8*, 3226–3239.

Guillette, L. J. (1992). Morphology of the reproductive tract in a lizard exhibiting incipient viviparity (*Sphenomorphus fragilis*) and its implications for the evolution of the reptilian placenta. *Journal of Morphology*, *212*, 163–173.

Guillette, L. J. (1993). The evolution of viviparity in lizards. *Bioscience*, *43*, 742–751.

Guillette, L. J., Fox, S. L., & Palmer, B. D. (1989). Oviductal morphology and egg shelling in the oviparous lizards *Crotaphytus collaris* and *Eumeces obsoletus*. *Journal of Morphology*, *201*, 145–159.

Guillette, L. J., & Jones, R. E. (1985). Ovarian, oviductal, and placental morphology of the reproductively bimodal lizard, *Sceloporus aeneus*. *Journal of Morphology*, *184*, 85–98.

Harvey, P. H., & Pagel, M. D. (1991). *The comparative method in evolutionary biology*. Oxford: Oxford University Press.

Heulin, B., Garnier, D., Surget-Groba, Y., & Deunff, J. (2008). Plasma levels of estradiol during vitellogenesis and early gestation in oviparous and viviparous *Lacerta* (*Zootoca*) *vivipara*. *Amphibia-Reptilia*, *29*, 135–139.

Heulin, B., Ghielmi, S., Vogrin, N., Surget-Groba, Y., & Guillaume, C. P. (2002). Variation in eggshell characteristics and in intrauterine egg retention between two oviparous clades of the lizard *Lacerta vivipara*: Insight into the oviparity-viviparity continuum in squamates. *Journal of Morphology*, *252*, 255–262.

Heulin, B., Stewart, J. R., Surget-Groba, Y., Bellaud, P., Jouan, F., Lancien, G., & Deunff, J. (2005). Development of the uterine shell glands during the preovulatory and early gestation periods in oviparous and viviparous *Lacerta vivipara*. *Journal of Morphology*, *266*, 80–93.

Hoffman, L. H. (1970). Placentation in the garter snake, *Thamnophis sirtalis*. *Journal of Morphology*, *131*, 57–87.

Jerez, A., & Ramírez-Pinilla, M. P. (2003). Morphogenesis of extraembryonic membranes and placentation in *Mabuya mabouya* (Squamata, Scincidae). *Journal of Morphology*, *258*, 158–178.

Kiernan, J. A. (2008). *Histological and histochemical methods - theory and practice* (4th ed). Oxfordshire: Scion Publishing.

Linville, B. J., Stewart, J. R., Ecay, T. W., Herbert, J. F., Parker, S. L., & Thompson, M. B. (2010). Placental calcium provision in a lizard with prolonged oviductal egg retention. *Journal of Comparative Physiology B*, *180*, 221–227.

Maddison, W. P., & Maddison, D. R. (2017). Mesquite: A modular system for evolutionary analysis. Version, 3, 2. Retrieved from https://mesquiteproject.org

Mathies, T., & Andrews, R. M. (1995). Thermal and reproductive biology of high and low elevation populations of the lizard *Sceloporus scalaris*: Implications for the evolution of viviparity. *Oecologia*, *104*, 101–111.

Mathies, T., & Andrews, R. M. (2000). Does reduction of the eggshell occur concurrently with or subsequent to the evolution of viviparity in phrynosomatid lizards? *Biological Journal of the Linnean Society*, *71*, 719–736.

Midford, P. E., Garland, T., & Maddison, W. P. (2011). PDAP: PDTREE Package for Mesquite, Version 1.16. Available at http://mesquiteproject.org/pdap_mesquite/index.html.

Murphy, B. F., Brandley, M. C., Murphy, C. R., & Thompson, M. B. (2012). Morphology and development of the placentae in *Eulamprus quoyii* group skinks (Squamata: Scincidae). *Journal of Anatomy*, *220*, 454–471.

Murphy, B. F., & Thompson, M. B. (2011). A review of the evolution of viviparity in squamate reptiles: The past, present and future role of molecular biology and genomics. *Journal of Comparative Physiology B*, *181*, 575–594.

Orme, D., Freckleton, R. P., Thomas, G., Petzoldt, T., Fritz, S. A., Isaac, N., & Pearse, W. (2015). Package "caper": Comparative analyses of phylogenetics and evolution in R. R package version 0.5.2 Available at: http://CRAN.R-project.org/package=caper. Last accessed 1 September 2017.

Ortiz, E., & Morales, M. H. (1974). Development and function of the female reproductive tract of the tropical lizard, *Anolis pulchellus*. *Physiological Zoology*, *47*, 207–217.

Packard, G. C., & DeMarco, V. G. (1991). Eggshell structure and formation in eggs of oviparous reptiles. In In D. C. Deeming & M. W. J. Ferguson (Eds.), *Egg Incubation: Its effects on embryonic development in birds and reptiles* (pp. 53–70). Cambridge: Cambridge University Press.

Packard, G. C., Tracy, C. R., & Roth, J. J. (1977). The physiological ecology of reptilian eggs and embryos, and the evolution of viviparity within the class reptilia. *Biological Reviews of the Cambridge Philosophical Society*, *52*, 71–105.

Packard, M. J., & Hirsch, K. F. (1986). Scanning electron microscopy of eggshells of contemporary reptiles. *Scanning Electron Microscopy*, *4*, 1581–1590.

Packard, M. J., Packard, G. C., & Boardman, T. J. (1982). Structure of eggshells and water relations of reptilian eggs. *Herpetologica*, *38*, 136–155.

Pagel, M. D. (1992). A method for the analysis of comparative data. *Journal of Theoretical Biology*, *156*, 431–442.

Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature*, *401*, 877–884.

Palmer, B. D., DeMarco, V. G., & Guillette, L. J. (1993). Oviductal morphology and eggshell formation in the lizard, *Sceloporus woodi*. *Journal of Morphology*, *217*, 205–217.

Paolucci, M., Di Fiore, M. M., & Ciarcia, G. (1992). Oviduct 17β-estradiol receptor in the female lizard, *Podarcis s. sicula*, during the sexual cycle: Relation to plasma 17β-estradiol concentration and its binding proteins. *Zoological Science*, *9*, 1025–1035.

Perkins, M. J., & Palmer, B. D. (1996). Histology and functional morphology of the oviduct of an oviparous snake, *Diadophis punctatus*. *Journal of Morphology*, *227*, 67–79.

Picariello, O., Ciarcia, G., & Angelini, F. (1989). The annual cycle of oviduct in *Tarentola m. mauritanica* L. (Reptilia, *Gekkonidae*). *Amphibia-Reptilia*, *10*, 371–386.

Purvis, A. (1995). A composite estimate of primate phylogeny. *Philosophical Transactions of the Royal Society of London Series B*, *348*, 405–421.

Qualls, C. P. (1996). Influence of the evolution of viviparity on eggshell morphology in the lizard, *Lerista bougainvillii*. *Journal of Morphology*, *228*, 119–125.

R Development Core Team. (2016). R: A language and environment for statistical computing. Retrieved from https://www.r-project.org

Revell, L. J. (2010). Phylogenetic signal and linear regression on species data. *Methods in Ecology and Evolution*, *1*, 319–329.

Revell, L. J. (2012). phytools: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, *3*, 217–223.

Robert, K. A., & Thompson, M. B. (2000). Energy consumption by embryos of a viviparous lizard, Eulamprus tympanum, during development. *Comparative Biochemistry and Physiology Part A Molecular Integration & Physiology*, *127*, 481–486.

Rojas, C. A., Barros, V. A., & Almeida-Santos, S. M. (2015). Sperm storage and morphofunctional bases of the female reproductive tract of the snake *Philodryas patagoniensis* from southeastern Brazil. *Zoomorphology*, *134*, 577–586.

Scartozzoni, R. R. 2009. *Reproductive strategies and feeding ecology of water snakes of the tribe Hydropsini (Dipsadidae, Xenodontinae)*. (Thesis). University of São Paulo, São Paulo

Sever, D. M., & Hamlett, W. C. (2002). Female sperm storage in reptiles. *Journal of Experimental Zoology*, *292*, 187–199.

Shine, R. (1985). The evolution of viviparity in reptiles: An ecological analysis. In C. Gans, & F. Billett (Eds.), *Biology of the reptilia* (Vol. 15, pp. 605–694). New York: John Wiley & Sons.

Shine, R., & Thompson, M. B. (2006). Did embryonic responses to incubation conditions drive the evolution of reproductive modes in squamate reptiles? *Herpetological Monographs*, *20*, 159–171.

Siegel, D. S., Miralles, A., Chabarria, R. E., & Aldridge, R. D. (2011). Female reproductive anatomy: Cloaca, oviduct, and sperm storage. In R. D. Aldridge, & D. M. Sever (Eds.), *Reproductive biology and phylogeny of snakes* (pp. 347–409). Enfield: Science Publishers.

Siegel, D. S., & Sever, D. M. (2008). Seasonal variation in the oviduct of female *Agkistrodon piscivorus* (Reptilia: Squamata): An ultrastructural investigation. *Journal of Morphology*, *269*, 980–997.

Stewart, J. R. (1985). Placentation in the lizard *Gerrhonotus coeruleus* with a comparison to the extraembryonic membranes of the oviparous *Gerrhonotus multicarinatus* (Sauria, *Anguidae*). *Journal of Morphology*, *185*, 101–114.

Stewart, J. R., & Brasch, K. R. (2003). Ultrastructure of the placentae of the natricine snake, *Virginia striatula* (Reptilia: Squamata). *Journal of Morphology*, *255*, 177–201.

Stewart, J. R., Heulin, B., & Surget-Groba, Y. (2004). Extraembryonic membrane development in a reproductively bimodal lizard, *Lacerta* (*Zootoca*) *vivipara*. *Zoology*, *107*, 289–314.

Stewart, J. R., Mathieson, A. N., Ecay, T. W., Herbert, J. F., Parker, S. L., & Thompson, M. B. (2010). Uterine and eggshell structure and histochemistry in a lizard with prolonged uterine egg retention (Lacertilia, Scincidae, *Saiphos*). *Journal of Morphology*, *271*, 1342–1351.

Stewart, J. R., & Thompson, M. B. (2009). Placental ontogeny in Tasmanian snow skinks (genus *Niveoscincus*) (Lacertilia: Scincidae). *Journal of Morphology*, *270*, 485–516.

Symonds, M. R. E., & Blomberg, S. P. (2014). A primer on phylogenetic generalised least squares. In L. Z. Garamszegi (Ed.). *Modern phylogenetic comparative methods and their application in evolutionary biology* (pp. 105–130). Berlin, Heidelberg: Springer.

Thompson, M. B., Adams, S. M., Herbert, J. F., Biazik, J. M., & Murphy, C. R. (2004). Placental function in lizards. *International Congress Series*, *1275*, 218–225.

Thompson, M. B., & Speake, B. K. (2006). A review of the evolution of viviparity in lizards: Structure, function and physiology of the placenta. *Journal of Comparative Physiology B*, *176*, 179–189.

Tinkle, D. W., & Gibbons, J. W. (1977). The distribution and evolution of viviparity in reptiles. *Miscellaneous Publications Museum of Zoology University of Michigan*, *154*, 1–55.

Uetz, P., & Hošek, J. (2017). The reptile database. Retrieved from https://www.reptile-database.org.

Van Dyke JU, Beaupre S. J. (2011). Bioenergetic components of reproductive effort in viviparous snakes: Costs of vitellogenesis exceed costs of pregnancy. *Comparative Biochemistry and Physiology. Part A Molecular Integration & Physiology*, *160*, 504–515.

Vleck, C. M., & Hoyt, D. F. (1991). Metabolism and energetics of reptilian and avian embryos. In D. C. Deeming & M. W. J. Ferguson (Eds.), *Egg incubation: Its effects on embryonic development in birds and reptiles* (pp. 285–306). Cambridge: Cambridge University Press.

Weekes, C. H. (1927). Placentation and other phenomena in the scincid lizard *Lygosoma* (*Hinulia*) *quoyi*. *Proceedings of the Linnean Society of New South Wales*, *52*, 499–554.

Weekes, C. H. (1935). A Review of placentation among reptiles with, particular regard to the function and evolution of the Placenta. *Proceeding of the Zoological Society of London*, *105*, 625–645.

Young, L. J., Godwin, J., Grammer, M., Gahr, M., & Crews, D. (1995). Reptilian sex steroid receptors: Amplification, sequence and expression analysis. *Journal of Steroid Biochemistry and Molecular Biology*, *55*, 261–269.

Zaher, H. (1999). Hemipenial morphology of the South American xenodontine snakes, with a proposal for a monophyletic Xenodontinae and a reappraisal of colubroid hemipenis. *Bulletin of the American Museum of Natural History*, *240*, 1–168.

Zehr, D. R. (1962). Stages in the normal development of the common garter snake, *Thamnophis sirtalis sirtalis*. *Copeia*, *1962*, 322–329.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Braz HB, Almeida-Santos SM, Murphy CR, Thompson MB. Uterine and eggshell modifications associated with the evolution of viviparity in South American water snakes (*Helicops* spp.). *J Exp Zool B Mol Dev Evol*. 2018;330:165–180. https://doi.org/10.1002/jez.b.22800