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Evolution of Eggshell Structure in Relation to Nesting Ecology in Non-Avian Reptiles

Running Head: Evolution of eggshell structure and nesting ecology in reptiles

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Abstract

Amniotic eggs are multifunctional structures that enabled early tetrapods to colonize the land millions of years ago, and are now the reproductive mode of over 70% of all terrestrial amniotes. Eggshell morphology is at the core of animal survival, mediating the interactions between embryos and their environment, and has evolved into a massive diversity of forms and functions in modern reptiles. These functions are critical to embryonic survival and may serve as models for new antimicrobial and/or breathable membranes. However, we still lack critical data on the basic structural and functional properties of eggs, particularly of reptiles. Here, we first characterized egg shape, shell thickness, porosity, and mineralization of eggs from 91 reptile species using optical images, SEM, and μ CT, and collected data on nesting ecology from the literature. We then used comparative analyses to test hypotheses on the selective pressures driving their evolution. We hypothesized that eggshell morphology has evolved to protect shells from physical damage and desiccation, and, in support, found a positive relationship between thickness and precipitation, and a negative relationship between porosity and temperature. Although mineralization varied extensively, it was not correlated

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with nesting ecology variables. Ancestral state reconstructions show thinning and increased porosity over evolutionary time in squamates, but the opposite in turtles and crocodilians. Egg shape, size, porosity and calcification were correlated, suggesting potential structural or developmental tradeoffs. This study provides new data and insights into the morphology and evolution of reptile eggs, and raises numerous questions for additional research.

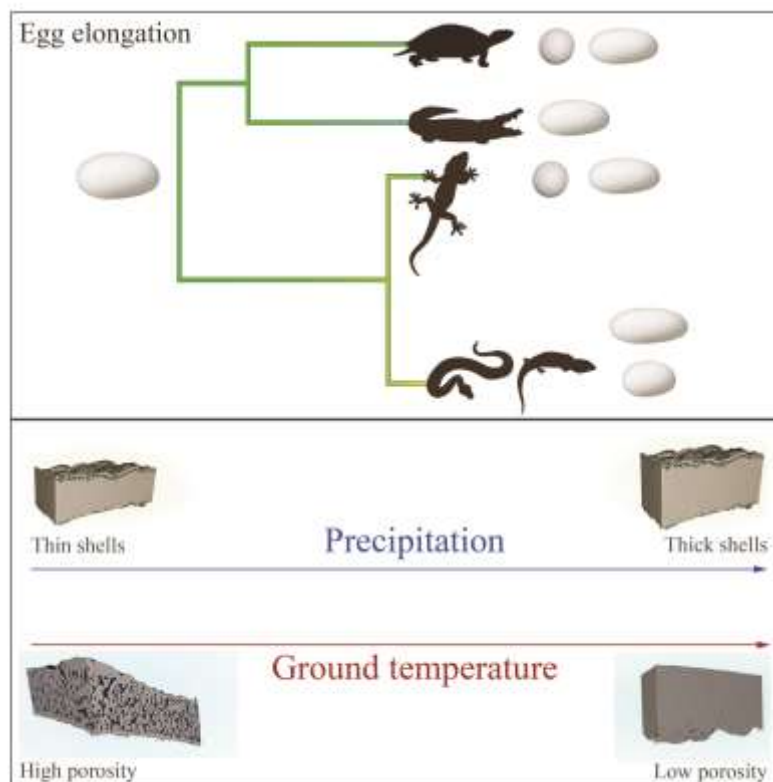
Keywords: egg shape, micro-CT, calcification, nest architecture.

Research Highlights

Reptile eggs have changed over evolutionary time, spherical eggs arose independently in turtles and geckos. Shells have become thinner and more porous in squamate reptiles, but thicker in testudines and crocodilians.

Eggshell thickness and porosity have evolved in relation to environmental factors and nest type.

Graphical abstract



1 INTRODUCTION

Amniotic eggs are multifunctional structures that enabled early tetrapods to colonize diverse terrestrial habitats millions of years ago, and represent the reproductive mode of over 70% of terrestrial amniotes (King & Lee 2015). They mediate the interactions between embryos and their environment and provide three critical functions: a) protection from the microbial and physical environment, b) exchange of water and respiratory gases, and c) provision of calcium to the developing embryo.

All of these functions are based on egg morphology. Egg design in birds is an excellent example of adaptive response to environmental challenges. Egg size and shape, for example, vary depending on whether the bird incubates in open or enclosed nests (Nagy, Hauber, Hartley & Mainwaring 2019), reflecting specific needs for protection against mechanical damage in vulnerable nests (e.g. Birkhead, Thompson, Biggins & Montgomerie 2019) and thermal requirements inside the nest. Climatic conditions at incubation sites have strong effects on egg morphology. For example, round eggs have a higher heat capacity compared to elongated eggs (Turner 2002) and are more common in hot and dry environments, where long eggs gain and lose heat faster (Duursma, Gallagher, Price, & Griffith 2018). Eggshell structure has also evolved in part as a response to ambient conditions during incubation including temperature, humidity and altitude (Board 1982, Carey 1980). Eggshell components, such as the outermost cuticle, show large variation among avian taxa and play an important role in the adaptation of birds to various environments with varying risk of microbial contamination (D'Alba, Maia, Hauber & Shawkey 2016).

Egg shape in Reptilia (*sensu stricto* Modesto & Anderson 2004) varies from spherical to elongated and has been hypothesized to influence incubation efficiency and mechanical strength (Barta & Székely 1997), heat gain and water balance (Duursma et al. 2018). All oviparous reptiles produce eggs with some sort of protective shell and provide a physical (and chemical) barrier to microbial infection and to physical disturbance. Although typically eggshells are classified as “hard” or “soft” and/or “pliable” (Packard & Packard, 1980) their structure can range widely between these extremes (Packard & DeMarco 1991), especially in non-avian reptiles. In turtles for example, Kusuda et al. (2013) determined that at least six eggshell types exist on basis of the structure of the calcium carbonate layers. Even though

the validity of a binary classification of shell type has been called into question (Kusuda et al. 2013, Deeming 2018), this nomenclature is still used to this day in comparative studies of eggshell morphology (e.g. Pike, Andrews & Du 2012, Hallman & Griebeler 2015, Deeming 2018).

Numerous pores and air gaps span the shell enabling exchange of water and gases needed for embryonic survival and growth. Shells also incorporate calcium to varying degrees, in part as a reservoir for the embryo, which together with the degree of shell porosity can substantially affect both mechanical and respiratory functions (Rice 1989, Deeming 2018). Largely through variation along these axes of shape, hardness, porosity, and calcification, eggshells have evolved into an enormous diversity of forms presumably attuned to their environment (Deeming 2002). However, the selection pressures driving this evolution have not been clearly identified, and we lack fundamental knowledge of both eggshell forms and functions. This is particularly true in non-avian reptiles, whose eggs have received considerably far less attention than those of birds.

Birds lay their eggs in diverse environments but incubate their eggs with parental heat, thus parental incubation patterns and nest characteristics play a crucial role in shaping the existing diversity in egg morphology and eggshell functional properties (Deeming 2002, Duursma et al. 2018, Nagy et al. 2019). In contrast, most non-avian reptiles do not parentally incubate their eggs and bury their eggs in soil or in mounds of decaying vegetation (e.g. crocodilians; Shine 1988). Although a large number of squamates lay eggs containing embryos at intermediate developmental stages due to partial uterine egg retention (Shine 1983) or are viviparous, most reptile eggs, remain exposed to the external environment for periods as long as 9 to 12 months (e.g. Varanidae, Thompson & Pianka 2001; Sphenodontidae, Thompson 1990). Consequently, their embryos develop under a microclimate that is completely determined by local hydrologic, climatic and edaphic factors. How do they do this? The

mechanisms underlying the remarkable functionalities of reptilian eggs are intriguing. It is in this context, that comparative analyses offer a powerful approach to understanding these mechanisms, by testing structure-function hypotheses across a broader range than is typically possible within a single species.

The selective pressures on egg morphology can be both abiotic (including humidity, temperature, altitude and nest substrate properties) and biotic (including microbes and incubation behaviors). Eggs in drier habitats need to conserve water, while those in wetter habitats need to minimize the risk of egg flooding (where shell pores become occluded by water potentially leading to embryo asphyxiation). Ambient temperature can have profound effects on reptile eggs, both because eggs are generally not incubated, and in some cases have temperature-dependent sex determination. Altitude also imposes particular challenges to eggs; eggs laid in high altitude face a dangerous increase in gas diffusion and as a result, adaptations that increase the resistance to diffusivity, such as the reduction of pore size in avian eggs (Rahn, Carey, Balmas, Bhatia, & Paganelli, 1977, Carey 1980) or thickening of the eggshell could be advantageous. In terms of nest location, eggs on rocky substrates should be less brittle than those on sand. The most critical biotic factor affecting eggs is likely microbes that would find in the egg a nutrient-rich food source should they cross the eggshell barrier through either a crack or pore. Egg porosity must thus be both high enough for efficient water and gas exchange and low enough to protect against microbial infection. The response to these sometimes competing selection pressures govern the evolutionary patterns of eggshell morphology.

Here, we first perform comparisons of egg and eggshell morphometrics across lineages of non-avian reptiles and quantify the egg and adult size allometry of different groups. Then we

use a comparative functional morphological approach to improve our understanding of the broad evolutionary patterns of eggshell morphology in non-avian reptiles and to elucidate its relationships with specific nesting ecologies. We focus on two functional hypotheses to explain the evolution of eggshell characteristics: 1) permeability modulation, and 2) protection from environmental damage.

1) In contrast to eggs laid in water, those laid on land are in constant danger of desiccation. Soft eggshells may have evolved convergent into calcified eggshells in different amniote lineages (Sander 2012, Stein et al. 2019) because non-calcified eggshells lose water faster than calcareous shells (Ar 1991, Ackerman, Seagrave, Dmi'el, & Ar, 1985) and increased shell mineralization and/or thickness would allow embryos to survive in a wider range of hydric conditions. This in turn would enable colonization of arid environments. However, non-avian reptiles vary considerably in eggshell calcification, and a vast number of reptiles successfully reproduce using eggshells with incipient or no calcification (Packard and DeMarco 1991). We predict that eggshell thickness and calcium content will be higher in arid environments relative to humid habitats. Similarly, because eggshells predominantly made of fibers transfer gases and water quickly we expect to see fibrous (soft) shells in humid environments (i.e. with low risk of dehydration).

2) Thickness, porosity and the degree of calcification likely determine the function of eggshells as protective barriers helping protect eggs from physical damage by, e.g., increasing resistance to fracture or by preventing the passage of microorganisms into the egg content. Calcification protects eggs from microbial contamination, as microbes do not easily degrade minerals. If infection were a significant selection pressure, we would then predict that eggshells are thicker and/or more calcified in species with long periods of incubation and/or

in environments with high risk of microbial infection (e.g. tropical, humid). Similarly, we predict that less porous eggshells would offer more protection against contaminants in wet and warmer environments and that the performance of eggshells varies with nest type (where substrates can vary from rocky substrates to soft sand to decaying vegetation and compact soil).

2 MATERIALS AND METHODS

2.1 Sample collection and preparation

We obtained complete eggs from 91 species of non-avian reptiles: crocodiles (n=5), turtles (13) and squamates (n =73), through museum loans (The Steinhardt Museum of Natural History, Tel Aviv, and the Museum für Naturkunde, Berlin), donations from two Belgian zoos (Zoo Antwerpen and Zoo Planckendael) and private breeders. We only used samples that had no sign of embryonic development. The type of preservation (e.g. fixation plus ethanol or freezing) and time since collection could potentially affect eggshell structure. For two species (*Agama agama* and *Iguana iguana*) we had specimens obtained from both, museum collections (fixated) and from private breeders or zoos (fresh). A comparison of eggshell thickness and diameter of shell fibers between fixated and fresh samples showed no perceivable differences (supplementary online material, Figure S1). For a complete list of specimen sources and identification numbers see supplementary online material, Table S1a. After all tests were performed, remaining material from egg samples was either returned to respective museum collections or deposited in the collection of the EON unit of the Biology Department at the University of Ghent and available on request from the corresponding author.

After photographing each egg (see below), we cut small fragments ($\sim 1\text{cm}^2$) of each eggshell specimen and thoroughly rinsed them with ethanol (museum specimens) or water and then ethanol (fresh eggshells) and kept them in plastic tubes in 70% alcohol for subsequent analyses. The remaining egg samples were either returned to the museum collections or kept at -40°C in the laboratory.

2.2 Characterization of egg size and morphology

To obtain accurate measures of egg size and shape (elongation) we took photographs of each under standardized conditions of illumination and egg position (horizontal). We measured egg size using ImageJ 2.10 software (Rueden, Schindelin & Hiner 2017) and calculated egg elongation as length divided by width. We then calculated egg volume using the equation $V = \pi * LW(3c^2 + 14c + 35)/210$ (Maritz & Douglas 1994), where L is egg length, W is egg width, and c is the bicone (parameter representing bluntness at the ends of the egg), which was assessed by comparing the shape of each egg with models in (Maritz & Douglas 1994).

To image shells, and more specifically to estimate calcium content and distribution, using Scanning Electron Microscopy, we mounted one small piece of shell from each species, taken from the equator of each egg, on an aluminium stub, sputter-coated them with gold/palladium and imaged them on a SEM (FlexSEM 1000; Hitachi) at an accelerating voltage of 10 kV. Measurements of dimensions of all shell components were made on the micrographs using ImageJ software.

To determine the proportion of mineral content of eggshells we used standard histological staining coupled with optical microscopic observation. First, we embedded small shell fragments using a gradual increase in concentrations of epoxy resin, we then trimmed and cut sections (of 3.5μ thickness) of each sample using a Leica UC-6 ultramicrotome (Leica Microsystems, Germany). We then used alizarin red S staining to evaluate the mineral

deposition of eggshells (Lazcano et al. 1993, Valentine 2017). Alizarin red S selectively stains calcite, amorphous calcium carbonate, aragonite, hydroxyapatite and other calcium phosphates (Lee, Hodson, & Langworthy, 2008, Lazcano et al. 1993). We imaged the stained sections using a Leica optical microscope at 40x magnification. Eggshell mineralization was indicated by the presence of bright red areas on the stained sections on the shells. Using the stained eggshell micrographs we then used ImageJ software to calculate the areas stained in red relative to the entire eggshell area to obtain a proportion of calcium and its location in each shell sample.

We assessed porosity on a subset of eggshell samples from 50 species using X-ray computed tomography (see below). One shell fragment per species (~0.5 cm x 0.5 cm) was sampled from the equator of each egg. We processed and visualized CT-scans of each eggshell using Dragonfly software (ORS Inc., Montreal). In a first step we filtered the reconstructed 3D-images using a median filter to remove noise. Then, we created separate regions of interest for shell and air spaces and segmented the volume data using the threshold method; as a result the greyscale images are segmented to binary images containing the voxels composed of either eggshell or air and from which the corresponding volume was obtained. We calculated porosity (P) as the ratio between the volume of the air spaces (V_a) to the total volume of the sample (volume of shell ' V_s ' plus volume of air ' V_a '), expressed in percentage: ($P = V_a / (V_s + V_a) * 100$).

2.3 X-ray micro computed tomography (μ CT)

We obtained full 3D-image datasets from 50 eggshell samples using Synchrotron X-ray microCT. Tomography was performed at Beamline 8.3.2 of the Advanced Light Source at Lawrence Berkeley National Laboratory. 20keV X-rays illuminate the sample, and detection

is done with a LuAG:Ce scintillator, Olympus microscope objectives in an Optique Peter lens system, and a PCO.edge sCMOS detector, with a resulting voxel size of ~0.65 microns. Around 2000 projection images were collected as each sample was rotated over 180 degrees. TomoPy and Xi-CAM were used to perform tomographic reconstruction.

2.4 Nesting ecology and life history data

For each species we compiled information on incubation length, clutch size and nest type using taxonomic databases (Animal Diversity Web, IUCN 2014, Meiri 2018) and published articles on individual species. When incubation length was available as a range we used their average value. We obtained adult mass values for squamates from the dataset by Feldman Sabath, Pyron, Mayrose, & Meiri (2016). For crocodiles and testudines, we retrieved the maximum confirmed weight for each analyzed species from literature.

We defined nest type categories as: burrow (a tunnel-like excavation in the ground of at least 30 cm in depth), mound (eggs laid in a pile of mud and vegetation), hole or crevice (shallow cavity in a tree or between rocks), exposed (superficial oviposition on ground or attached to external surfaces) and parental sheltering (when the mother remains coiled around the eggs). For two species, *Oligodon cyclurus* and *Elaphe hodgsoni* we did not find information about the type of nest where they lay eggs, and for four species, *Elaphe hodgsoni*, *Blanus cinereus*, *Gerrhosaurus skoogi* and *Scincus scincus* data on incubation length was not available.

We obtained the latitude and longitude centroids to represent the geographical distribution of each species from Roll et al. (2017). For each of the species centroids we extracted two climatological variables for a 30-year period to represent average environmental conditions encountered by a species within its geographic range: annual precipitation in mm and annual mean ground temperature (°C) (temperature at the Earth's surface including the vegetated ground coverage) from the NASA Langley Research Centre (LaRC) POWER Project

(<https://power.larc.nasa.gov/data-access-viewer/>). The use of centroids to derive climate data has limitations and can include important inaccuracies (Park & Davies 2017), particularly when centroids originate solely from large occurrence location databases or administrative areas (Cheng et al. 2020). The centroids used in this study were calculated by combining species range maps, field guides and regional specialist data (see methods in Roll et al. 2017) and therefore effectively represent specific geographical distribution. In addition, we checked that each species centroid did not fall on a location outside the habitat reported for that species (e.g. in the ocean). In four species where this was the case (supplementary online material, Table S1a), we relocated the centroid to the nearest location on land within the species habitat. We recorded the altitude range at which each species occurs from specific accounts at the Animal Diversity Web (<https://animaldiversity.org>) and published papers (for the complete list of references used per species see supplementary online material, Table S5). As information of species distribution across an altitudinal gradient is scarce and not detailed, we first looked into the vertical distribution ranges of our selected species; then, as we could not detect a clear clustering, we noted the maximum (3200 m) and minimum (0 m) altitude occurrences within our dataset and we set their average (1600 m) as a cut point. All species distributed below 1600 m were classified as “Low” and above it as “High”.

2.5 Phylogeny and Statistical analyses

We obtained phylogenetic information across the non-avian reptiles in our sample from the Timetree of Life project (<http://www.timetree.org>; Kumar, Stecher, Suleski, & Hedges 2017). Given an input list of taxa, this public knowledge-based platform returns a time calibrated, ultrametric tree retrieved from published genetic-based time estimates from scientific literature (Hedges et al. 2015) including the most current published calibrated phylogenies by Zheng & Wiens 2016 (for Squamata), Oaks 2011 (Crocodylia), Chiari, Cahais, Galtier &

Delsuc 2012 and Pereira, Sterli, Moreira & Schrago 2017 (Testudines). We interpolated species without any genetic data if at least one member of their genus had genetic information. The tree used in analysis is available in the supplementary online material, Table S1b).

To initially visualize how the data are distributed and to avoid possible auto-correlations in preparation for the subsequent analyses, we produced a correlation matrix among our variables (supplementary online material, Figure S2). Next, to examine evidence for phylogenetic signals in eggshell morphology traits, we used the function “phylosig” in the Phytools R package (Revell, 2012) to compute Pagel’s Lambda coefficients. This showed that there is a strong phylogenetic signal in eggshell thickness ($\lambda = 0.88$; p-value $<10e-20$) and calcium content ($\lambda = 0.76$; p-value $<10e-10$) but not for eggshell porosity ($\lambda = 0.30$; p-value 0.06).

We used the “fastAnc” and “contMap” functions, both implemented in Phytools (Revell, 2012), to estimate and visualize on the tree the ancestral states (95% CI) using maximum likelihood of the three continuous morphological eggshell traits for the last common ancestor of non-avian reptiles.

Since there was a strong phylogenetic signal, we used the phylogeny-informed Phylogenetic Generalized Least Squares (PGLS) implemented in the R package “caper” (Orme et al. 2018) to test for associations between ecological variables and eggshell morphology. First we log-transformed eggshell thickness and porosity to reduce their skewness, and for each of the 3 generated global models (i.e., one for each eggshell morphology trait treated as the outcome variable) we checked for the homogeneity and normal distribution of the residuals. Clutch size and adult (log) body mass were strongly correlated ($r = 0.74$, $p < 0.01$), so we chose to use

only adult mass in building the global models. Following the recommendations of IT approaches (Burnham, Anderson & Huyvaert 2011, Grueber, Nakagawa, Laws, & Jamieson 2011) we constructed the global models with just the biologically relevant covariates that served to test our predictions for the permeability and protection hypotheses. Specifically, we included for each of our dependent variables (i.e. eggshell thickness, calcium content or eggshell porosity) adult (log) mass, nest type, incubation length, egg elongation, altitude, annual precipitation and ground temperature as independent variables (representing nesting ecology and life history). We also examined potential interactions between nest type and precipitation, nest type and ground temperature. For better interpretation of parameter estimates, we standardized effect sizes on two SD after model averaging (Gelman 2008). We then used the ‘dredge’ function in the MuMIn R package (Barton, 2019) to generate a complete set of models for each dependent eggshell trait. Next, from each model set, we identified candidate models that included a top model set following the criterion by Burnham & Anderson (2002), by selecting the top 2AIC_C of models. We then model averaged the parameter estimates and their 95% C.I. for each variable in the top model set using the zero-averaging method (Grueber, Nakagawa, Laws, & Jamieson 2011) which is recommended when the aim is to determine which factors (among multiple) have the strongest effect on a response variable (Nakagawa and Freckleton 2011).

3 RESULTS

3.1 Phylogenetic regressions

As a first step, we investigated to what extent key parameters of egg structure and morphology covary. We found a positive relationship between the adult (log) mass of an individual and egg volume ($r = 0.40$; $p < 0.001$, $\lambda=0.87$; Figure 1a) and eggshell thickness ($r = 0.22$; $p = 0.01$, $\lambda=0.87$), with heavier (larger) individuals producing larger eggs that have

thicker eggshells. When we examine this relationship for Squamata, Testudines and Archosauria separately we find that the positive relationship between adult mass and egg volume is maintained for the former two, but not for Archosauria. There was also a significant negative association between adult mass and egg elongation ($r = -0.26$, $p = 0.01$, $\lambda=0.74$; Figure 1b). For shell thickness, the relationship with egg volume is also clade specific as it remains highly positive for Squamata, but not for Testudines and Archosauria. We did not find a relationship between egg volume and shell thickness ($r = 0.05$; $p = 0.27$, $\lambda=0.79$). Thicker eggshells exhibit higher calcium amounts ($r = 0.19$, $p = 0.04$, $\lambda=0.80$) but thickness does not vary significantly with porosity ($r = 0.14$; $p=0.81$, $\lambda=0.98$).

Egg elongation does not vary significantly with porosity ($r = 0.18$, $p = 0.11$, $\lambda=0.98$) but more elongated eggs have a significantly lower calcium proportion ($r = -0.23$; $p = 0.01$, $\lambda=0.54$; Figure 2), compared to more spherical eggs. Finally, we found a significant relationship between calcium content and porosity of an eggshell ($r = 0.39$; $p = 0.003$, $\lambda=0$).

Interestingly, we found considerable variation in the patterns of calcification within eggshells (Figure 3). Calcium is found as a compact, uniform layer and constitutes the main eggshell component in all Crocodiles, also in members of Cryptodira within Testudines and gecko species belonging to families Gekkonidae, Phyllodactylidae and Sphaerodactylidae (in these gekkos the calcified shell was always observed under an organic layer). Among all other squamates, the arrangement of calcium within the shell is non-uniform; for example, calcium is found as a continuous but nebulous crust layer on Diplodactylidae and Eublepharidae geckos, as crystals sparsely embedded irregularly along the shell (e.g. *Heloderma horridum*, Figure 3) or only on the surface of the shell (e.g. *Natrix natrix*, Figure 3) often forming rosette or star-like crystals (e.g. *Hydrosaurus weberi*; supplementary online material, Figure S3). In some squamates we also observe calcium diffusely forming part of a matrix embedding shell

fibers (e.g. *Eumeces schneideri*, Figure 3). Lastly, we failed to detect any calcium in the shell of several species (predominantly snakes; e.g. *Morelia spilota*).

3.2. Ancestral character state reconstruction

Based on our ancestral character state reconstructions, it is highly likely that the most recent ancestor of Reptilia had moderately thick eggshells (227 μm [46 μm , 310 μm] 95% C.I.) and an intermediate level of calcium (48.9 % calcium [17%,80%] 95% C.I; Figure 4) in their eggshells. Over time, eggshells decreased in thickness across Squamata, but became thicker in Crocodilia and some turtle groups (Figure 5). Full calcification occurred independently several times (in turtles, crocodiles and three times within Gekkonoidea, Figure 4).

Our results also suggest that the ancestor likely had shells with a low level of porosity (10.6% [1.5%,24%] 95% C.I. Figure. 6), which progressively increased in some squamates, particularly in Serpentes, and slightly decreased or remained low in Testudines and Crocodilia.

Moreover, we found that the ancestor most likely presented a moderate-low elongation factor (width (cm) / length (cm); 1.58 [0.94, 2.21] 95% C.I.; Figure S4) and over time most groups kept this ratio constant but not in Gekkonoidea and Testudines where more spherical-shaped eggs evolved independently. Interestingly, more spherical eggs appear to be phylogenetically linked with higher calcium content.

3.3 Eggshell morphology in relation to nesting ecology

Eggshell thickness

Four variables (incubation length, adult mass, annual precipitation and ground temperature) were included in the top models (those with $\Delta \text{AIC} \leq 2$) from the phylogenetic generalized least squares models tested (supplementary online material, Table S2). Consistent with the

protection hypothesis, we found that species nesting in environments with higher precipitation produce eggs with thicker eggshells. Moreover, we found that eggshell thickness significantly increased with adult body mass but not with incubation length or ground temperature (Table 1).

Calcium content

None of the variables tested produced models with strong support to explain the calcium content of shells (supplementary online material, Table S2). We found little evidence that calcium content is associated with climate variables or life history parameters (Table 2).

Eggshell porosity

All seven predictor variables were present in the set of best models to explain eggshell porosity (supplementary online material, Table S3). Under both, the permeability and the protection hypotheses, we expected that porosity would decrease with precipitation, temperature and altitude. Partially supporting our predictions, we found that eggshells are significantly less porous in environments with elevated temperature compared to cooler habitats (Table 3). We also found a significant association between porosity and nest type and an interaction between ground temperature and nest type: eggs laid in burrows decreased in porosity with elevations in temperature; in contrast, eggs laid inside mounds or piles of decaying vegetation increased in porosity with increasing ground temperature (Table 3, Figure S5). Similarly, the interaction between nest type and annual precipitation was associated with eggshell porosity, where only eggs laid in mounds markedly decrease in shell porosity as precipitation increases.

4 DISCUSSION

We used micro-ct, and scanning electron microscopy to characterize the morphology of diverse reptilian eggshells, and comparative analyses to test hypotheses on their potential functions in nesting ecology. We hypothesized that eggshell thickness, porosity, and calcium content are optimized to protect shells from physical damage and desiccation (i.e. respectively the protection and permeability hypotheses). Consistent with the protection hypothesis, we found a positive relationship between thickness and precipitation, and consistent with the permeability hypothesis, a negative relationship between porosity and temperature. Surprisingly, despite high variability, calcium content was not correlated with any nesting ecology variable. However, the versatile distribution of calcium within the eggshells and its covariance with egg shape call for new functional explanations for calcification patterns. Ancestral state reconstructions show that eggs became thinner and more porous over evolutionary time in squamates, and vice versa in turtles and crocodilians. More generally, egg shape and size were correlated with porosity and calcification, suggesting potential structural or developmental tradeoffs. Such tradeoffs will be of substantial interest for additional research.

Water is essential to embryonic growth and survival, and eggshell porosity plays a critical role in its regulation. Unlike avian eggs, many (non-fully calcified) reptile eggs absorb water that reaches the embryo via air spaces (pores) between the fibers. Perhaps for this reason, many squamate eggs have much less albumen (the primary source of water for nestling archosaurs) than do other reptile eggs (Deeming & Ruta 2014). By contrast, pores modulate the diffusive capacity of eggshells (Paganelli 1980) and can contribute to desiccation via evaporative water loss, and, depending on their size, may serve as channels for movement of bacteria to the yolk and embryo (Berrang, Cox, Frank, & Buhr, 1999). We therefore predicted that egg porosity would be lower in hot environments, to avoid excessive water loss and in

rainy habitats to prevent bacterial infection. Indeed, shell porosity declined with increasing ground temperature, suggesting that desiccation risk may have shaped the pore structure of eggshells. Intriguingly, in species that nest in tunnels, porosity also declined with ground temperature. In these deep burrows, increases in ground temperature may lead to dry microclimatic conditions surrounding the eggs. Reduction in the level of porosity of these shells would prevent excessive dehydration via a mechanism similar to that observed in avian eggshells laid at high altitudes and dry environments (Rahn, Carey, Balmas, Bhatia & Paganelli, 1977, Carey 1980). Egg porosity increases with temperature in mound nests, where gas flux is strongly influenced by the respiration of microorganisms. Increased water content and temperature in the mound increase rates of microbial respiration, thereby impeding gas diffusion in eggs (Seymour, Vleck & Vleck 1986). Thus, increased porosity in these nests might have evolved to counteract impeded gas exchange when ground temperature is high. An increased sample size of porosity and direct measurements of microhabitat variables will enable more conclusive testing of these hypotheses.

In contrast to our predictions, porosity was not related to precipitation. However, we found that eggs have thicker shells in rainier environments. Wetter habitats can be conducive to egg flooding and asphyxiation, and might impose a higher risk of contamination by microbes (Board 1979, Cook, Beissinger, Toranzos, Rodriguez & Arendt, 2005). A thicker eggshell may thus be a physical barrier to lower both of these risks. However, we found no evidence that shell thickness varies with nest type or duration of incubation, suggesting no differential protection from damage or breakage in various types of substrates or in relation to the duration of egg exposure to the elements. Perhaps the suppleness of reptile eggs lowers their risk of cracking relative to avian eggs. In this case, we would broadly expect to find less-calcified, and hence suppler, eggs on hard substrates. Alternatively, and independently of

eggshell thickness, variation in chemical composition of shells could be particularly important in providing diverse kinds of protection. For example, mucopolysaccharides, such as those forming the mantle and/or matrix surrounding eggshell fibers in some reptile species (Palmer & Guillelte 1991; this study: *Orthriophis hodgsoni*, Figure S6) and birds (Board & Sparks 1991) could be critical for increasing eggshell resistance to desiccation, abrasion or mechanical impact (Wotton 2004). These diverse functions, which depend on the interaction of polysaccharide chains with water, are commonly seen in many organisms including bivalves, echinoderms, lungfish and amphibian (Wotton 2004). Unfortunately, with the exception of a handful of studies on the amino acid composition of shells (e.g. Sexton Bramble, Heisler, Phillips & Cox 2005, Choi, Han, Kim, & Lee, 2018) the chemistry of reptile eggshells remains poorly understood. It will therefore be necessary to acquire a deeper understanding of the differences in composition of the organic matrix of shells before we can elucidate how specific eggshell phenotypes function in different nesting environments.

With a few exceptions (8% of spp), all species in our study incorporate calcium in their shells. However, we found no correlates of calcification with any ecological factors. Thus, the functional relevance of increased shell calcification remains unclear. Increased calcification does not seem to be a requirement to optimize protection from desiccation, mechanical damage or microbial attack. However, reptiles place calcium in diverse locations in the egg (Figure. 3). That calcium can be deposited as distinctive crystals, as matrix embedding proteinaceous fibers, or as an amorphous crust on top of eggshells. That the location of these calcific forms within the eggshell seems indiscriminate suggest that calcium deposition is a labile trait, and that the degree of mineralization (which would correspond to their levels of stiffness) likely reflects a continuum (Kusuda et al. 2013, Deeming 2018) rather than a bimodal categorization of eggshell morphology.

Location of calcium would likely affect the material properties of the egg, as a superficial calcium layer, for example, should have different effects than calcium diffused throughout the shell. Although such variation should be experimentally confirmed, a more meaningful measurement of calcification that includes not only amount but location of calcium is needed to test its ecological relevance. More broadly, it draws into question the traditional distinction between “hard” and “soft” shelled eggs. This intuitive scheme, which recently has come into renewed use in a lively debate over the ancestral condition of eggs as inferred by fossils (Stein et al. 2019, Legendre et al. 2020), may be over-simplified, and reduce a grayscale continuum into black-and-white while ignoring important morphological and, potentially, functional information. Furthermore, calcium and protein content in the shell and therefore their rigidity is known to decrease with the progression of incubation (Cox, Koob, Mecham, & Sexton, 1984). A more nuanced view of egg stiffness and composition will be valuable for future investigations into the deep evolutionary history of eggs. Exploring this variation through materials property testing of eggshells in conjunction with biomimetic replicas with varying calcification patterns will be a critical next step towards understanding the evolution of hardness in eggs.

We found that the most recent common reptile ancestor likely had a moderately calcified eggshells, supporting the idea that early amniotic eggs were already mineralized (Kohring 1995) and that through evolution biomineralization increased to the fully calcified structures in some lineages (e.g. birds, crocodiles, some turtles and geckos). Our results do not indicate clear improvements in egg protection with this shift in shell calcification. Rather, increased biomineralization may be a mechanism for shedding toxic metal ions (e.g. Cu, Pb ; Park Jeong, Yang, Kim, & Lee, 2007; Yalçın-Özdilek, Özdilek & Sangün 2011), and may simply be related to the abundance of calcium in the environment (Deeming 2018) or to increasing

concentrations of atmospheric oxygen (Stein et al. 2019). Archosaur and turtle embryos use calcium in the eggshell for bone development (Romanoff & Romanoff, 1967; Packard & Packard 1984, [Packard & Packard, 1989](#), Packard & Packard, 1991), while evidence suggests that other reptile embryos obtain calcium predominantly from the yolk (Packard & Packard 1984) or even the environment (Simkiss 1962). Thus, increased calcification may also reflect a shift in resource allocation to the shell. Kratochvíl and Frynta (2006) noted that in geckos the presence of spherical eggs and their degree of calcification are correlated, suggesting a functional relationship between the two traits. Our broader analysis similarly revealed that reptile eggs are, with a few exceptions, non-spherical and fully calcified shells may reflect other factors, including their lack of pelvic canal and distinct behaviors such as flight and incubation.

In non-avian reptiles, the pelvic limitation hypothesis suggests that the size of the pelvic opening during egg expulsion (Kratochvíl & Frynta 2006, Deeming 2018) constrains females to increase egg size by length but not width (Congdon & Gibbons 1987). As expected, we found an allometric relationship between egg and adult body sizes. And in line with the pelvic limitation hypothesis, we found that smaller females enlarge eggs by increasing egg length relative to width. Turtles may additionally optimize egg packing inside burrows by laying spherical eggs (Moll 1979).

Our results show that nesting ecology has affected the evolution of aspects of reptile egg morphology, in particular shell porosity and thickness. Other components such as calcification and overall shape appear to have been driven by other selective pressures and constraints. Moreover, they highlight the paucity of fundamental knowledge we currently have on this topic. Our understanding of reptile eggs is limited relative to that of bird eggs,

despite their rich diversity of forms and remarkable functionality that enables them, to nourish and protect nestlings from predation, microbial infection, water loss, flooding and other insults without interference by parents. We hope that this work will inspire new research on both their proximate and ultimate aspects.

Data availability

The data that support the findings of this study are openly available in Zenodo at <http://doi.org/10.5281/zenodo.4585220>

Micrographs and 3D images of eggshells produced in this study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Relationship between eggshell thickness and nesting ecology in 89 species of non-avian reptiles. Results from phylogenetic least square regressions after model averaging. Eggshell thickness was log-transformed prior to analyses. Effect sizes standardized on 2SD, following Gelman 2008.

Thickness variable	estimate	Unconditional s.e.	95%CI	Relative importance
intercept	4.14	0.62	(2.97, 5.35)	
Adult mass	0.36	0.07	(0.22, 0.51)	1
Rain	0.16	0.05	(0.05, 0.26)	0.96
Incubation length	0.01	0.04	(-0.08, 0.11)	0.82
Ground Temperature	-0.05	0.06	(-0.18, 0.06)	0.54
Altitude	0.0001	0.02	(-0.04, 0.05)	0.28

high vs low	
Nest*	0.21

*variable not included in the top set of models

Table 2. Summary results of the PGLS analyses, after model averaging, exploring the association between eggshell calcium content and nesting ecology in 89 reptile species. Effect sizes standardized on 2SD, following Gelman 2008.

Calcium variable	estimate	Unconditional s.e.	95%CI	Relative importance
intercept	68.62	22.35	(24.82, 112.43)	
Adult mass	-0.12	0.11	(-0.33, 0.12)	0.64
Rain	0.004	0.02	(-0.04, 0.04)	0.12
Incubation length	0.01	0.06	(-0.10, 0.13)	1
Ground Temperature	-0.01	0.02	(-0.04 0.04)	0.1
Altitude	0.009	0.03	(-0.06 0.07	0.24
high vs low				
Nest*				

*variable not included in the top set of models

Table 3. Association between eggshell porosity and nesting ecology variables in 50 species of non-avian reptiles. Results from phylogenetic least square regressions after model averaging. Effect sizes standardized on 2SD, following Gelman 2008.

Porosity variable	estimate	Unconditional s.e.	95%CI	Relative importance
intercept	26.25	7.69	(11.19, 41.31)	
Adult mass	0.001	0.02	(-0.04, 0.04)	0.16
Rain	0.02	0.05	(-0.08, 0.13)	1
Incubation length	-0.04	0.02	(-0.08, 0.01)	0.82
Tground	-0.61	0.19	(-0.97, -0.24)	1
Altitude				0.31
high vs low	0.01	0.02	(-0.04, 0.04)	
Nest*				1
mound	-4.55	0.98	(-6.48, -2.66)	
crevice	-2.66	0.78	(-4.18, -1.13)	
exposed	-2.73	0.80	(-4.30, -1.16)	
parental	-2.52	0.79	(-4.07, -0.97)	
Nest: Tground				1
mound: Tground	0.29	0.06	(0.17, 0.41)	
crevice:Tground	0.12	0.04	(0.05, 0.19)	
exposed:Tground	0.12	0.04	(0.05, 0.19)	
parental:Tground	0.11	0.04	(0.04, 0.18)	
Nest: Rain				1
mound:rain	-0.73	0.20	(-1.13, -0.33)	
crevice:rain	-0.04	0.06	(-0.15, 0.07)	
exposed:rain	-0.05	0.06	(-0.16, 0.07)	
parental:rain	-0.04	0.06	(-0.16, 0.07)	

* nesting burrow was the reference category

Figure legends

Figure 1. Associations between adult (log) body mass and (log) egg size (volume) and elongation ratio (length/width) for 91 species of crocodilians (orange), testudines (purple) and squamates (green). Scatterplots are based on raw data points, therefore much of the variation can be accounted to phylogenetic effects. Slopes estimated by PGLS (black lines) represent the relationships between the predictor and the response variable: a) (log) egg volume $\sim 0.47(\log)\text{mass}-0.66$ and b) Elongation $\sim -0.13(\log)\text{mass} + 2.01$.

Figure 2. Association between calcium content of eggshells and egg shape (elongation ratio; length/width) for 91 species of crocodilians (orange), testudines (purple) and squamates (green). Evolutionary regression slope estimated by PGLS (black line) represents the observed relationship between the predictor and the response variable (Egg elongation $\sim -0.005\text{calcium}+1.78$).

Figure 3. Optical microscope images of representative eggshells of non-avian reptiles showing the distribution of calcium (areas in red) within each sample. Scale bars: a) *Morelia spilota* and b) *Natrix natrix* -100 μm , c) *Pelusios carinatus* and d) *Heloderma horridum* - 60 μm e) *Varanus griseus*, 100 μm , f) *Eumeces schneideri* 30 μm .

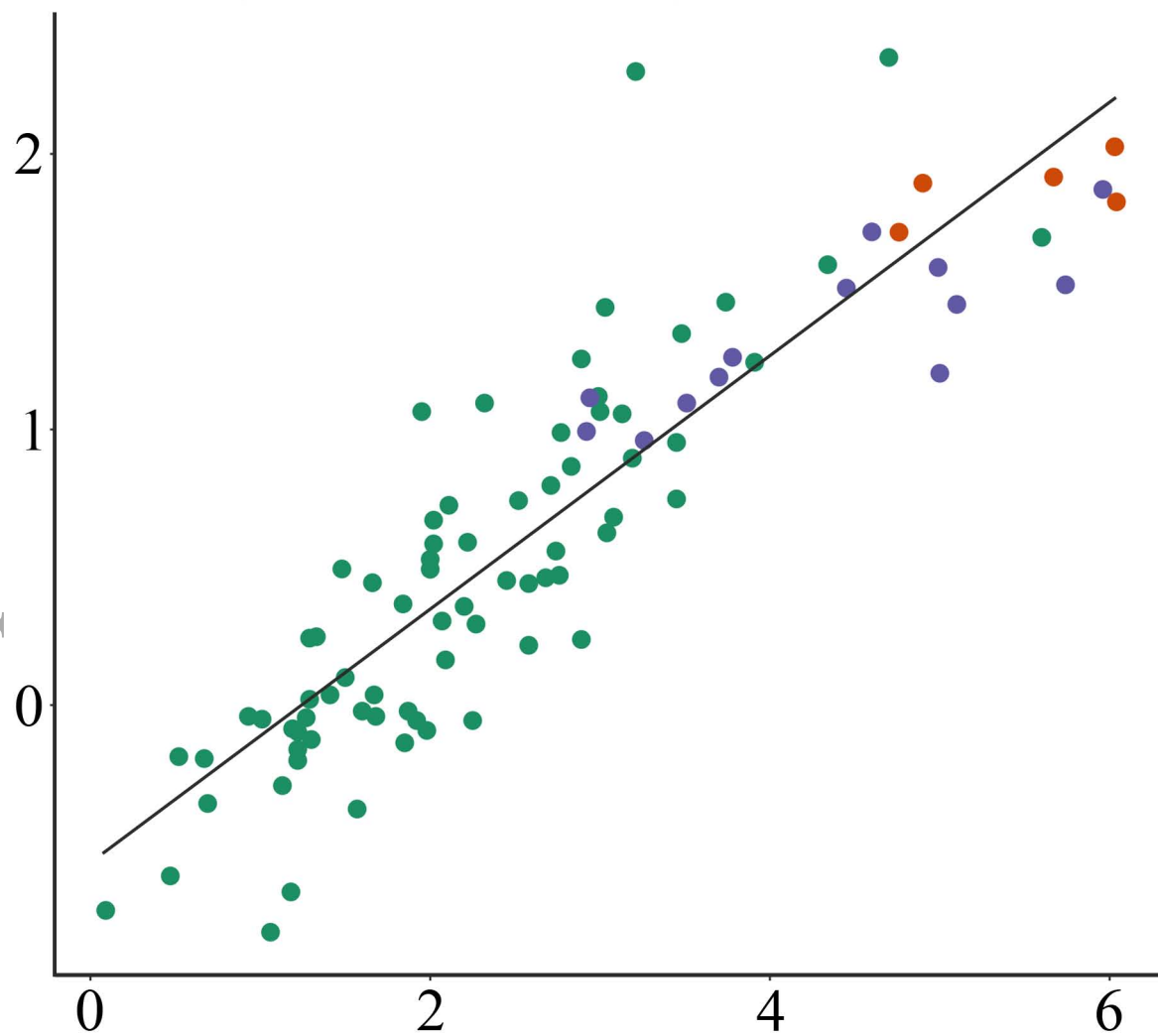
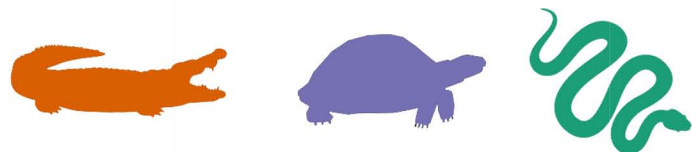
Figure 4. Maximum likelihood estimates of ancestral states of degree of calcification in non-avian reptile eggshells. The scale bar indicates calcium content from low (hot colors) to high (cool colors). Changes along branches calculated by interpolation (Revell, 2013). Colored circle symbols to the right of each branch tip depict states of distribution of calcium within

the eggshell as seen in Figure 3. Scanning electron micrographs at the bottom show representative eggshell samples increasing, from left to right, in their degree of calcification. Scale bars: a) *Macroprotodon cucullatus* -25 μ m, b) *Oligodon purpurascens* -50 μ m, c) *Testudo marginata* 500 μ m d) *Caiman crocodilus* - 500 μ m and e) *Gekko petricolus* -100 μ m.

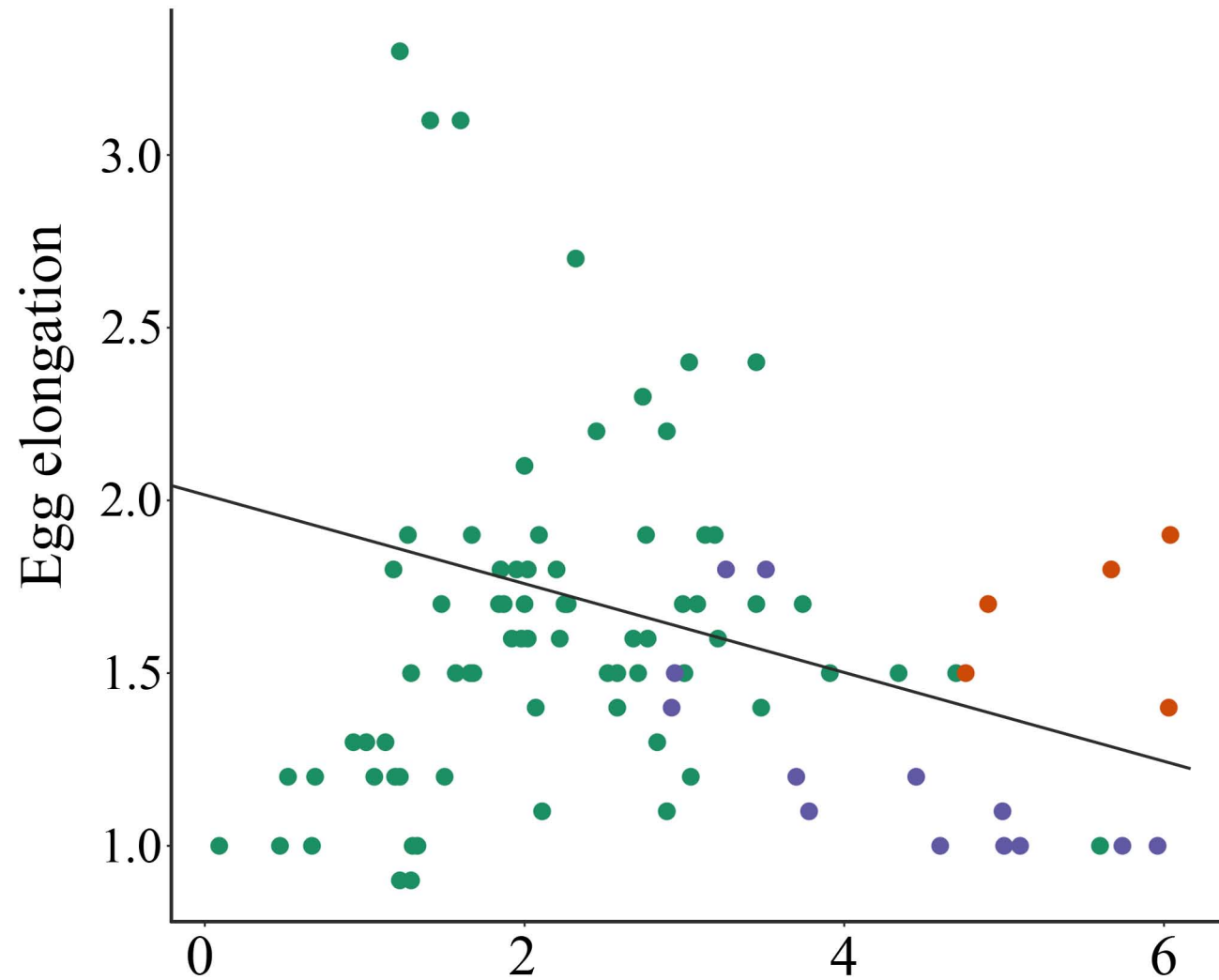
Figure 5. ContMap cladogram illustrating maximum likelihood estimated ancestral eggshell thickness. Branch colors correspond to estimates of thickness, with red indicating thinner and blue indicating thicker shells.

Figure 6. Maximum likelihood estimates of ancestral states of porosity levels in non-avian reptile eggshells. The scale bar indicates the percentage of porosity from low (hot colors) to high (cool colors). Changes along branches calculated by interpolation (Revell, 2013). At the bottom, 3D models of representative eggshell samples obtained from micro CT data demonstrate increasing degrees of porosity (air gaps colored dark blue) from left to right electron. a) *Crocodylus niloticus*, b) *Gehyra fehlmanni*, c) *Hydrosaurus weberei* d) *Elaphe hodgsoni* and e) *Natrix natrix*.

a)



b)





Egg elongation

3.0

2.5

2.0

1.5

1.0

0

25

50

75

100

Calcium content (%)

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