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INVITED PAPER

Hydric Environment and Chemical Composition Shape Non-avian Reptile Eggshell Absorption

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Synopsis The amniotic egg fulfils a critical role in reproduction by serving as an interface between the external environment and the embryo. Because non-avian reptiles are rarely incubated, they must be heated by, and absorb water from, the oviposition site for the developing embryo. The mechanisms by which they absorb sufficient, but not excess, water and how these mechanisms vary with local habitat is largely unknown, despite its significance to their evolution. Here, we first performed histology, Fourier-transform infrared spectroscopy and dynamic vapor sorption experiments to elucidate the mechanisms of eggshell absorption for 56 reptile species. Then, we used phylogenetic comparative analysis to test the hypothesis that the absorptive capacity of reptile eggshells increases with aridity of the environment. We found that water absorption increases in the presence of a superficial mucopolysaccharide layer and decreases with increased calcium content. We found that eggs from arid environments have highly absorbent eggshells, but only in species with weakly calcified shells. Our results suggest that reptile eggshells have over evolutionary time tuned absorptive capacity to environmental moisture level. Since these eggs often must sustain conflicting constraints, they may serve as inspirations for new biomimetic materials, such as water filtering membranes or humidity sensors.

Introduction

The amniotic egg provides important functions in reproduction, and the eggshell itself functions as an interface between embryo and environment. Roughly 70% of all terrestrial vertebrates (19,000 species), including the vast majority of non-avian reptiles, use eggs to reproduce across a wide range of environments (Modesto and Anderson 2004; King and Lee 2015). However, while most birds incubate and create microhabitats for their eggs (Hansell 2000; Deeming 2002), non-avian reptiles generally do not, and are therefore often directly exposed to, and completely reliant on, ambient factors at the site of oviposition (Shine 1988; Deeming 2004). Selection by environmental challenges both abiotic (i.e., ambient temperature, altitude, and humidity) and biotic (including microbial infections and predation) has significantly shaped egg phenotype (morphology, structure, and composition), in non-avian reptiles (Packard and Hirsch 1986; Pike et al. 2012; Deeming 2018; D'Alba et al. 2021). The precise mechanisms behind these associations between form and function, however, are still unclear.

Non-avian reptile eggshells generally consist of two or three layers, each consisting of multiple integrated organic and inorganic compounds (Packard and Hirsch 1986; Schleich and Kästle 1988; Packard and DeMarco 1991). The innermost layers, the boundary layer and the *membrana testacea* are composed of multiple layers of protein fibers with varying arrangements; these fibers are composed of keratins and/or collagen (Zheng et al. 2014; Chang and Chen 2016; Álvarez et al. 2022) and can be either randomly intermingled or mat-like, and

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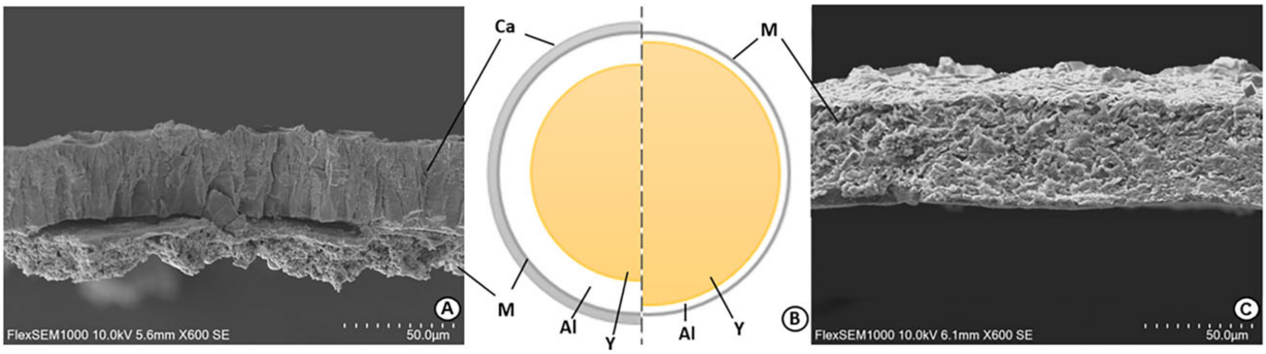

Fig. 1 Microstructure of eggshells in cross section (inner boundary not shown): **(A)** strongly calcified eggshell of *Hemidactylus turcicus*; **(B)** simplified model of a strongly calcified eggshell containing large amounts of albumen (left) and a weakly calcified eggshell containing negligible amount of albumen (right); and **(C)** weakly calcified eggshell of *Eumeces schneideri*; Ca, outer calcium layer; M, fibrous membrane; Al, albumen; and Y, yolk.

loosely arranged or aligned in parallel layers (Packard and DeMarco 1991; Chang and Chen 2016). Moreover, these fibers may be embedded in a matrix of proteins and carbohydrates leading to various configurations (e.g., porosities at different scales) (Palmer et al. 1993; D'Alba et al. 2021). In addition, eggshells often have mineral constituents (predominantly calcium carbonate either in calcitic or aragonitic form (Simkiss 1962; Silyn-Roberts and Sharp 1985;) that can form a third, superficial crystalline layer, as in crocodiles and most turtles (Fig. 1A). The structure of squamate (lizards, snakes, and amphisbaenians) eggshells is highly diverse, with a vast majority having a distinct outer layer of calcium carbonate. In others, the eggshell is less calcified, and the calcium carbonate does not form a distinctive outer layer, but it is dispersed throughout the shell (Packard and DeMarco 1991; Schleich and Kästle 1988; Choi et al. 2018; D'Alba et al. 2021; Legendre et al. 2022). Traditionally, reptile eggs have been classified as either rigid (crocodiles, some turtles, and most geckos), pliable (turtles), or flexible, parchment-type eggs (most squamates and some turtles) (Fig. 1C). However, recent advances in the field (Kusuda et al. 2013; D'Alba et al. 2021; Legendre et al. 2022) have shown that these classifications are oversimplified and in need of re-evaluation. Kusuda et al. (2013), for example, showed that six eggshell types exist within turtles alone. Selection for different functions has likely driven this variation in eggshell structure.

Water management is one such critical function, as water is essential for embryonic development. In most eggs, it is maternally sourced via albumen (Tracy 1980; Packard et al. 1982; Thompson 1987; Andrews 2015), but some have absorbent shells that capture moisture from the environment (Ackerman et al. 1985). Variation in the extent of shell calcification (used for bone development by the embryo), together with the degree of shell porosity can substantially affect both mechanical and respiratory functions (Deeming 2018). Mineralized material is a barrier to water exchange (Grigg 1987), thus fully calcified shells are less permeable and generally neither require nor allow acquisition of environmental water. However, some water uptake occurs in some species (Ferguson 1982; Amos and Rahn 1985; Andrews 2015). Similar to avian eggs, these strongly calcified eggs are most often produced with sufficient albumen to provide the embryo with water during development (Packard et al. 1982). In contrast, many squamate eggs (e.g., Bearded dragon, *Pogona vitticeps*; Corn snake, *Pantherophis guttatus*) have little albumen (<5% in weight, personal observation Gerben Debruyn, Deeming and Ruta 2014). Therefore, water most be sourced externally, and this absorption is facilitated by a scant mineralization that should render eggshells highly permeable (Packard et al. 1982; Deeming and Birchard 2007).

The process of mineralization of reptile eggshells is not completely understood, but as in birds, amorphous calcium may precipitate within an organic matrix of mucopolysaccharides and proteins in the oviduct that induce the formation of crystals (Simkiss and Tyler 1959; Rodríguez-Navarro et al. 2015). The incorporation of mucopolysaccharides either diffusely or as a superficial layer on top of the shell, as seen in many species of both non-avian reptiles (Simkiss and Tyler 1959; Palmer and Guillette 1991; Campos-Casal et al. 2020) and birds (Deeming and Ferguson 1991) could increase resistance to abrasion and mechanical impact (Wotton 2004) and also help avoid desiccation (Campos-Casal et al. 2020). This is because mucopolysaccharides form hydrogen bonds with water molecules that hydrate the surrounding tissue (Wotton 2004). In addition, less-calcified squamate eggs have more proline in their eggs than highly calcified gecko eggshells, a potential additional mechanism to increase water retention (Sexton et al. 2005).

The hydric environment strongly influences incubation success and hatchling size (Tracy 1980; Packard et al. 1982; Delmas et al. 2008; Booth and Yu 2009; Gatto and Reina 2022). Eggs developing on a substrate with a high water potential (i.e., more water present) yielded larger hatchlings and higher hatching success. However, the hydric environment does not affect hatching success for species with highly calcified eggs as much. While these eggshells are less permeable, they still naturally lose water during incubation, as in bird eggs (Rahn et al. 1979; Grigg 1987; Ar 1991). Importantly, eggshells that absorb large amounts of environmental water can potentially also lose it rapidly, leading to desiccation and embryonic death unless impeded by other structural components of the eggshell, its chemical composition, the size of the egg, or the water potential of the surrounding substrate (Tracy and Snell 1985). For example, eggshell porosity, which in reptiles declines with increasing ground temperature to avoid desiccation via evaporative water loss (D'Alba et al. 2021) could be a strong determinant of eggshell permeability. Thus, a complex suite of egg and eggshell properties make each species sensitive to their hydric environment to different extents (Belinsky et al. 2004; Deeming and Ferguson 1991).

The large diversity of reptile eggshells and the wide range of environmental challenges that these eggs face, make it critical to determine such form–function relationships. Elucidating these relationships will not only help understand how their performance may help survival in specific environments but may also serve as inspiration for materials with high wettability for application in gas adsorption, water treatment, separation, smart textiles, and biomedicine.

Here, we tested the hypothesis that the water absorbance capacity of eggshells is tuned to moisture content of their environment. We further identify potential eggshell structure parameters that could influence water vapor absorption. Our predictions were as follows:

(1) Influence of the native environment: We predict that eggshells laid in warmer and more arid environments absorb increasingly more water vapor, particularly in weakly calcified eggshells that rely on environmental water (Andrews 2015). Similarly, we predict that the sorption properties vary with nest type (open vs closed nests) as this affects the degree to which the egg is influenced by the surrounding climate (Deeming 2018; Nagy et al. 2019).

(2) Influence of eggshell structure: We predict that degree of calcification will strongly affect eggshell water sorption properties. That highly calcified eggs do not absorb as much water as weakly calcified eggshells are known, but we predicted gradual variation in the sorption properties linked to eggshell mineralization, even within the group of weakly calcified eggshells. In addition, we predicted that the greater surface area of highly porous and thick eggshells enables greater water vapor sorption. Finally, we predicted that eggshells with a superficial layer of mucopolysaccharides absorb more water vapor, as they bind water easily (Wotton 2004).

To assess these predictions, we first investigated the structure (via optical and electron microscopy) and chemical composition (via differential staining and Fourier-transform infrared spectroscopy (FTIR) of reptile eggshells across a broad sample of species. We then quantified water vapor absorption using dynamic vapor sorption (DVS). Lastly, we conducted phylogenetic comparative analyses to test hypothesized relationships between eggshell structure, water vapor absorption, and the native environment.

Material and methods

Sample collection and preparation

We acquired eggs or eggshell fragments of 56 non-avian reptiles (5 crocodile-, 9 turtle-, and 42 squamate species) from museum loans (The Steinhardt Museum of Natural History, Tel Aviv, Israel, and the Museum für Naturkunde, Berlin, Germany), donations from Belgian Zoos (Zoo of Antwerp and Zoo Planckendael), and two private breeders. Experiments were performed on all 56 species unless stated otherwise (Supplementary Table S1). We only used eggs that showed no embryonic development. Individual eggs vary in size, shape, yolk, and albumen content, all of which may affect their material properties. To control for these effects, and limit our tests to the eggshell itself, we cut small fragments ($\sim$1 cm^2) from a standardized position on the equator of each egg, rinsed it in ethanol and kept it in a vial in 70% ethanol until testing.

The DVS method requires only small samples (2.50 ± 0.50 mg) (Van der Schueren et al. 2013), allowing us to include species for which whole fresh eggs were not available and from a wide range of environments and egg morphologies.

Dynamic vapor sorption

To measure water vapor sorption of the eggshell, we cut the eggshells of 56 species (Supplementary Table S1) into pieces of (2.50 ± 0.50 mg) and characterized them using the Q5000SA DVS apparatus (TA instruments). We put the samples on metalized quartz sample pans followed by drying at 35°C and 0% relative humidity (RH) and then increased the RH in steps of 15% at a constant temperature of 25°C until 90% RH to make it comparable between samples. The next step was only started after equilibrium (weight change percentage <0.05 for 45 min), or after 900 min.

Scanning electron- and light microscopy

To visualize the eggshells, we mounted a piece of eggshell on an aluminium stub and sputter coated it with gold/palladium (10 mm thick layer). Micrographs were then taken on a scanning electron microscopy (SEM, FlexSEM 1000; Hitachi)

To visualize the location of mucopolysaccharides in the eggshells, we embedded each sample in epoxy resin using a 5-day protocol following the methods described by D'Alba et al. (2021). Then, we trimmed each resin block and sectioned each sample therein, to a thickness of 3 μm using a Leica UC-6 ultra-microtome (Leica Microsystems, Germany). Next, we performed Alcian blue staining to evaluate the mucopolysaccharide and glycoproteins contents (Dong et al. 2012). Using a DM1000 led optical microscope (Leica optical microscope), we imaged the stained egg sections and determined whether the mucopolysaccharides were either present as a distinct layer superficially on the eggshell, or together with calcium (diffuse throughout the eggshell or with crystals). We used the reported values of calcium content from D'Alba et al. (2021), who used Alizarin red to stain calcite, amorphous calcium carbonate, aragonite, hydroxyapatite, and other calcium phosphates (Lazcano et al. 1993; Lee et al. 2008).

Nesting ecology and life-history data

We used the reported values for porosity (ratio between the volume of air spaces to the total volume), shell thickness (μm), average ground temperature (°C) from D'Alba et al. (2021). Similarly, we categorized nest type as either open or closed, for ease of analysis, based on the categorization by D'Alba et al. (2021). We obtained the unitless variable aridity index (AI), which is defined as the ratio between precipitation and the ability of the atmosphere to remove water through evapotranspiration (Zomer et al. 2022) or each species centroid. Note that a higher AI indicates a more humid environment and low AI represents a higher aridity.

Phylogenetic analysis

We first evaluated the distributions of the data by plotting a histogram and examined the data to detect any correlated variables using a correlation matrix. Variables were scaled using the scale function of the programming software R (R Core Team 2013).

Using TimeTree of Life (http://www.timetree.org), we acquired the phylogenetic information of all reptiles in our dataset as described in detail by D'Alba et al (2021). To examine whether the variables exhibit a phylogenetic signal, we used the "phylosig" function in the Phytools package in R to calculate the Pagel's Lambda (Revell 2012; R Core Team 2013). We found a significant phylogenetic signal for all variables except average ground temperature (Supplementary Table S3). Accordingly, we use the R package "caper" to conduct a phylogenetic generalized least squares (PGLS) (Orme et al. 2018). Afterwards, we also tested for normal distribution and homogeneity of the residuals and calculated the phylogenetic signal for the residuals of our model ($\text{lambda} = 6.96\text{e-}05$, $p = 1$), indicating that our model adequately accounts for the phylogenetic structure in the data (Revell 2010).

To test our hypothesis on water sorption of eggshells we included shell type, calcium content, location of mucopolysaccharides, nest type, average ground temperature and the interactions between shell type and AI, and shell type and average ground temperature in the PGLS model as explaining variables (Table 1). Porosity and shell thickness were excluded from the model as they were separately significantly correlated with calcium content (P-value $= 0.0072$ and 0.0069, respectively), so we decided to only use calcium content in the model. Next, we used the "dredge" function (MuMln R package, Barton and Barton 2015) to determine which variables better explained the variation and ranked them using Akaike information criterion (AIC). Then, we selected the top models (delta AIC < 2, Burnham and Anderson 2004) and model averaged the parameter estimations and the 95% CI for each variable in the top models with the zero-averaging method (Grueber et al. 2011). This method identifies the multiple response variable with the strongest effect (Nakagawa and Freckleton 2011).

Fourier-transform infrared spectroscopy

To determine the chemical composition of both the inner and outer side of an eggshell, we recorded the infrared spectra using a FTIR spectrometer equipped with a universal attenuated total reflectance (ATR) attachment (diamond crystal) from Thermo Scientific. We recorded two spectra for each species (46 species in total): one from the inside of the egg and one of the outside (Supplementary Fig. S1) with a wavenumber range from 4000 cm^{-1} to 400 cm^{-1} (Supplementary Fig. S3). For each eggshell, we took 32 scans and applied a resolution of 4 cm^{-1}. Peaks around 3000 cm^{-1}, which we attribute to both collagen and keratin could be partly caused by the presence of –OH (water) in the sam-

Table 1 Model summary of relationship between water absorption of reptile eggshells (56 species) and both native environment and eggshell structure parameters

Variable	Estimate	Std. Error	95% CI	RI
(Intercept)	**0.96699**	**0.24231**	**(−1.442, −0.492)**	
Aridity index	−0.0528	0.09975	(−0.248, 0.143)	1
Eggshell type	**1.13449**	**0.29818**	**(0.550, 1.719)**	**1**
Mucopolysaccharide layer	**0.39922**	**0.15905**	**(0.0874, 0.711)**	**1**
Calcium	**0.29336**	**0.13742**	**(−0.563, −0.024)**	**1**
Aridity index: eggshell type	**0.28287**	**0.12937**	**(−0.536, −0.029)**	**1**
Average temperature	0.03486	0.06444	(−0.161, 0.091)	0.24
Nest type	0.06985	0.1717	(−0.267, 0.406)	0.22
Average temperature : Eggshell type	Not included in top set of models			0

Results from PGLS analysis. Variables in bold indicate statistical significance.

ples. To rule out the influence of water, all samples were stored in ethanol and dried at room temperature before the FTIR measurements were taken. Additionally, FTIR spectra of both pure collagen and keratin confirm that this peak can be attributed to collagen and keratin (Cardamone 2010; Riaz et al. 2018). To distinguish any grouping in the chemical composition of the eggshells, we performed a principal component analysis (PCA) on the FTIR curves to reduce the dimensionality of the data into two principal components summarizing the variation of the chemical composition of the eggshells. The first two principal components explain 76.57% of the total variance of all eggshells and we visualized it on a PC biplot.

Results

Water absorption—DVS

Overall, there is high variability in water vapor absorption among reptile eggshells. Eggshells from crocodiles, most turtles, and most gecko species absorb little moisture (<10% at 95RH), while squamates absorb much more (Fig. 2). According to IUPAC classification, the sorption profile of reptile eggshells shows a type III isotherm, which describes the adsorption on both macro-porous and non-porous adsorbents, where the mass increases exponentially (Sangwichien et al. 2002). Especially at the higher relative humidities, we observed a very steep slope.

Mucopolysaccharide content

Mucopolysaccharides were found in eggshells of all species but in varying locations. It was always detected whenever calcium was present, either diffusely throughout the eggshell (Fig. 3B) or together with calcium crystals (Fig. 4B). Interestingly, in some species, we also found mucopolysaccharides as a continuous superficial layer on top of the eggshell (Fig. 4A–C), even without the presence of calcium.

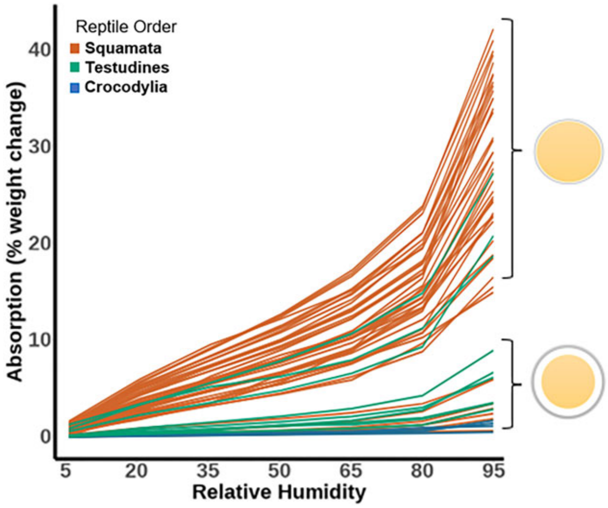

Fig. 2 Sorption profile at 5–95% relative humidity of reptile eggshells. Showing the absorption on the *Y*-axis and the relative humidity on the *X*-axis. Bottom group containing highly calcified eggshells containing sufficient albumen for the development of the embryo. Top group weakly calcified eggshells with generally lower amounts of albumen.

Influence of eggshell structure, composition, and native environment on water sorption

All variables except interaction effect between average ground temperature and eggshell type were present in the best set of models ($\Delta\text{AIC} \leq 2$, Table 1). We found that eggshell type (i.e., weakly vs strongly calcified) correlated significantly with water sorption of the eggshells. More specifically, the highly calcified eggshells of crocodiles, many turtles, and some geckos absorb less water vapor than the weakly calcified eggshells. In addition, calcium content strongly influences eggshell performance: as eggshell calcifica-

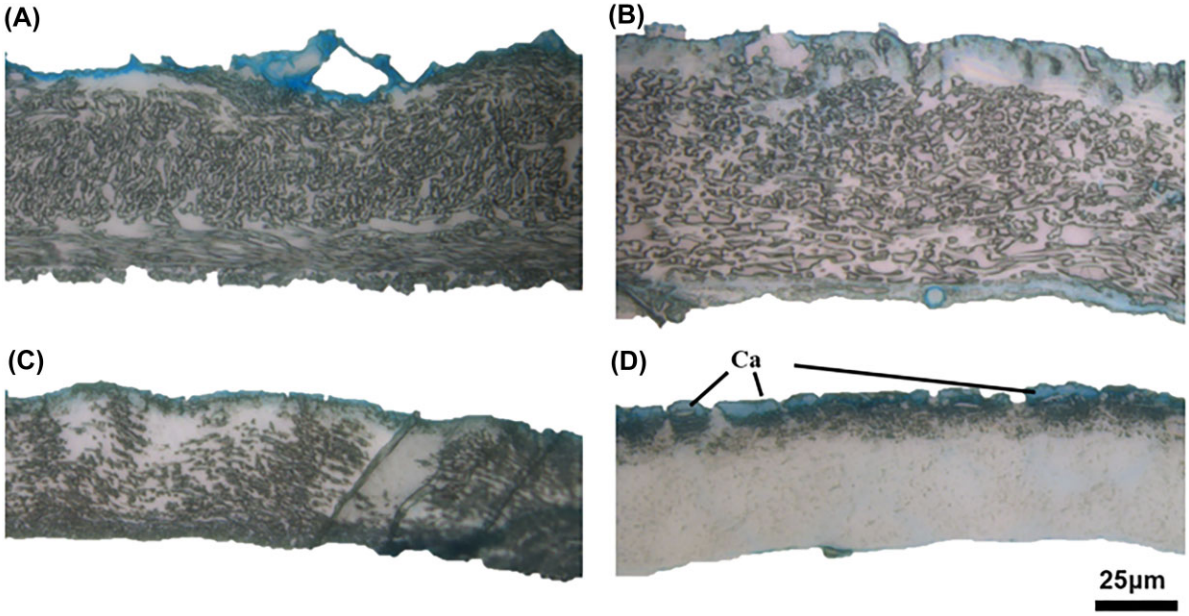

Fig. 3 Optical micrographs of reptile eggshells stained (Alcian blue) cross sections, showing the different ways mucopolysaccharides (blue stain) can be present. As a superficial layer in *Acanthocercus atricollis* **(A)** and *Coleonyx mitratus* **(C)**. Together with calcium, either diffuse throughout the eggshell in *Ovophis monticola* **(B)** or in calcium crystals e.g., *Eublepharis macularius* **(D)**. Ca indicates calcium crystals on the surface of the eggshells. Eggshells are oriented with their outer surface at the top.

tion increased, absorption of water vapor significantly decreased. The presence of a superficial layer of mucopolysaccharides on the outside of the eggshell significantly increased water sorption. Moreover, as predicted eggshells of species inhabiting more humid environments (high aridity index) absorb significantly less water when compared to species from arid environments, however, this was only true for those with weakly calcified eggshells. We did not find a significant relationship between the sorption properties of highly calcified eggshells and the aridity index. We found no effect of average ground temperature or nest type on water vapor sorption (Table 1).

Chemical composition of eggshells

Surface analysis of both in- and outside of reptile eggshells by ATR-FTIR revealed their chemical composition (Supplementary Figs. S1–S5) and complemented the staining data. FTIR spectra providing the chemical fingerprints for some representative reptile eggshells are shown in Fig. 4A. The peaks of keratin, collagen, and calcium carbonate, only calcite not aragonite, were confirmed by comparing them to pure material (Supplementary Fig. S7) and their peaks (including aragonite) were also compared with previously published data (Zhang et al. 2012; Rodríguez-Navarro et al. 2013; Chang and Chen 2016; Elejalde-Cadena et al. 2023).

A distinct absorption band centered around 3275 cm^{-1} is typically associated with –OH and $-NH_2$ from water and proteins. The broad bands around 2920 and 2850 are assigned to –CH stretching of proteins (i.e., keratin and collagen) and lipids. Two peaks around 1635 cm^{-1} and 1525 cm^{-1} are ascribed to the presence of the amide I and amide II bands, respectively. These peaks combined with the amide III band around 1300–1200 cm^{-1} and 1157 cm^{-1} from C–H bonds indicate the presence of keratin and collagen. The spectra of both keratin and collagen are very similar and, since the shell membrane consists of keratin and collagen, we predicted a mixture of both (Chang and Chen 2016). However, the peak around 1743 cm^{-1}, attributed to the stretching vibration of carbonyl ($C = O$) bonds (Tizo et al. 2018), is not seen in the FTIR of keratin but only in our measurements for collagen (Supplementary Fig. S7). This could be an indication of higher amounts of collagen in eggshells of some species. These peaks are visible in the Snouted cobra (*Naja anullifera*), Diamond python (*Morelia bredli*), Corn snake (*Pantherophis guttatus*, Fig. 5A), and two species of lizard's Common chuckwalla (*Sauromalus obesus*) and the Egyptian spiny lizard (*Uromastyx aegyptia*) (Supplementary Figs. S1–S4).

Additionally, in almost all species, we find a broad band from 1100 to 990 cm^{-1} that is associated with polysaccharides, together with a smaller peak around

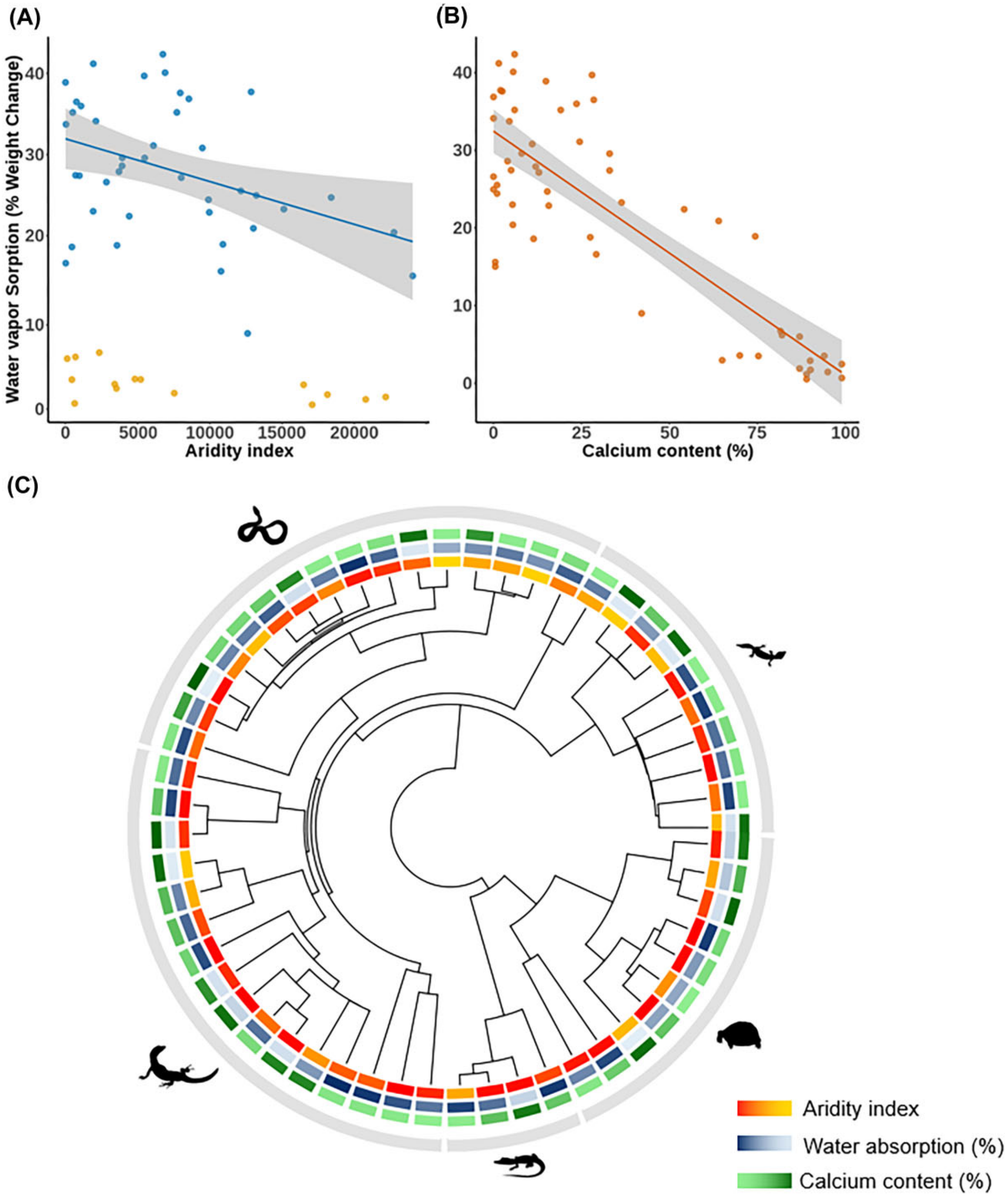

Fig. 4 Water absorption by eggshells in relation to environmental aridity and calcium content. **(A)** Negative significant relationship between the water vapor absorption (% mass change) and Aridity index (unitless) in the native environment of the eggshells for species with weakly calcified eggs (blue points), no relationship for species with strongly calcified eggs (yellow points). **(B)** Negative significant relationship between water absorption and calcium content of eggshell (for both weakly and highly calcified eggs together). **(C)** Phylogenetic tree of the species in the study with color coded bars for the variables aridity (most inner band: dark red to light yellow), water sorption (middle band: dark to light blue), and calcium content (outer band: light to dark green).

1240 cm^{-1} that can be ascribed to both amide III band and sulphate bonds, potentially indicating that the polysaccharides were somewhat sulphated. Such chemical compositions are found in (1) both the inner and outside of weakly calcified eggs of squamates species like the arboreal rat snake (*Gonyosoma oxycephalum*) and grass snake (*Natrix natrix*) and (2) the inside of highly calcified eggshells such as the Nile crocodile (*Crocodylus niloticus*) and the hawksbill turtle eggs (*Dermochelys coriacea*).

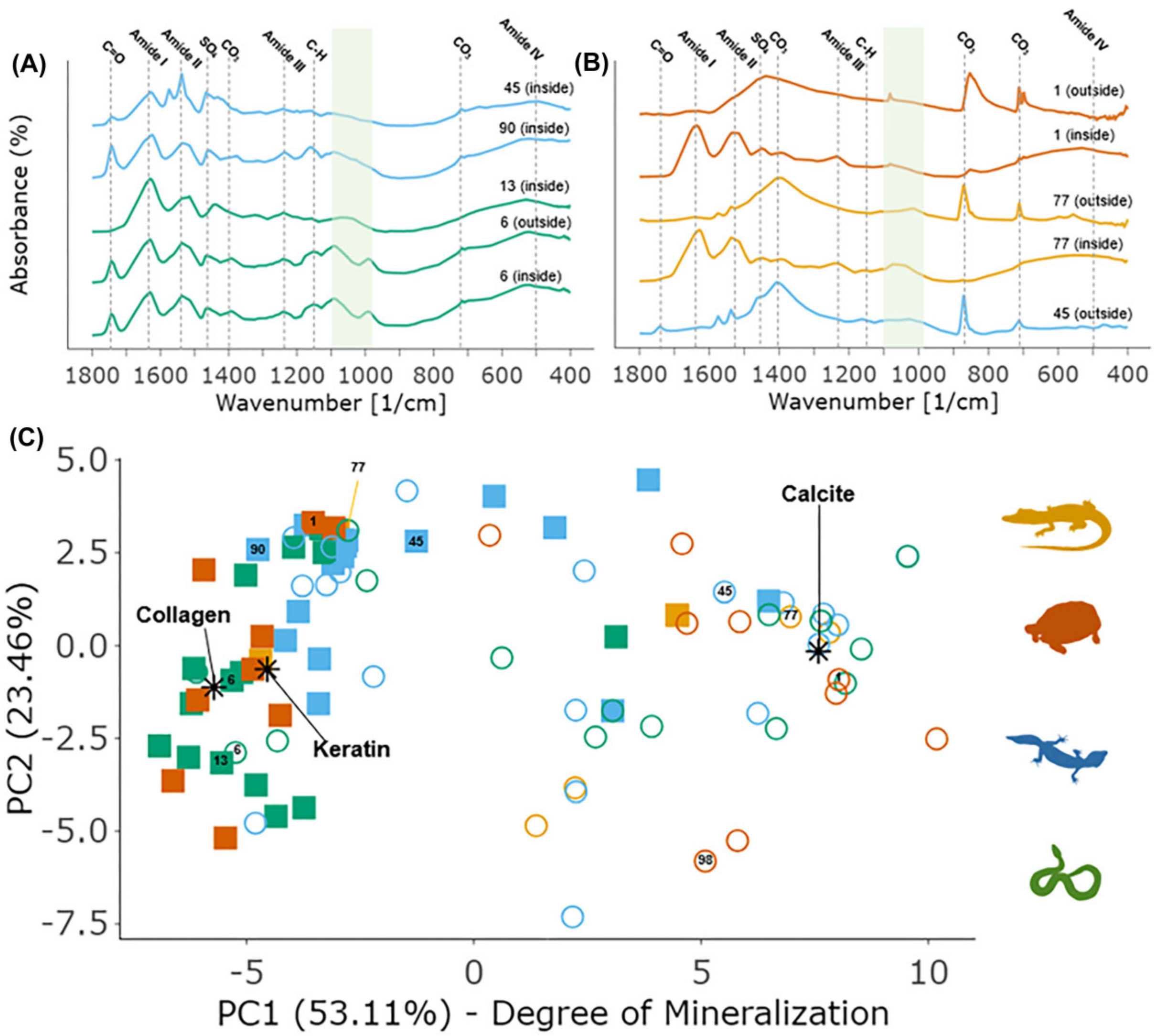

Fig. 5 **(A, B)** Representative FTIR spectra from the in- and outsides of reptile eggshells, number corresponds to collection number (Supplementary Table S1). Dotted line indicates the most important peaks and green band indicates polysaccharides. **(C)** Principal component biplot showing the first and second principal component of the FTIR curves from reptile eggshells and measurements from pure collagen, keratin, and calcite. Squares indicate measurements from the outside- and circles indicate measurements from the inside of an eggshell. Orange = testudines, blue = lizards, yellow = crocodylia, and green = snakes.

Another peak around 1000 cm^{-1} could also indicate the presence of a phosphate band, but this overlaps with the polysaccharide band, and thus we cannot be certain to which group it is linked. Simultaneous peaks associated with proteins (amides), polysaccharides, and sulphates also indicate the presence of glycoproteins, also seen in bird eggs and are the main organic components of the cuticle (Rodríguez-Navarro et al. 2013).

Some crocodiles in our dataset, such as the Nile crocodile (*Crocodylus niloticus*), Dwarf crocodile (*Osteolaemus tetraspis*), and the saltwater crocodile (*Crocodylus porosus*) showed additional phosphate bands around 687 and 557 cm^{-1} associated to amide IV band (Supplementary Fig. S5). Traces of phosphate are also seen in squamates such as the Western Mexico Whiptail (*Aspidoscelis costatus*), Common chuckwalla (*Sauromalus obesus*), and the grass snake (*Natrix natrix*) (Supplementary Figs. S1–S4). In turtles, we only found these peaks in the loggerhead turtle (*Caretta caretta*).

Signature peaks of calcium carbonate groups ($CaCO_3$) are observed on the outside for most reptile eggs in our dataset. These are from aragonite crystals in testudines, and from calcite for all squamates and crocodylia. Calcite peaks are visible in 1417, 874, and 713 cm^{-1}. Only in Testudines did we find calcium carbonate crystals in the form of aragonite (Fig. 5A, Supplementary Fig. S6). Typical aragonite peaks (1465, 1083, 854, 712, and 700 cm^{-1}) are seen in all turtles. None of the species displayed any Vaterite peaks (1087, 876, and 744 cm^{-1}, Fig. 5A).

Next, first and second principal components of the FTIR curves show two noticable clusters: the first containing mainly the spectra from the outside of an eggshell and the second measurements from the inside (Fig. 5B). This distinction in chemical composition is also observed in the microscopic images (Fig. 1). The outside of the eggshell most often has some degree of calcification, while the inner membrane mainly consists of keratin and collagen fibers.

In most species with highly calcified eggs such as crocodiles, turtles, and some squamates (mainly geckos), the outside surface of the eggs display very intense carbonate peaks, while polysaccharides- and keratin peaks are very weak and in some cases absent (Fig. 5A). The spectra resemble almost pure calcium (Rodríguez-Navarro et al. 2013). However, inside of the eggs, the carbonate peaks are weak and shows more intense peaks for keratin and polysaccharides (Supplementary Fig. S4).

However, in some squamates, the chemical composition of the inner and outer eggshell surfaces is similar, e.g., Snouted Cobra (*Naja annulifera*) and the European blind snake (*Xerotyphlops vermicularis*) incorporate little calcium in their eggshell (Supplementary Figs. S4 and S5).

Discussion

Our results support our hypothesis that selection to optimize water uptake for embryonic development has shaped eggshell structures and composition. First, from a mechanistic perspective, we found that eggshell permeability decreases with the level of mineralization. Reptile eggs have evolved two main strategies to minimize desiccation risk in arid or other less favourable environments (e.g., saltwater) or minimize asphyxiation risk in wet environments. The first is seen in highly calcified eggshells, e.g., Persian gecko (*Hemidactylus luqueorum*), Saltwater crocodile (*Crocodylus porosus*), and the red-footed tortoise (*Chelonoidis carbonarius*) that absorb almost no water vapor (<5%), and experience a net loss of 10.5–12.5% water during incubation (Thompson 1987) through embryonic development and an increase in water vapor pressure inside the egg. As in birds, the albumen is the main source of water for the embryo, and hence additional water absorption, and highly sorptive eggshells, are not needed (Rahn et al. 1979; Grigg 1987; Ar 1991). The second mechanism is seen in the weak shell calcification of most squamates and some turtles. These eggshells are permeable and allow water vapor to pass through the pores (i.e., air spaces) of the eggshell (Ackerman et al. 1985; Belinsky et al. 2004). While this is well documented already, we also found large variation in moisture absorption within these weakly calcified eggshells ranging from 15% in William's mud turtle (*Pelusios Williamsi*) and European blind snake (*Xerotyphlops* Vermicularis) up to 40% in species such as the green iguana (*Iguana Iguana*), desert monitor (*Varanus griseus*), and ball python (*Python regius*). Since these species lack or only have a small amount of albumen (Deeming and Ruta 2014; personal observation GD), they must obtain water from the environment (Tracy 1980; Adams et al. 2010).

From a more ultimate perspective, we found that within these partially calcified eggshells, species from arid environments absorb more moisture than those from humid environments, supporting the prediction that water vapor absorption is tuned to environmental conditions. Partial calcification of eggshells in dry environments, and the resulting increase in environmental water uptake, may therefore be a result of selection on desiccation resistance. This is especially true for reptile species that lack or have minimal albumen (Tracy 1980; Thompson 1987; Deeming and Ruta 2014). Adding albumen could be costly for the female, particularly in arid environments, so eggs that can obtain water from the environment could be a critical adaptation. The disconnect between water vapor absorption and environmental aridity in highly calcified eggshells is not surprising as these species do not need to absorb water from the environment.

What causes the observed variation in water absorption? We predicted that eggshell structure and composition, i.e., the incorporation of mucopolysaccharides, and different quantities of calcium could ensure that the embryo receives an adequate amount of water during development.

In line with this prediction, and with previous research (Tracy 1980; Packard et al. 1982; Grigg 1987; Palmer and Guillette 1991; Belinsky et al. 2004; Andrews 2015), we found that weakly calcified eggshells are up to 10× more absorptive than strongly calcified ones. More specifically, and unique to this study we show that even within the category of weakly calcified eggshells (Grigg 1987; Andrews 2015), small increases of eggshell mineralization result in decreased water uptake by the eggshell itself.

Moreover, mucopolysaccharides also shape the sorption properties of eggshells. These were typically found diffusely throughout the eggshell, together with calcium, or as a distinct layer on top of the eggshell (Fig. 3). Such a superficial layer increases the water vapor sorption by effectively binding water molecules and helps to retain the absorbed water. This is also the case in bivalves, echinoderms, and amphibian species (Wotton 2004). Their presence in all eggshells illustrates their significance, but also carries risks. Since microbes easily digest sugars, a thin layer of them on top of the egg could promote bacterial growth and infection. Further, enhanced absorption may also enhance desiccation by extracting water from inside the egg: depending on the water vapor pressure difference the eggshell can absorb water from- or lose water to the environment (Ackerman et al. 1985; Snell and Tracy 1985). In birds, mucopolysaccharides play an important role in eggshell mineralization (Hincke et al. 2012), but only a few studies have considered the role of mucopolysaccharides in reptile eggs (Simkiss and Tyler 1959; Campos-Casal et al. 2020). Even though it has been proposed that they have a similar biomineralization function, they can also play a role in desiccation resistance.

Even though nest type affects environmental exposure of eggs, and thus water loss, it was not correlated with eggshell absorbance. However, our climate variables were extracted from species occurrence centroids, and the aridity and temperature surrounding nests (microclimate) may be different. Such precise data are beyond the scope of this study but holds a great potential for further studies. Additonally, most of the highly calcified eggs, which absorb very little water vapor, are either buried (e.g., crocodiles or many turtles) or glued on a substrate (many geckos) (D'Alba et al. 2021) and thus, a detailed examination of their microclimate would be more informative than broad scale climatic variables.

PCA of the FTIR curves enabled us to distinguish broad patterns in the chemical composition of reptile eggshells. It further confirms that for most reptiles, the outside has a different chemical composition than the inside (Packard and Hirsch 1986; Schleich and Kästle 1988; Packard and DeMarco 1991). While the outside has a higher content of calcium, mostly in the form of carbonate, the inside is richer in proteins, mucopolysaccharides, and phosphates. Optical microscopy and SEM images clearly shows the typical reptile eggshell has a multilayered structure, in which the outer layer is more calcified, with variable amounts of calcium depending on the species (Fig. 1A–C; Kusuda et al. 2013; D'Alba et al. 2021). By contrast, the inner layers consist of multiple layers of protein fibers. Previous research has shown by staining that these fibers mainly consist of keratin and/or collagen (Chang and Chen 2016; Álvarez et al. 2022). The FTIR spectra showed that some species potentially had high density of lipids in their eggshells (e.g., Snouted cobra, Egyptian spiny lizard, Fig. 5A). Lipids can be present in the cuticle in different relative proportions and could be a form of protection against penetration of bacteria (Samiullah and Roberts 2014; D'Alba et al. 2017; Kulshreshtha et al. 2022), or indicating a higher content of collagen as both collagen and lipids have similar peaks around 2920 and 2850 cm^{-1}. It should be noted that the FTIR spectra look very similar to that of pure collagen, however it could also be a collagen-like protein (Rabinowitz and Shapiro 1972; Riaz et al. 2018).

Interestingly, on visual inspection, it seems that some lizards are more closely clustered together compared to snakes, showing that the chemical compositions of lizard species are more similar to each other than to snakes (Fig. 5C). It is difficult to interpret these just using FTIR spectra and future research could determine the potentially subtler differences in chemical composition between lizard and snake eggs, these differences may influence eggshell functioning. For example, eggs might benefit from a higher proportion of collagen to increase flexibility of the eggs. Since eggs can often be very elongated (D'Alba et al. 2021), having a more flexible egg is an advantage since eggs that are elongated and highly calcified could be more prone to breakage when stress is applied along the short axis. Incorporating extreme amounts of calcium could also be costly for the female (Deeming 2018). However, future research on tensile strength and puncture tests in reptile eggs could improve our understanding of the mechanics of eggs with a high content of collagen.

In species with strongly calcified eggshells, we found the typical distinct difference between the chemical composition of the inner- and outside of their eggshell (Supplementary Fig. S5) (Packard and Hirsch 1986; Schleich and Kästle 1988; Packard and DeMarco 1991). The outer eggshell showed to be highly calcified, while the inner membrane is proteinaceous. Like previous research, when calcium carbonate was present, it was in the form of calcite for most squamates, and only in turtles was it in the form of aragonite (Silyn-Roberts and Sharp 1985; Al-Bahry et al. 2011). The presence of vaterite is described in some squamates such as the Chinese cobra (*Naja atra*) (Zheng et al. 2014), but we did not detect vaterite in any of our species. The outer layer of most turtles such as the loggerhead turtle egg (*Caretta caretta*) and red-footed tortoise (*Chelonoides* carbonaria) resembled a pure aragonite spectrum (Baird and Solomon 1979; Silyn-Roberts and Sharp 1985). For the pancake turtle (*Malacochersus tornieri*), the outer surface also contained proteins. Similarly, to snake eggs, the incorporation of proteins such as collagen could increase the flexibility of the egg, since spherical eggs like turtles are known to be more fracture resistant, whereas elongated eggs have a higher tensile strength (Chang and Chen 2016). Additionally, species such as the saltwater crocodile (*Crocodylus porosus*) and common agama (*Agama agama*) showed especially high peaks of phosphate. Interesting is that some species show an additional peak at 600 cm^{-1} on the outside of their eggshell, these peaks could be an indication of crystalline apatitic phosphate in the cuticle or calcium phosphate as this has been documented in the eggshell of several reptiles (Schleich and Kästle 1988; Campos-Casal et al. 2020). Further testing will enable us to identify the effects of these varying chemistries on material properties of the eggs.

Here, we show that reptile eggshell chemistry and morphology are likely co-adapted at least in part to their hydric environment. Increased knowledge of structure–function relationships not only promote the development of theoretical bases for surfaces with special wettability, but also provide important strategies and design principles with the aim of mimicking natural structures and functionalities (Kong et al. 2019).

Conclusion

We found that reptiles eggshells absorb more water when they are gradually less calcified, and have a superficial layer of mucopolysaccharides. This was mostly seen in species native to arid environments. It could be a critical adaptation as females do not have to provide sufficient water to the embryo for developments such as seen in the highly calcified eggs of crocodiles, most turtles, and most geckos. Instead, weakly calcified eggshells can absorb water from their environment and transport this inside the egg for the embryo. This could be advantageous, especially in arid environments, where water is scarce. Future studies should, however, explore the potential cost associated with highly absorptive eggshells, including trade-offs with increased microbial infection.

Author contributions

G.D., K.D.C., and L.D.A. conceived the study; G.D., J.G., E.S., M.W., and W.X. refined methodology and collected the data; G.D. and M.P.J.N. performed statistical analyses; G.D. and L.D.A. wrote the manuscript draft; M.D.S., K.D.C., and L.D.A. provided advice and acquired funding. All authors contributed to editing the final paper.

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Supplementary data

Supplementary data available at *ICB* online.

Conflict of interest

The authors declare no conflicts of interest.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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