Received: 4 June 2017 | Revised: 6 September 2017 | Accepted: 15 September 2017
DOI: 10.1002/jmor.20756

RESEARCH ARTICLE

WILEY JOURNAL OF morphology

The rhinoceros among Serpents: Comparative anatomy and experimental biophysics of Calabar burrowing python (*Calabaria reinhardtii*) skin

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Funding information
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Abstract

The Calabar burrowing python (*Calabaria reinhardtii*) has a unique combination of marked thickness of the integumentary layers, a highly organized lamellate arrangement of the dermal collagen bundles, and a reduction in the size of the interscale hinge region of the integument. Biomechanical testing demonstrates that the skin of *C. reinhardtii* is more resistant to penetration than the skin of other snakes. The laminar arrangement of the collagen bundles provides for penetrative resistance, even while maintaining the flexibility characteristic of snake skin. Considering the life history of this species, it is hypothesized that the specialized integument of *C. reinhardtii* is a passive defensive mechanism against penetrative bites from maternal rodents and predators.

KEYWORDS
collagen, defense, dermis, penetration

1 | INTRODUCTION

Snakes, like all other vertebrates, have a skin consisting of an outer, ectodermally derived, epidermis, and an inner, mesodermally derived, dermis (Chernova, 2009; Maderson, 1964). One of the characteristic features of a squamate reptile, the monophyletic group which includes snakes and lizards (Wiens et al., 2012), is the presence of thickened ectodermal scales (Chang et al., 2009). In terms of their material composition and basic histological properties there is little variation among snake scales either interspecifically or between different regions of the same snake (Baden & Maderson, 1970; Klein & Gorb, 2012), although, there are marked differences between the scales and the interscale, or hinge, regions (Abo-Eleneen & Allam, 2011).

The structural diversity of snake scales is most evident at the finer (electron microscopic) level, where patterns of pits, projections, and ridges collectively termed microdermatoglyphs have been described (Price, 1982; Price & Kelly, 1989). Microdermatoglyphic features have been used as taxonomic characters (Beyerlein, 1998; Stille, 1987) but they seem to have greater utility as indicators of frictional interactions during locomotion (Berthé, Westhoff, Bleckmann, & Gorb, 2009; Gower, 2003).

Although much is known about variation of the scale epidermis, less is known about morphological variation of the dermis in snakes; however, the dermal thickness and collagen fiber organization do vary between species (Jayne, 1988; Pockrandt, 1937). Lillywhite (2014) noted in passing that the strength of the connection between the dermis and underlying tissues seems to vary between species, so some species are much easier to skin than others. Perhaps the best known dermal specialization in snakes occurs in some asiatic natricines which sequester prey-obtained steroids within specialized dermal nuchal glands (Mori et al., 2012; Smith, 1938).

Previous functional analyses of snake skin have centered on the cytology of skin shedding (Alibardi & Toni, 2006; Maderson, 1965), and cutaneous fluid balance (Lillywhite & Maderson, 1982; Tu, Lillywhite, Menon, & Menon, 2002). Less attention has been paid to the mechanical properties of snake skin. Differences in tensile properties between prepyloric and postpyloric skin have been attributed to shared characteristics in Macrostomata (Rivera, Savitzky, & Hinkley, 2005). Jayne (1988) showed that the mechanical properties of snake skin varied between different species and between different longitudinal and circumferential locations within an individual. Deformation associated with cyclic stress/strain cycles (or ratchetting) in snake skin (Zhu & Kang, 2015), distension of lower jaw skin (Close & Cundall, 2014), and mechanics of locomotion (Hazel, Stone, Grace, & Tsukruk, 1999; Klein, Deuschle, & Gorb, 2010) have been explored with varying emphases on the biomechanical properties of the skin.

The vertebrate integument can protect the organism from physical threats. Species lacking bony spicules or osteoderms rely on the spatial arrangement of the integumentary components, particularly the collagenous bundles of the dermis, to increase the strength of the skin (Bruet, Song, Boyce, & Ortiz, 2008; Yang et al., 2013). For example, the white rhinoceros (*Ceratotherium simum*) is protected against penetration from the horns of conspecifics by a proliferation of dermal collagen bundles arranged in organized, overlapping, layers (Shadwick, Russell, & Lauff, 1992). A counter-example exists within gekkonid lizards where the integument is "fragile" and specialized to break off upon contact (Bauer, Russell, & Shadwick, 1989).

The Calabar burrowing python (*Calabaria reinhardtii*) is a semi-fossorial inhabitant of the leaf litter/rainforest floor in equatorial Africa (Angelici, Luiselli, Mercy, Effah, & Luiselli, 2000; Luiselli & Akani, 1998). Despite the popular name "burrowing python," repeated morphological and molecular analyses have demonstrated that *Calabaria* is, in fact, a boid, closely related to Erycinae radiation of ground or sand boas (Kluge, 1993; Noonan & Chippindale, 2006). *Calabaria* raids rodent burrows to preferentially forage for newborn and juvenile rodents; in doing so, it frequently suffers defensive bites from the parental rodents (Lehmann, 1971; Luiselli & Akani, 1998; Luiselli et al., 2002). When attacked or threatened, rather than fleeing, *C. reinhardtii* relies on passive defensive behaviors including coiling, hiding its head, and elevating its tail (which is more brightly colored than the rest of the body) in a posture that has been interpreted as cephalic mimicry (Greene, 1973; Lehmann, 1971). This defensive repertoire would seem to increase the likelihood of a damaging rodent bite (Lehmann, 1971). The present study was undertaken to explore how the integument of *C. reinhardtii* was able to withstand these penetrative bites. To do this we: (1) compare the histological structure of the integument of *C. reinhardtii* with a sample of snakes chosen for their phylogenetic, ecological, and behavioral similarities; and (2) document the comparative biomechanical properties of the integument, to test for functional consequences of the observed morphology.

2 | MATERIAL AND METHODS

2.1 | Morphology

Three adult (SVLs of 69, 77, and 87 cm) *Calabaria reinhardtii* (Schlegel, 1851) were obtained commercially. The snakes were euthanized (A.T. Still University IACUC Protocol # 175) and four skin samples extracted from each specimen: (1) dorsal midline within 5% of SVL from the caudal tip of the quadrate; (2) lateral surface within 5% of SVL from the caudal tip of the quadrate; (3) lateral surface at midbody; and (4) dorsum of the tail distal to halfway between the cloaca and the tail tip. Midbody was exactly 50% of SVL of the snake. Sample sites were chosen to allow for comparison along the cranial-caudal length of the body, to reflect the higher incidence of attacks on the dorsal surface of the head and tail in snakes (e.g., Wüster et al., 2004), and the importance of the lateral body surface when moving through burrows (e.g., Marvi & Hu, 2012). Each skin sample was approximately 1 cm^2 and included multiple complete scales and portions of the underlying skeletal muscle. Samples were not used if gross examination revealed scars, infection, or any other abnormality. The samples were fixed in 10% neutral-buffered formalin for 72 hr at 4°C. Postfixation the samples were dehydrated through an ethanol series, cleared, then embedded in paraffin; all histological procedures were standardized for all of the skin samples examined. Serial sections were cut (in all three planes) at 10 μm. Mounted sections were stained with Van Gieson's trichrome or Vehoeff's elastin stain (staining protocols followed Luna, 1968).

Two additional skin samples from the above regions were used for scanning electron microscopy to visualize the arrangement of scalation and collagen fibers in the cross-section area. Samples were fixed as described above, dehydrated through absolute alcohol, then, critical point dried in liquid carbon dioxide. Once fully dried the specimens were mounted and sputter-coated with palladium, then viewed with a JSM-6100 SEM (JEOL USA Inc, Peabody, MA).

2.2 | Morphological comparisons

For this study, we compared the qualitative and quantitative features of the skin of *C. reinhardtii* to four different comparison groups: (1) a phylogenetic group made up of Boidae, and particularly Erycinae, species; (2) an ecological group composed of semi-fossorial species; (3) species that perform cephalic mimicry; and (4) random species not belonging to the above groups.

For the phylogenetic samples, we examined single specimens of the boids *Acrantophis dumerilli* (SVL = 65 cm; Jan, 1860) and *Candoia aspera* (SVL = 58 cm; Gunther, 1877); from the boid subfamily Erycinae we examined one specimen of *Charina bottae* (SVL = 34 cm; Blainville, 1835), three specimens of *Eryx colubrinus* (SVLs of 68, 68, and 70 cm; Linneaus, 1758), and three specimens of *Lichanura trivirgata* (SVLs of 54, 54, and 67 cm; Cope, 1861).

The ecological comparative group consisted of species that move through burrows or that burrow through substrate. This group included single specimens of *Acrantophis dumerilli*, *Cemophora coccinae* (SVL = 38 cm; Jan, 1863), *Charina bottae* and *Psammophis schokari* (SVL = 73 cm; Forskal, 1775), as well as three specimens each of *Eryx colubrinus*, *Lichanura trivirgata*, and *Xenopeltis unicolor* (SVLs of 60, 90, and 90 cm; Reinwardt, 1827).

Sampled species that perform cephalic mimicry include single specimens of *Calamaria septentrionalis* (SVL = 45 cm; Boulenger, 1890), *Charina bottae*, and three specimens of *Xenopeltis unicolor*. Random species not belonging to the above groups included single specimens of *Farancia abacura* (SVL = 83 cm; Holbrook, 1836), *Lytorhynchus diadema* (SVL = 61 cm; Duméril, Bibron, and Duméril, 1854), and *Thamnophis sirtalis* (SVL = 55 cm; Linnaeus, 1758), as well as three specimens of *Crotalus atrox* (SVLs of 82, 86, and 92 cm; Baird & Girard, 1853). The specimen of *Calamaria septentrionalis* was obtained from the Royal Ontario Museum (ROM 35602); all other specimens were obtained live from commercial dealers. Specimens were euthanized, skin samples were removed from the neck and midbody and fixed loose in petri dishes (to avoid any stretching), processed histologically (as described above), and quantified as described below.

2.3 | Morphological quantification

Representative sections of the integument from each region of every species were photographed using a DM 4000B microscope (Leica Microsystems Inc., Buffalo Grove, IL). The digital images were then imported into ImageJ (NIH) and the following features quantified (Figure 1): (1) thickness of the epidermis at midscale; (2) thickness of the superficial dermis at midscale; (3) thickness of the deep dermis at midscale; (4) cranial-caudal length of the hinge at midscale; and (5) cranial-caudal length of the scale (incomplete in Figure 1).

Statistical comparisons were made using only the five species for which three specimens each had been sampled with SPSS V.20 (IBM Corp, Armonk, NY). One-way ANOVA tests were run separately for thickness of epidermis, superficial dermis and loose dermis, and α was adjusted with Bonferroni correction. There was significant difference in body size among the main comparison species [one-way ANOVA; $F(4,10) = 3.9$, $p = 0.037$]; however, Tukey's post hoc analysis demonstrated that none of the comparison species had significantly different SVLs than *C. reinhardtii* (the only significant difference was between *C. atrox* and *L. trivirgata*).

The hinge region between scales is normally defined as the portion of the integument covered by a single layer of thin epidermis, including the portion that "doubles-back" folding under the scale (Lillywhite, 2014). This region, which can be exposed by manually stretching the snake's skin, includes elastin fibers and is generally interpreted as providing flexibility to the snake. The relative degree to which the hinge region extends deep to the scale can be determined by what is herein defined as the support index which is the fraction of the scale that is not located superficial to a hinge. The lower the value for the support index, the greater the flexibility of the scale.

Complete (dorsal-ventral) serial sections were digitally photographed, then 3D reconstructions were performed using the BioVis3D software system (BioVis3D, Montevideo, Uruguay).

2.4 | Penetration experiments

Three adult specimens each of *C. rheinhardtii*, *L. trivirgata*, *E. colubrinus*, *X. unicolor*, and *C. atrox* were obtained commercially. All snakes were euthanized through cardiac excision once fully anesthetized using isoflurane. The housing, care, and use of these live snakes followed all applicable national guidelines and was approved by the Institutional Animal Care and Use Committee of A.T. Still University (A.T. Still University IACUC Protocol # 175). From each specimen, two skin samples (each 1 cm^2) were excised from three body regions: (1) dorsal midline within 5% of SVL from the caudal tip of the quadrate; (2) lateral surface within 5% of SVL from the caudal tip of the quadrate; (3) lateral surface at midbody. Midbody was exactly 50% of SVL of the snake. These are the same three regions that were surveyed histologically. No samples were used if gross examination revealed scars, infection, or any other abnormality. The skin samples were excised with care being taken to avoid any tears or cuts in the skin itself.

The skin samples were placed on a vertical metal plate. A force transducer (FT03, GRASS Instruments) was coupled to a mechanical infusion pump (model 901, Harvard Apparatus). The force transducer was coupled to a strain gage amplifier (P122 GRASS Instruments) the output of which was sampled (at 10 kHz) using MiDas (Xcitex Inc., Woburn, MA) data acquisition software. A hypodermic needle was attached to the force transducer, and the syringe pump used to drive the needle against the middle of a scale while the load associated with penetration was recorded. Two needles were used separately, one sharp and one blunt, to simulate different kinds of potential physical stress the snake may endure in the wild. A fresh sharp 18 gauge needle was used for each trial, the same mechanically blunted (18 gauge) needle was used for each trial. Force (*F*) was measured via the force transducer, and displacement (*L*) was quantified by multiplying the velocity and time during compression. A linear curve is typical of all datasets, and its slope (*K*) was quantified with linear regression. *K* was compared using mixed ANOVA, with different regions of the body as within-subject factor and species as between-subject factor, and Tukey's post hoc analysis was performed. Bonferroni correction was applied to α as two separate tests were conducted on sharp and blunt needles. The statistical analysis was performed in SPSS V.20 (IBM Corp, Armonk, NY).

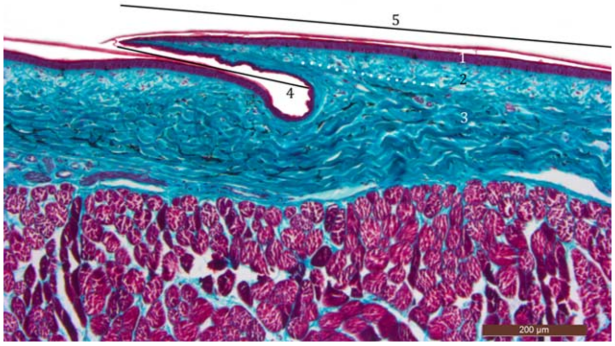

FIGURE 1 Histological section through the integument of *Charina bottae* illustrating the measurements taken as part of this study: (1) thickness of the epidermis; (2) thickness of the superficial dermis; (3) thickness of the deep dermis; (4) cranial-caudal length of the hinge; and (5) cranial-caudal length of the scale (incomplete in this image). A white dashed line marks the boundary between the superficial and deep dermis

3 | RESULTS

3.1 | Morphology

Regardless of species or where the samples were collected on the snake's body, all of the skin samples exhibited the same three basic layers including a superficial epidermis, a middle layer of (generally "loose") dermis, and a deep dermal layer characterized by denser collagen bundles. Each layer appeared to be composed of the same basic tissue types. For example, in all of the species the deep dermis consists predominantly of type I collagen organized in distinct bundles. Despite these general similarities, there were quantitative and qualitative differences among the species.

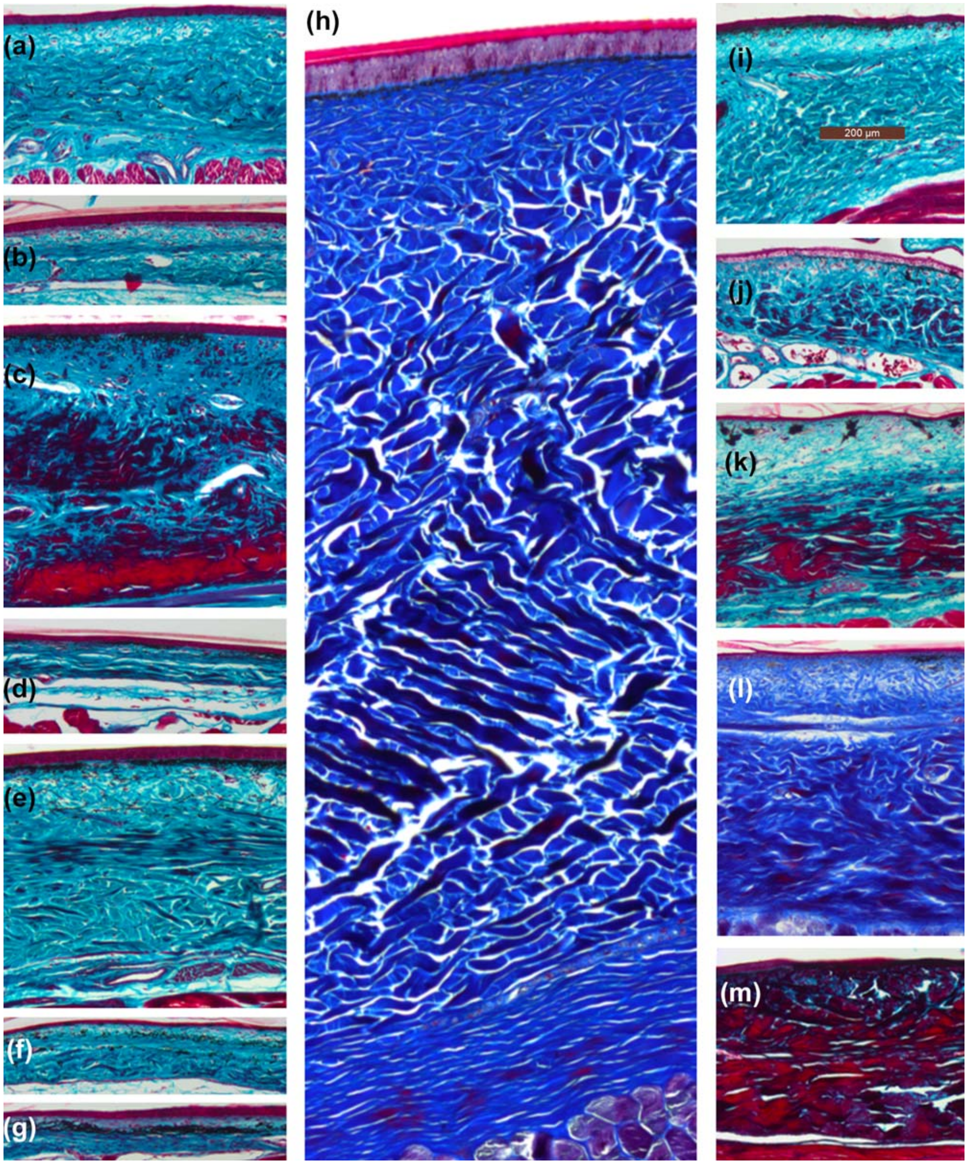

FIGURE 2 Interspecific variation in the relative thickness of the component layers of the snake integument; all images are from the lateral surface of the snake's midbody and all are shown at the same scale [scale bar in (i) is 200 μm]. (a) *Charina bottae*; (b) *Cemophora coccinea*; (c) *Calamaria septentrionalis*; (d) *Lytorhynchus diadema*; (e) *Farancia abacura*; (f) *Psammophis schokari*; (g) *Thamnophis sirtalis*; (h) *Calabaria reinhardtii*; (i) *Acrantophis dumerilli*; (j) *Candoia aspera*; (k) *Crotalus atrox*; (l) *Eryx colubrinus*; (m) *Xenopeltis unicolor*

The integument of *Calabaria reinhardtii* was thicker than that of any other species examined (Figure 2). This held true for all of the integumentary sampling sites and includes the epidermis, superficial dermis and deep dermis (Table 1). The epidermis of *C. reinhardtii* was 76 μm thick, which was 4.4 times greater than the mean value of all species examined (17.4 μm, Table 1). There were significant differences in thickness of epidermis among the five main study species [one-way ANOVA; $F(4,10) = 5{,}572.077$, $p = 9.32 \times 10^{-12}$]. Tukey's post hoc analysis demonstrated that the epidermis of *C. reinhardtii* was significantly thicker than those of the four primary comparison species (*Crotalus atrox*, *Eryx colubrinus*, *Lichanura trivirgata*, and *Xenopeltis unicolor*). The epidermis of *E. colubrinus* was significantly thicker than that *C. atrox*, *L. trivirgata*, and *X. unicolor*. The epidermis of *L. trivirgata* was significantly thicker than *C. atrox*; the epidermal thickness of *X. unicolor* was not significantly different from the above two species, lying somewhere in the middle. Although not tested statistically, the epidermal thickness of *C. reinhardtii* far exceeds those of the species represented by a single specimen (Table 1).

The superficial dermal layer of *C. reinhardtii* was 142.8 μm thick, which was 3.2 times thicker than the mean value of all species examined (45.3 μm, Table 1). There were significant differences in the thickness among the superficial dermis of the five main study species [one-way ANOVA; $F(4,10) = 548.74$, $p = 1.15 \times 10^{-11}$). Tukey's post hoc analysis demonstrated that the superficial dermis of *C. reinhardtii* was significantly thicker than those of *C. atrox*, *E. colubrinus*, *L. trivirgata*, and *X. unicolor*. The superficial dermis of *E. colubrinus* was significantly thicker than those of *C. atrox*, *L. trivirgata*, and *X. unicolor*. The superficial dermis of *C. atrox* and *L. trivirgata* was significantly thicker than

TABLE 1 Interspecific variation in the thickness of the integumentary layers of the species examined

	Epidermis	Superficial dermis	Deep dermis
Acrantophis	18.6	70.0	366.8
***Calabaria* (3)**	**76.0, 3.5**	**142.8, 8.1**	**1,898.9, 88.5**
Calamaria	12.7	8.9	86.0
Candoia	17.8	9.4	138.8
Cemophora	21.6	10.3	187.3
Charina	16.9	39.3	196.6
Crotalus (3)	10.9, 1.0	84.5, 0.7	287.2, 4.2
Eryx (3)	23.7, 2.3	123.3, 2.6	650.1, 6.5
Farancia	26.2	82.9	379.3
Lichanura (3)	16.9, 0.9	85.9, 1.8	603.2, 10.6
Lytorhynchus	13.2	6.3	63.0
Psammophis	10.1	53.1	79.0
Thamnophis	24.5	9.4	70.9
Xenopeltis (3)	12.7, 0.7	5.2, 0.5	185.9, 5.8
Mean	17.4	45.3	253.4

All measurements are in microns. For species in which three specimens were examined, the reported values are mean, standard deviation. The mean values listed in the bottom row exclude *Calabaria reinhardtii*.

that of *X. unicolor*, but not significantly different from each other. Although not tested statistically, the thickness of superficial dermis of *C. reinhardtii* far exceeds those of the species represented by a single specimen (Table 1).

The clearest quantitative difference in the integument was found in the thickness of the deep dermis. The deep dermis of *C. reinhardtii* was approximately 7.5 times thicker than the mean value for all the species examined, and was over 30 times thicker than the deep dermis of *Lytorhynchus diadema* (Table 1). There were significant differences in thickness among the deep dermis of the five main study species [one-way ANOVA; $F(4,10) = 877.5$, $p = 1.11 \times 10^{-12}$]. Tukey's post hoc analysis demonstrated that the deep dermis of *C. reinhardtii* was significantly thicker than those of all of the other main study species. The deep dermis of *E. colubrinus* and *L. trivirgata* was thicker than those of *C. atrox* and *X. unicolor*, but not significantly different from each other. The thickness of deep dermis of *C. atrox* and *X. unicolor* was not significantly different from each other. The other species examined (Table 1) all had deep dermal thicknesses that were less than what was found in *C. reinhardtii*.

In addition to the quantitative differences, there was a qualitative difference in the deep dermis of *C. reinhardtii* relative to the other species examined. In most of the species examined the collagen bundles in the deep dermis were more randomly arranged, with only a suggestion of an organized laminar arrangement (Figure 2). In *Eryx colubrinus* and *Lichanura trivirgata* the deep dermis was more laminar (Figure 2) but still exhibited large amounts of randomly oriented collagen bundles. In *C. reinhardtii* the majority of the deep dermis was organized into alternating layers of collagen bundles (Figure 2).

Scanning electron microscopy (Figure 3a,b) and three-dimensional reconstruction (Figure 3c) of the collagen bundles within the deep dermis of *C. reinhardtii* reveal that the alternating layers are oriented nearly perpendicular to the adjacent layers. The alternating layers were both oriented approximately 45 degrees to the long (cranial-caudal) axis of the snake's body; so, in one layer the collagen bundles are directed

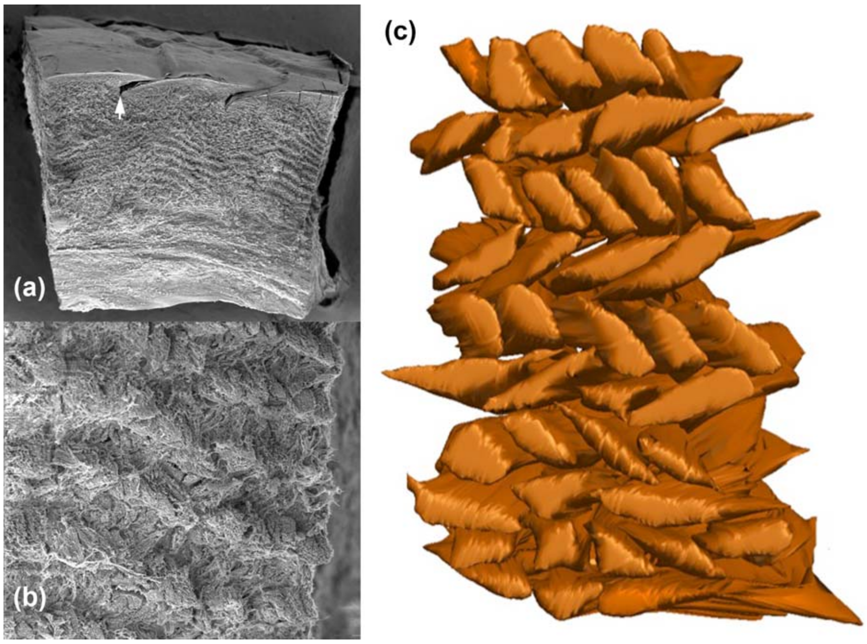

FIGURE 3 The laminar arrangement of the deep dermis of *Calabaria reinhardtii*. (a) Scanning electron micrograph (20x) showing the scales and hinge region (vertical arrow) and the lamellar structure of the deep dermis. (b) scanning electron micrograph (100 x) showing the alternating layers of collagen bundles in the deep dermis. (c) 3D reconstruction of collagen bundles from sequential histological sections

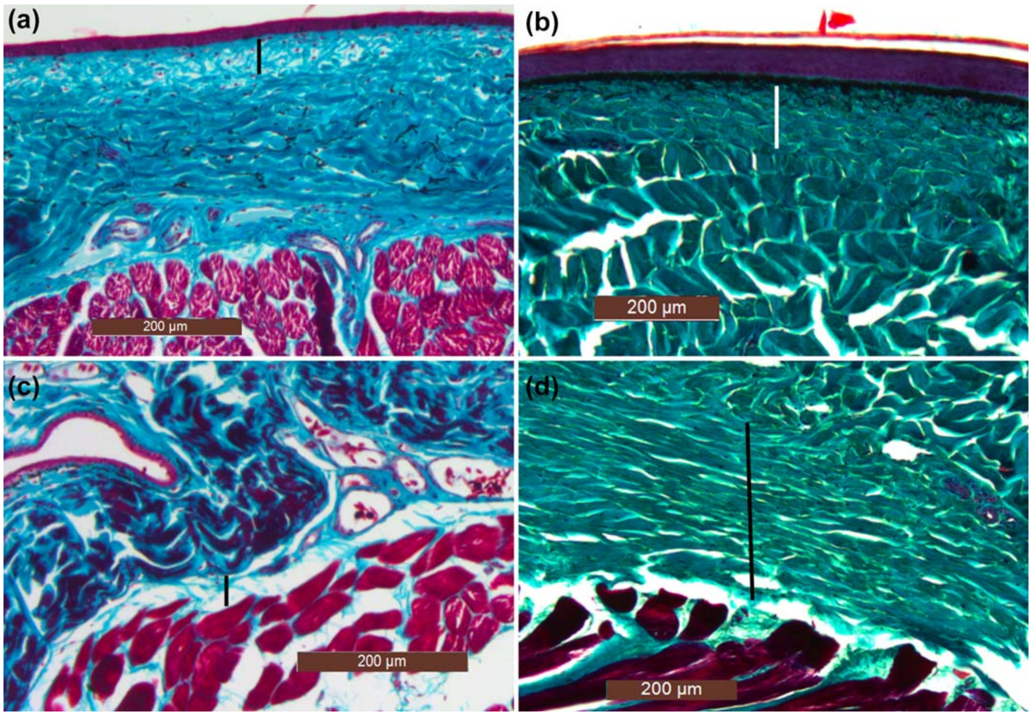

FIGURE 4 Interspecific variation in the histological structures of the boundaries of the deep dermis, all illustrated with tissue from the dorsal surface of the neck region. The superficial boundary (a & b; vertical line) ranged from a distinct zone of lower density collagen bundles with a varied orientation as in *Charina bottae* (a) to a dense array of collagen bundles which integrated into, but were distinct from, the deeper dermis as in *Calabaria reinhardtii* (b). The lower boundary (C&D) ranged from a small amount of loose connective tissue integrating with the epimysium as in *Candoia aspera* (c) to a thick group of well-organized collagen bundles that integrated with the epimysium and anchored to the vertebral processes as in *Calabaria reinhardtii* (d)

caudo-ventrally, while in the adjacent layers they are directed caudodorsally. The collagen bundles laminar arrangement did not appear to vary over the depth of the dermis, nor was there any obvious difference between reconstructions on the neck and midbody of the *C. reinhardtii*.

The boundaries of the deep dermis are different in *Calabaria* than in any of the other species examined. There is a clearly demarcated superficial dermis located between the deep dermis and the epidermis; this superficial dermal layer is distinct, in part, because it has a more random arrangement of smaller collagen bundles which are present in lower density (Figure 4a). In *C. reinhardtii* the superficial dermal layer can still be distinguished, but it has a higher density of collagen bundles (Figure 4b) and these bundles are well-organized, although in a fashion distinct from the alternating pattern characteristic of the deep dermis. The deep dermis abuts the axial musculature. In the species examined, excluding *Calabaria*, there were no collagenous specializations at this lower boundary (Figure 4c). However, in *C. reinhardtii* there was a layer of dense collagen bundles located between the deep dermis and the epimysium of the skeletal muscle (Figure 4d). The deep collagen layer had an organization distinct from that of the deep dermis (which it integrated into) and could be traced back to attachment points on the vertebrae and ribs.

Calabaria reinhardtii combines a significantly (*t*-test, $p = 0.00015$) larger than average scale with a non-significantly (*t*-test, $p = 0.404$) smaller than average hinge, to yield a support index larger than those of all other species examined, and significantly (*t*-test, $p < 0.0001$) greater than the mean (Table 2). When comparing the species, the higher support index of *C. reinhardtii* means that there is relatively less free margin to each scale (Figure 5). Attempts to manually stretch the skin of live *C. reinhardtii* failed to produce noticeable displacement of the scales, and no interscale or hinge region was revealed. Elastin was found in the hinge region of every species examined including *C. reinhardtii*. No differences were observed in the relative amount or location of the elastin fibers among the species included in this study.

Cross-sections through the tail of *C. reinhardtii* showed that deep dermal collagen bundles are prominent in this region, and there is a close integration of the dermis with the underlying muscle and skeletal tissue (Figure 6).

3.2 | Penetration experiments

Analysis of the penetration data revealed neither significant interaction between body location and species, nor significant differences in penetration slopes between different locations of the body. There was a significant difference (Figure 7) in compression slopes between species [Sharp needle: $F(4, 10) = 28.903$, $p < 0.001$, partial $\eta^2 = 0.920$; Blunt needle: $F(4, 10) = 78.903$, $p < 0.001$, partial $\eta^2 = 0.969$]. Tukey's post hoc analyses demonstrated that slope of the penetration curve for *C. reinhardtii* was significantly greater (Sharp needle: $p < 0.0005$; Blunt needle: $p < 10^{-6}$) than those of the other four main comparison species (*E. colubrinus*, *L. trivirgata*, *C. atrox*, and *X. unicolor*).

TABLE 2 Interspecific variation in the support index of the species examined

	Scale Length	Hinge length	Support index
Acrantophis	1,555	685	0.56
Calabaria	**3,466**	**864**	**0.75**
Calamaria	1,236	532	0.57
Candoia	790	442	0.44
Cemophora	2,015	864	0.57
Charina	1,168	371	0.68
Crotalus	2,755	1,584	0.43
Eryx	3,325	1,407	0.58
Farancia	4,023	1,612	0.60
Lichanura	1,874	587	0.69
Lytorhynchus	724	475	0.34
Psammophis	2,114	823	0.61
Thamnophis	1,076	683	0.36
Xenopeltis	3,225	1,586	0.51
Mean	1,991	896	0.53

The support index is the fraction of the scale length that is not undercut by a hinge. The mean values listed in the bottom row exclude *Calabaria reinhardtii*.

As expected, comparing the two penetration curves showed that it took significantly more force to drive the blunt needle into the skin than it did the sharp needle.

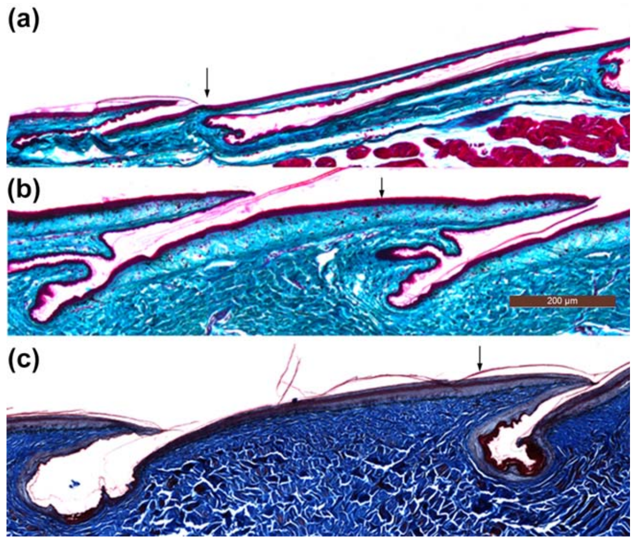

FIGURE 5 Interspecific variation in the relative size of the hinge region; the three images have been adjusted so that they have comparable scale lengths. All samples were taken from the lateral surface of the snake at midbody. The extent of the hinge (arrow) is greatest in *Lytorhynchus diadema* (a); moderate in *Acrantophis dumerilli* (b); and least in *Calabaria reinhardtii* (c). Scale bar is 200 μm

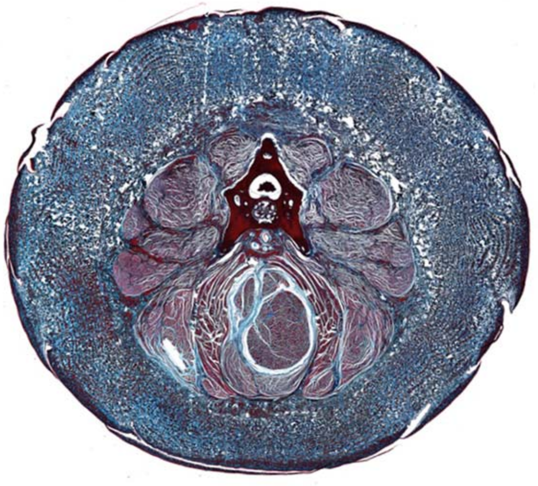

FIGURE 6 Cross section through the tail of *Calabaria reinhardtii* showing the prominence of the deep dermal collagen bundles, and the close integration of the dermis with the underlying muscle and skeletal tissue

4 | DISCUSSION

Interspecific variation in the thickness of the ophidian integument and the prominence of lamellar arrangement within the deep dermis exists for a diverse group of snakes (Jayne, 1988; Pockrandt, 1937), but the anatomy described here for *Calabaria reinhardtii* has not been reported in any other species. All specimens of *C. reinhardtii* examined exhibited similar degrees of dermal proliferation, and had thicker integument than other species examined (Table 1). Since the species used for statistical comparison were similar to *C. reinhardtii* in SVL, the unusual integument of *C. reinhardtii* is likely not the byproduct of body size.

The morphology of the interscale and hinge region of the snake integument has been previously described (Abo-Eleneen & Allam, 2011), and the role of interscale mobility (particularly of the ventral scutes) during some forms of locomotion has been detailed (Baum, Kovalev, Michels, & Gorb, 2014; Gans, 1974; Hu, Nirody, Scott, & Shelley, 2009; Lissmann, 1950). No previous study has quantified the features of the interscale region, or documented interspecific variation in the hinge region (Table 2, Figure 5). Our comparative results regarding the interscale and hinge regions on the dorsal-lateral surface of snakes, demonstrate that it may be linked to functional purposes other than locomotion.

A novel interpretation of the functional role of vertebrate integument was proposed when researchers found that collagenous fibers within the skin of sharks were storing elastic energy and, thus, reducing the metabolic costs of locomotion (Wainwright, Vosburgh, & Hebrank, 1978). Subsequent analysis (Alexander, 1987) demonstrated that for collagen fibers to function in elastic recoil they must be oriented at angles greater than 60° to the long axis of deformation. Subsequently, numerous cutaneous elastic recoil systems have been described (Pabst, 1996; Roberts & Azizi, 2011). The dermal collagen fibers in *C. reinhardtii* are unlikely to function in elastic energy storage. Not only are they oriented below the critical threshold value (Alexander, 1987), but

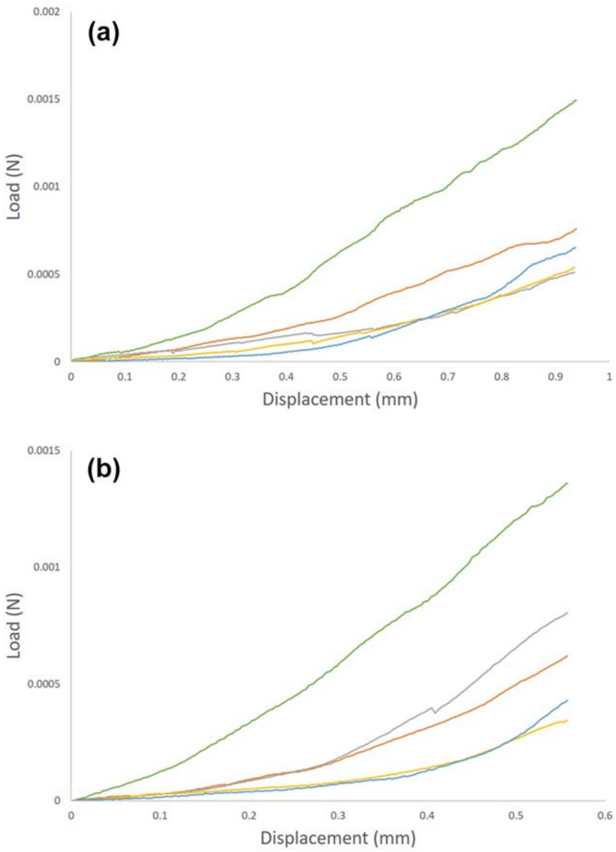

FIGURE 7 Penetration curves of skin samples taken from the midbody region of the snake and tested with a sharp (a) and blunt (b) needle. Note the distinct difference between *Calabaria reinhardtii* (green) and other snakes [*Xenopeltis unicolor* (red); *Crotalus atrox* (gray); *Eryx colubrinus* (yellow); *Lichanura trivirgata* (blue)]. For *C. reinhardtii*, where the skin is much thicker, only the initial portion of the curve is present; the curves presented for the other species represent penetration through the entire skin

cutaneous elastic recoil systems have evolved exclusively in organisms which use regular, even stereotyped, locomotor sequences, while locomotion in snakes tends to be episodic, highly variable, and substrate-dependent (Finkler & Claussen, 1999; Kelley, Arnold, & Gladstone, 1997).

The three morphological specializations reported from the integument of *C. reinhardtii*, that is, proliferation of the integument and particularly the deep dermis, lamellate organization within the dermal collagen bundles, and reduction of the interscale/hinge region, are not synapomorphic (shared derived) features of snakes, boids, or erycine snakes. Morphological and molecular analyses place *Calabaria* within the Boidae radiation, and within, or closely related to, the Erycinae (Kluge, 1993; Noonan & Chippindale, 2006). The species examined as part of this study include three members of the Erycinae (*C. bottae*, *E. colubrinus*, and *L. trivirgata*) as well as two other boid snakes (*A. dumerilli* and *C. carinata*). Neither the other Erycinae examined, nor the other boids, had the combination of integumentary features observed in *C. reinhardtii*. The deep dermal layers of two of the other erycines (*E. colubrinus* and *L. trivirgata*) examined were thicker than the average for all of the species examined (Table 1), but still significantly thinner than *C. reinhardtii* and lacked the other integumentary features found in that species.

Calabaria reinhardtii is a semi-fossorial species that inhabits the loose leaf litter on the jungle floor in equatorial Africa (Angelici et al., 2000; Luiselli & Akani, 1998). Relating the integumentary specializations found in *C. reinhardtii* to this habitat preference is difficult since other sampled species (i.e., *E. colubrinus*) move through a presumably more abrasive sand/soil substrate and lack the same specializations. Furthermore, these integumentary specializations were not found in *X. unicolor* (Table 1) which is another semi-fossorial snake favoring loose leaf litter, although in South-East Asia, not Africa (Orlov, 2000).

When threatened a *C. reinhardtii* relies on passive defense including head hiding and cephalic mimicry (Lehmann, 1971). Greene (1973) surveyed and categorized the different types of cephalic mimicry in snakes. *Calamaria septentrionalis* exhibits the same type of cephalic mimicry, but lacks the integuementary specializations seen in *C. reinhardtii*. Of the 76 snake species Greene reported to use a form of defensive tail display, only *C. reinhardtii* has been described as having integumentary specializations (Table 1).

The integument of *Calabaria* can be best understood within the context of the behavioral ecology of this species. *C. reinhardtii* raids rodent nests (which are mainly subterranean) and preferentially ingest juvenile and newborn rodents (Lehmann, 1971; Luiselli & Akani, 1998; Luiselli et al., 2002). Understandably, this predatory style leaves the snake vulnerable to retaliatory/defensive attacks from parental rodents. Biting is the primary defensive behavior in rodents, and rodent bites can inflict significant tissue damage to snakes, even leading to death (Hartman, 1922; Rosskopf & Woerpel, 1981; White et al., 2011). Field studies of *C. reinhardtii* have reported the snakes being attacked by adult, presumably parental, rodents (Lehmann, 1971).

As described previously, *Calabaria reinhardtii* has a suite of integumentary specializations including: (1) proliferative thickening of the integument and particularly the deep dermis; (2) a more structured lamellar arrangement to the deep dermis; and (3) relative reduction of the interscale/hinge region which effectively maximizes scale overlap while minimizing scale mobility. Herein it is hypothesized that these integumentary specializations work collectively to minimize the potential trauma due to rodent bites. If the hypothesis that the integument of *C. reinhardtii* is a specialized defense against penetrative rodent bite is supported, then one would expect that these integumentary features are prominent in the tail given that *C. reinhardtii* effectively offers up its tail when threatened (Greene, 1973; Lehmann, 1971). In fact, the integument surrounding the tail of *C. reinhardtii* is, if anything, even more specialized showing the same features noted in the neck and midbody regions as well as prominent attachments to the vertebral processes (Figure 6).

The hypothesis that the integument of *Calabaria* is a specialization to resist defensive bites is supported by the results of the penetration tests. At all of the tested locations, for both a sharp and a blunt penetrator, it took significantly more force to penetrate the skin of *C. reinhardtii* than the skin of the other study species (Figure 7). Because there was no significantly different load/displacement moduli among the other species examined, it is likely *C. reinhardtii* is uniquely specialized to use its well-organized thick dermis as a means of protection.

An important functional design of skin in some species is to serve as dermal armor against predation. Jarman (1989) proposed that the key to dermal armor was increased thickness of collagen; subsequent study (Shadwick et al. 1992) demonstrated that it is the arrangement of the collagen, rather than the thickness, that confers the penetrative resistance. In mammals, a collagen-based dermal armor is best known in the white rhinoceros (Shadwick et al., 1992); regional dermal thickening has also been reported in other mammals (Jarman, 1989; Luck and Wright, 1964; Sathar, Ludo Badlangana, & Manger, 2010). Dermal armor has also been described from several fish species (e.g., Bruet et al., 2008; Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003; Yang et al., 2013). In the pirarucu (*Arapaima gigas*), the collagen fibrils in the dermis have a Bouligand arrangement, in which each successive layer of collagen bundles has an angular offset from the deeper layer (Lin, Wei, Olevsky, & Meyers, 2011; Zimmermann et al., 2013); this skin was resistant to penetration by a sharp piranha tooth (Yang et al, 2014).

Calabaria reinhardtii does not have a Bouligand-type arrangement of collagen bundles in the deep dermis, instead the fibers show an alternating arrangement of perpendicularly offset layers (Figure 3). Given the increased strength associated with the Bouligand-type arrangement, its absence from the dermis of *C. reinhardtii* may reflect a form of functional trade-off. In a true Bouligand-type system many of the collagen bundles would be directly aligned with, or just slightly offset from, the long (cranial-caudal) axis of the snake's body. It is along this axis that snakes exhibit the greatest range of movement both during coiling and locomotion. While a Bouligand-type arrangement could reduce locomotive flexibility, the 45° orientation of the collagen bundles found in *C. reinhardtii* enhance penetration resistance while only reducing circumferential expansion of the snake's body.

Circumferential expansion associated with prey ingestion (Jackson, Kley, & Brainerd, 2004) is one of the best-known features of snakes. Boid snakes, including members of the Erycinae, are macrostomate, reflective of their ability to ingest relatively large prey items (Cundall & Greene, 2000; Rodríguez-Robles, Bell, & Greene, 1999). The lower jaw of *Calabaria* is unusual in retaining a form of cartilaginous symphysis (Young, 1998). This symphyseal morphology, coupled with the dermal limits to circumferential expansion, may explain why *C. reinhardtii* exclusively forages on newborn/juvenile rodents (Lehmann, 1971; Luiselli & Akani, 1998; Luiselli et al., 2002).

A female snake's circumferential size typically increases during reproduction, which leads to reduction in locomotor performance directly related to reproductive output (Seigel, Huggins, & Ford, 1987). The reproductive biology of *C. reinhardtii* is also unusual among snakes in that the females produce only a small number of eggs (4 being most common), and the eggs are more cylindrical than round or ovate (Luiselli & Akani, 1998; Luiselli et al., 2002). It is possible that these reproductive specializations are a way of minimizing the expansion of body diameter typically associated with reproduction in snakes.

This study provides morphological and experimental evidence for the protective properties of the skin of *Calabaria reinhardtii*. This study represents the first evidence of a novel integumentary defense specialization among snakes. The existence of similarly structured skin, and evidence for defensive adaptation, in mammals (*Ceratotherium simum*), fish (e.g., *Polypterus senegalus*), and now reptiles (*Calabaria reinhardtii*), is a previously unreported example of convergent evolution, and offers bioinspiration for the development of synthetic armors.

ACKNOWLEDGMENTS

The authors wish to thank C. Montgomery (Truman State University) and P. Kondrashov (A.T. Still University) for their support of this research. Amy Lathrop and Robert Murphy of the Royal Ontario Museum generously provided the specimen of *Calamaria septentrionalis* examined for this study.

AUTHOR CONTRIBUTIONS

The authors jointly participated in every aspect of this study including design, data collection, analysis, and manuscript preparation.

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How to cite this article: Han D, Young BA. The rhinoceros among Serpents: Comparative anatomy and experimental biophysics of Calabar burrowing python (*Calabaria reinhardtii*) skin. *Journal of Morphology*. 2018;279:86–96. https://doi.org/10.1002/jmor.20756