

Multiple anti-predator mechanisms in the red-spotted Argentina Frog (Amphibia: Hylidae)

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Introduction

Anuran anti-predator mechanisms include passive ones that reduce the likelihood of being detected by the predator (e.g. cryptic colouration and secretive habits), as well as active mechanisms that reduce the chances of being consumed during an encounter with a predator (Wells, 2007). Usually, anurans employ both active and passive mechanisms in a sequential or hierarchical defence strategy (Toledo, Sazima & Haddad, 2011). Thus, an anuran may avoid predators by either crypsis or secretive habits but, once detected, active anti-predator mechanisms, such as aposematic colourations, defensive postures, and the release of toxic and distasteful skin secretions, begin to play a role (Wells, 2007; Toledo & Haddad, 2009; Toledo *et al.*, 2011).

The red-spotted Argentina Frog, *Argenteohyla siemersi* (Mertens, 1937), is a hylid treefrog found in Argentina, Paraguay and Uruguay (Cei & Pierotti, 1955; Klappenbach, 1961; Trueb, 1970a; Cajade *et al.*, 2010). Typically, it has rounded, coral-reddish spots on the legs, belly, flanks and vocal sacs. *Argenteohyla siemersi* is included in the tribe Lophiohyliini Miranda-Ribeiro, 1926, together with *Aparasphenodon*, *Corythomantis*, *Itapotihyla*, *Nyctimantis*, *Osteocephalus*, *Osteopilus*, *Phyllodytes*, *Phytotriades*, *Tepuihyla* and *Trachycephalus*,

Abstract

Anurans employ a wide variety of anti-predator mechanisms to defend themselves. In casque-headed hylids, defence is thought to be a complex combination of several anti-predator mechanisms. However, the defence traits of only a few species are known; some hypotheses have yet to be addressed, whereas others, already tested in some species, need to be tested in additional taxa. The anti-predator mechanism of the casque-headed frog, *Argenteohyla siemersi*, is described here. It is a complex mechanism consisting of (1) behavioural and ecological traits, including secretive and semi-phragmotic habits and posture; (2) morphological features, including cryptic and aposematic colourations, a skull covered with bony dermal spines and protuberances that are associated with two types of granular venom glands; and (3) physiological and chemical traits, such as a highly lethal skin secretion. Our results are compared with those of previous studies of defence mechanisms in casque-headed frogs in an effort to understand the mechanisms and evaluate their potential phylogenetic signal in this group of anurans.

which form a monophyletic clade (Faivovich *et al.*, 2005; Duellman, Marion & Hedges, 2016) known as ‘casque-headed frogs’ (Faivovich *et al.*, 2005). Like some other casque-headed frogs, *A. siemersi* (1) has a heavily ossified skull and sculptured dermal bone that is co-ossified with the overlying skin (Trueb, 1970a); (2) takes refuge in bromeliad leaf axils (Cei & Pierotti, 1955; Cajade, Marangoni & Gangenova, 2013); and (3) presumably displays phragmotic behaviour by backing into axils and flexing the head at a right angle to the body (Barbour, 1926; Firschein, 1951; Trueb, 1970b), as suggested by Trueb (1973) and Lutz & Barrio (1966). However, this behaviour has not been observed in *A. siemersi*. Both phragmotic behaviour and cranial co-ossification have been considered as devices to decrease water loss in *Aparasphenodon* and *Corythomantis* (Trueb, 1970b; De Andrade & Abe, 1997; Navas, Jared & Antoniazzi, 2002) or as means for the frogs to defend themselves from predators (Barbour, 1926; Trueb, 1970b). There is strong evidence supporting the ‘defence hypothesis’ in both of these taxa (Jared *et al.*, 2005, 2015; Mendez *et al.*, 2016).

The defence traits in casque-headed frogs are known for only a few species; consequently, some hypotheses have not been addressed, whereas others, already tested in some species, remain to be tested in others, such as *Argenteohyla siemersi*.

Based on our field and laboratory observations of the anti-predator mechanisms of *A. siemersi*, we: (1) report and describe the phragmotic behaviour (H1: phragmosis is a defensive behaviour, Jared *et al.*, 2005); (2) describe the skull morphology and histology of the skin on the head and body (H2: co-ossification has a defensive function, Jared *et al.*, 2005); (3) describe the muscular and bony morphology of the posterior region of the head (H3: 90° flexion of the head is correlated with enlargement of the rhomboideus anterior muscle) (Trueb, 1970b); and (4) analyse the toxic power of the skin secretions with an Ld 50-test (H4: the coral-reddish spots function as aposematic colouration, Toledo & Haddad, 2009).

Materials and methods

Specimens examined

Behavioural observations were conducted in the laboratory on two adult frogs collected at ‘Selvas de Montiel’ (30°53′42.73″ S, 59°34′43.71″W), Entre Ríos Province, Argentina, on 21 August 2012. In addition, we raised eight subadults from larval Stage 41 (Gosner, 1960) in the lab; these were collected on 5 November 2014 at Riachuelito (27°33′45.80″S; 58°34′48.67″W), Corrientes Province, Argentina. Samples for the study of the skull and muscle morphology, and histology of the skin of the head and body of adult *A. siemersi* were obtained from specimens in the Herpetological Collection of Museo de La Plata (MLP). We studied the toxicity of skin secretions of five adult *A. siemersi* collected at ‘Reserva Natural Rincon Santa María’ (27°31′31.62″S, 56°36′18.65″W) on 29 July 2015.

Behavioural observations

Field observations were carried during fieldwork related to other studies (Cajade *et al.*, 2010, 2013). The colour patterns were associated with several behavioural display contexts and classified according to Toledo & Haddad (2009). Phragmotic behaviour was observed in the field, as well as in eight frogs in the laboratory, where each individual was provided a plastic tube simulating a refuge. We touched the frog’s head with tweezers to simulate a predator. Each individual was stimulated at 10 s intervals for 5 min (= 300 s) and each response was recorded. We term the response of head flexion to a nearly 90° angle to the trunk of the frog ‘induced phragmosis’. When a frog engaged in this behaviour, we waited until it resumed its normal posture before stimulating it again. The variables recorded for each frog were time period (duration), frequency of occurrence, and presence/absence of both body and head secretions at the end of the experiment.

Morpho-histological analysis

Two frog heads were submerged in a 30% solution of sodium hypochlorite for 24 h to remove the soft tissue to study the dermal bone morphology. One head (MLP 5844; Appendix S1) was mounted on a metal stub and sputter-coated with gold and examined with a Zeiss Supra 40 with scanning electron microscope (SEM). The other (MLP 5843;

Appendix S1) was examined under a Leica EZ4D stereoscopic microscope, from which macroscopic, digital images were captured. To facilitate future comparisons with other casque-headed frogs, the sculptured details of cranial roof bones are described in detail using terminology adopted and modified from Witzmann *et al.* (2010).

To study cranial co-ossification, we decalcified two additional heads (previously fixed in 4% formalin solution) in 5% nitric acid for 96 h before immersing in 5% potassium alum for 24 h, washing them with running water for 24 h, and then processing them following traditional histological techniques for light microscopy. Skin samples were taken from different regions of the body for comparative examination, as follow: cranial vault and loreal region (Fig. 1); jaws; middle back and anterior back adjacent to occipital region of head; and hind limbs, including samples from the dark background and red-coral spots. Tissues were embedded in Paraplast Plus (Sigma) and sectioned at 7 µm with a Microm HM 325 microtome. Sections were stained with haematoxylin and eosin (H&E) or modified Masson’s trichrome (MT) stain for general cytology and histology. MT differs from the original Masson’s trichrome in which the acid fuchsin solution also contains Orange G and xylydine ponceau. In addition, the following histochemical stains were applied to select sections to determine the secretory products of the different gland types: periodic acid-Schiff (PAS; Kiernan, 1999) for neutral glycoconjugates, Alcian blue 8 GX at pH 2.5 plus haematoxylin (AB; Kiernan, 1999) for primarily carboxylated acidic glycosaminoglycans and Coomassie blue R250 (CB; Kiernan,

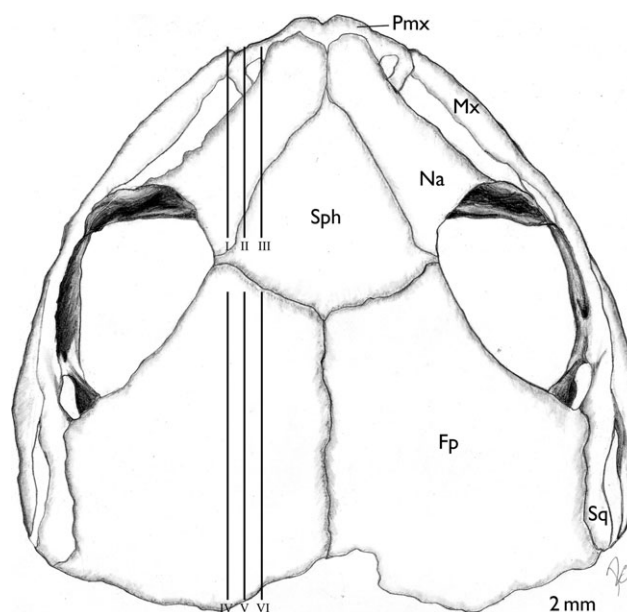


Figure 1 Dorsum of the skull of *Argenteohyla siemersi*. Black lines with roman numerals indicate locations of histological sections. Abbreviations: Na (nasal); Pmx (premaxilla); Mx (maxilla); Sph (sphenethmoid); Fp (frontoparietal); Sq (squamosal). Dermal sculpturing is not shown.

1999) for proteins. Mucous glands and cartilage were used as positive controls for PAS and AB; a keratinized portion of the epidermis was used as a positive control for CB. Stained sections were examined using a Zeiss Primo Star microscope, and images were captured using a Canon PowerShot A640 digital camera.

We made comparative observations of three additional skeletons of *Argenteohyla siemersi* (MLP 5845-5847), together with three *Hypsiboas raniceps* Cope, 1862 (MLP 2453, 4281, 3585) and three *Trachycephalus typhonius* (Linnaeus, 1758) (MLP 492, 3551, 3552; Appendix S1). These specimens were double-stained for bone and cartilage using the technique of Taylor & Van Dyke (1985), but the procedure was terminated before clearing to examine the cranial, axial and scapular muscles involved in head bending. Subsequently, the clearing process was completed to examine the morphology of certain cranial bones (e.g. sculptured bones and those in the occipital region). The two species of tree-frogs selected for comparison also use bromeliads as refuge, but they are not phragmotic.

Cutaneous secretion and lethality

Samples of skin secretions were extracted from adult frogs following the protocol of Jared *et al.* (2005). A single sample of milky secretion, obtained from the mix of all individual secretions, was centrifuged and the clear supernatant was collected, lyophilized, and stored at -70°C . The resulting dried secretion was used to determine lethality. For this, laboratory mice (male Balb/C isogenic) were donated by the Facultad de Veterinaria (Universidad Nacional del Nordeste, Argentina). Mice weighed between 18 g and 22 g, and were 7 or 8 weeks old. We followed the international animal welfare recommendations for the treatment and maintenance of mice and frogs (Stitzel, Spielmann & Griffin, 2002). The lethal toxicity of the secretion of *Argenteohyla siemersi* was assessed by intraperitoneal injection of various secretion concentrations (1, 1.8, 3.16, 5.6, 8.4, 10, 15 $\mu\text{g}/\text{animal}$) in 200 μL of 0.85% NaCl solution; negative control mice received only saline solution. Six animals were used for each secretion dose. The death-survival ratio was determined after 48 h, and Lethal Dose 50 (LD50) and its confidence limits were estimated using the Spearman-Kärber method (World Health Organization, 1981). LD50 was expressed as micrograms of toxin per mouse (20 ± 2 g) necessary to kill 50% of the population of tested animals.

Results

Studies and observations on behaviour

The frogs were observed either in reproductive activity or hidden inside a bromeliad. The bright coral-reddish colouration of *Argenteohyla siemersi* is concealed when the frog is in resting posture or inside the refuge (cf. plates in Cajade *et al.*, 2013). The bright colours (coral-reddish) are revealed when the frog jumps or swims, and are especially obvious in males calling from the water surface (Cajade *et al.*, 2010; cf.

plates in Cajade *et al.*, 2013). When the frog takes refuge (e.g. in a bromeliad), it is cryptic because retracts its limbs toward its body thereby hiding the bright colouration and exposing only the dark brown head and back of the body. If the frog is submerged in the water accumulated in a bromeliad, the head and body appear homochromic with the shadowy environment of the microhabitat; thus, the frog is difficult to detect, unless it is illuminated with a flashlight (Fig. 2).

Although phragmosis was not observed in *Argenteohyla siemersi* found inside bromeliads and umbellifers, the frogs usually adopt the phragmotic posture (with the head flexed to nearly 90°) as they are captured. During laboratory experiments, all frogs displayed induced phragmosis (Fig. 3). The frogs react to the stimulus in one of three different ways (Supplemental Material). (1) They flex the head to a nearly 90° angle to the body, but return to normal position immediately after the stimulus ends (brief phragmosis). (2) They flex the head to a nearly 90° angle to the body and remain in this position for varying periods of time (persistent phragmosis) (3) they do not display the phragmotic posture (i.e. moving the body and legs). All the frogs displayed persistent phragmosis at least once (Table 1). The mean number of stimuli



Figure 2 Refuge behaviour of *Argenteohyla siemersi* in a bromeliad (*Aechmea distichantha*) in natural (a) and artificially illuminated (b) light conditions. [Colour figure can be viewed at wileyonlinelibrary.com].

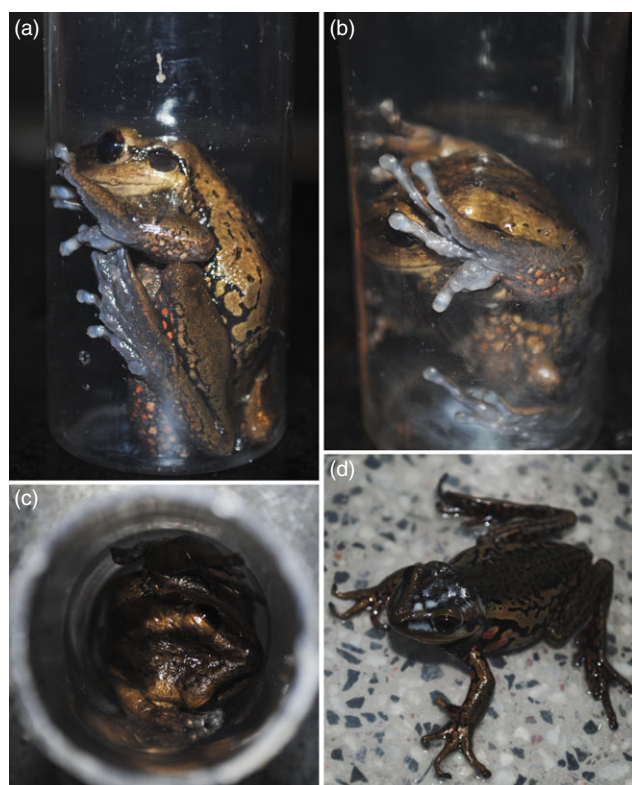


Figure 3 Induced phragmotic posture in *Argenteohyla siemersi* in the laboratory (a–c) and the milky secretion on the head induced by massaging the frog's head (d). [Colour figure can be viewed at wileyonlinelibrary.com].

Table 1 Summary of the laboratory experience conducted in *Argenteohyla siemersi* to induce phragmotic posture

Individual	Time experience (s)	Stimulus (n°)	Behavioural pattern (n°)	Duration (s)
1	300	16	12 (3)	12.8 ± 6.8 (5–25)
2	340	17	2 (14)	47.5 ± 13.4 (38–57)
3	410	11	6 (2)	26.8 ± 10.9 (14–45)
4	300	19	11 (7)	7.1 ± 4.9 (2–20)
5	314	12	10 (12)	22.5 ± 22.7 (8–72)
6	300	16	4 (2)	11.5 ± 13.3 (3–31)
7	300	14	8 (3)	21.8 ± 16.9 (4–51)
8	338	12	6 (5)	35.5 ± 23.5 (11–64)

made were 14.6 (SD = 2.85; min = 11, max = 17; $n = 8$) with a mean response number of persistent phragmosis of 7.3 (SD = 3.5; min = 2, max = 12; $n = 8$) and with a mean duration of the persistent phragmosis of 23.1 s (SD = 13.4 min = 2, max = 72; $n = 59$). Neither body nor head skin secretions were detected on the specimens during the experiment. Additionally, the laboratory-reared frogs usually displayed the phragmotic posture when they were manipulated by hand.

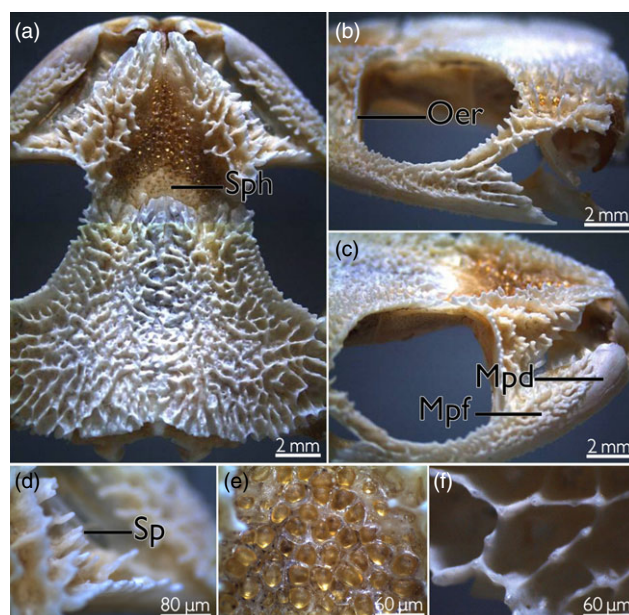


Figure 4 Cranial structure and dermal ornamentation of *Argenteohyla siemersi*. Regions without dermal structures: sphenethmoid (Sph; a), maxillary pars dentalis (Mpd; c). Dermal structures: spines (Sp; d), orbital sculptural ridge (Oer; b). Details: crystallized secretion of Type II granular gland (e). Vitreous protuberances (f). Skull table and sphenethmoid (a). Orbital sculptural ridge (oer; b). Pars facialis (Mpf) and pars dentalis (Mpd) of maxilla (c). Spines (Sp; d). [Colour figure can be viewed at wileyonlinelibrary.com].

Dermal skull

The cranium of *Argenteohyla siemersi* is strongly ossified with a casque of sculptured dermal bone that composes the skull table, except for a region immediately anterior to the frontoparietals (the endochondral sphenethmoid) (Fig. 3a), and the pars dentalis of the maxilla (Fig. 4c). Sculptured roofing bones are characterized by different patterns such as ridges, pits, emergent protuberances, spiny projections and pores. The skull table bears sculptured ridges (Fig. 4c), whereas the loreal region is covered with individual spiny projections in addition to ridges (Fig. 4d). The ridges can be interconnected and the intersections between them define rounded, polygonal pits of different sizes (Fig. 5a). The floor of the pit usually has one to three pores of variable sizes (Fig. 5a). The squamosal, along with the ventrolateral surface of the nasal and the adjacent pars facialis of the maxilla, the sculptured ridges are interconnected longitudinally, but lack transverse connections, to form a pattern of parallel, longitudinal pits of varying size. (Fig. 5a). A supraorbital flange, composed of the frontoparietal, extends laterally over the orbit, the sculpted margin of which is composed of the nasal, pars facialis of the maxilla and the zygomatic ramus of the squamosal. (Fig. 4b). There are emergent protuberances along the tops of the ridges (Fig. 5). The smooth surface of the protuberances has a vitreous appearance, whereas the rest of the sculptured dermal bone is wrinkled and opaque (Figs 4f and 5b).

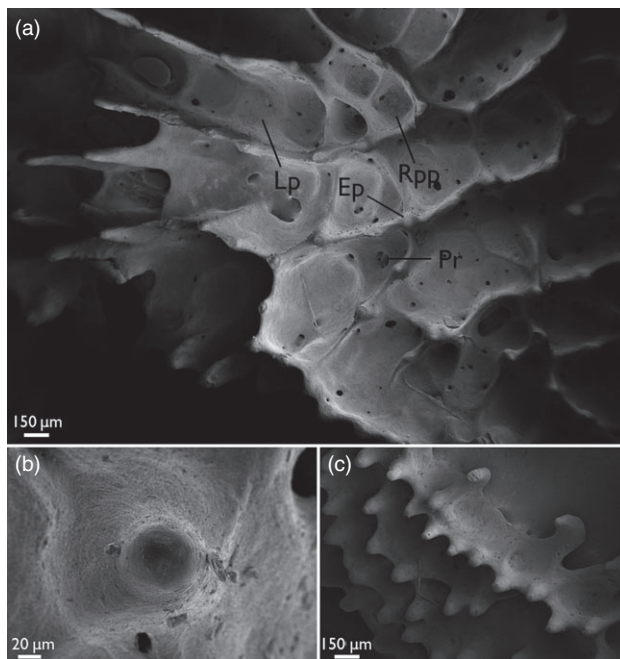


Figure 5 SEMs of cranial ornamentation of *Argenteohyla siemersi*. (a) Details of ornamentation, a protuberance (b), and a sculptural ridge (c). Abbreviations: Ep (emergent protuberance); Lp (longitudinal pit); Rpp (rounded polygonal pit); Pr (pores).

Body and head skin histology

The skin of adult *Argenteohyla siemersi* has four or five layers of epidermal cells; the superficial layers are partially keratinized (Figs 6 and 7). The dermis consists of a stratum spongiosum and a stratum compactum; the former bears three different dermal glands – viz., mucous glands and two types of granular glands (Figs 6 and 7). Mucous glands are distributed over the body and head, and are typical of those of other amphibians (Fig. 6a); mucocytes are only positive for detection of neutral glycoconjugates (Fig. 6b). The two types of granular glands are structurally similar, having a syncytial secretory compartment within the acinus, which is surrounded by myoepithelial cells. However, the types of granular glands differ in their locations and histochemical staining properties (Fig. 7). Type I granular glands have a granular, acidophilic content (Fig. 7a) and are only positive for detection of proteins (Fig. 7b). In contrast, Type II granular glands are bigger, have a content with striated appearance that fills the glandular lumen, stain positive for neutral glycoconjugates (Fig. 7c), and are highly positive for detection of proteins (Fig. 7d). Granular glands have a characteristic distribution that is summarized in Table 2.

In the cranial vault region, some parts of the stratum compactum are replaced by dermal bone, which fuses or co-ossifies the skin with underlying bones (e.g. frontoparietal, sphenethmoid, nasals, squamosals) (Fig. 8). Other parts of the cranium are not co-ossified; the stratum compactum is only

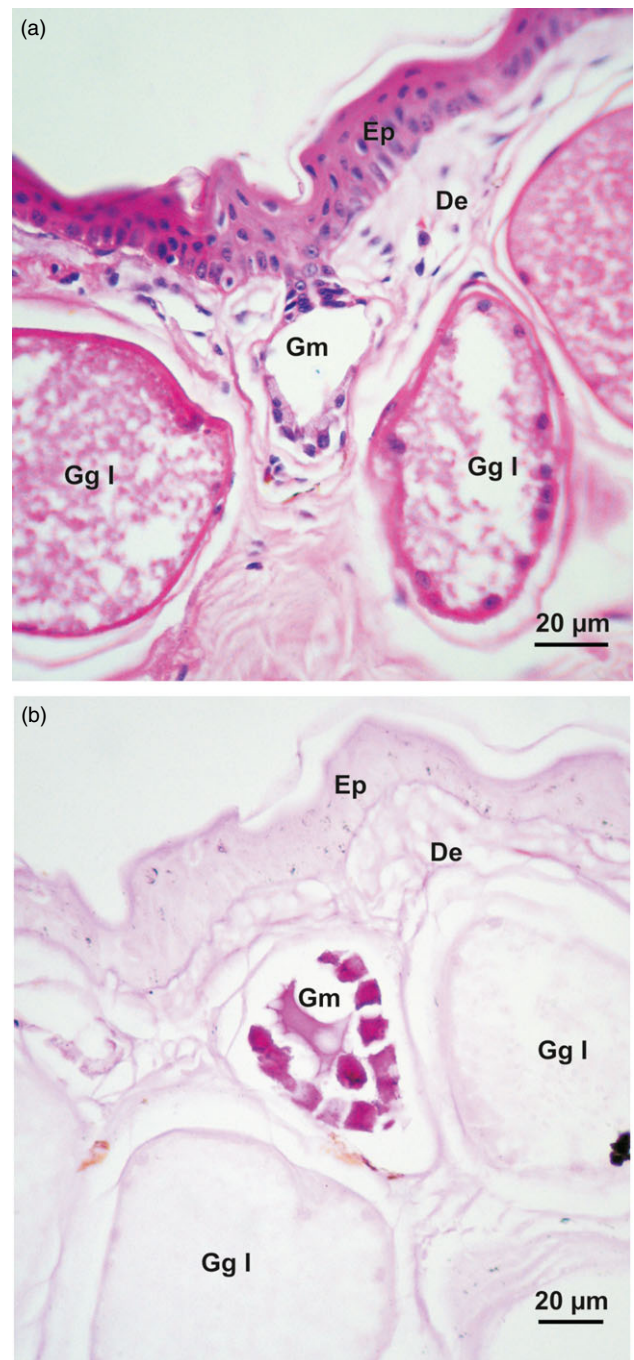


Figure 6 Skin glands of *Argenteohyla siemersi* from histological section number I (Fig. 1). (a) Detail of mucous (Gm) and granular glands (Gg) stained with H&E. Morphology is typical of anurans, with mucocytes (mucous cells) surrounding the lumen. (b) Detail of mucous and granular glands stained with PAS. Note that mucocytes and the lumen content stained positive for detection of neutral glycoconjugates. Abbreviations: De (dermis); Ep (epidermis); Gg I (Type I granular gland); Gm. [Colour figure can be viewed at wileyonlinelibrary.com].

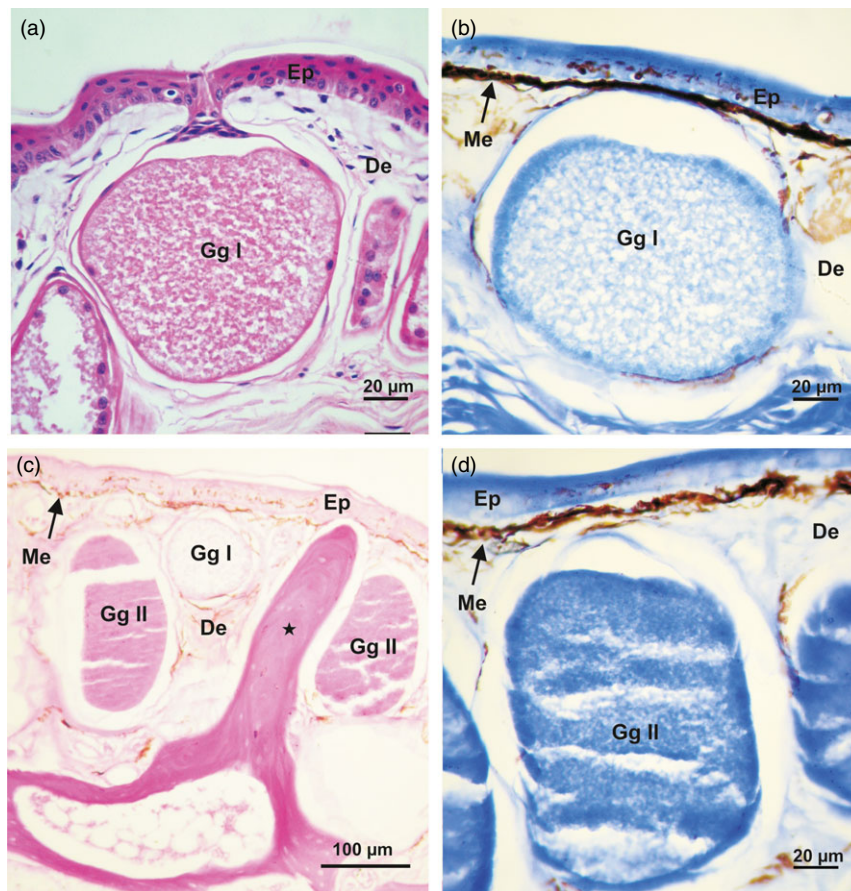


Figure 7 Different types of granular glands in the skin of *Argenteohyla siemersi* from histological section number II (Fig. 1). (a, b) Type I granular gland (Gg I) section stained with H&E (a) and CB (b). The gland content is acidophilic and granular in appearance, with a slight staining of glandular content for protein detection. (c) Section stained with PAS. The contents of a Type II granular gland (Gg II) are positive for detection of neutral glycoconjugates, and a bony spine (star) in the dermis reaches the epidermis (Ep). (d) Detail of Gg II highly positive for detection of proteins with CB. Abbreviations: De (dermis); Ep (epidermis); Gg I; Gg II; Gm (mucous gland); Me (melanophores); star (bony spine). [Colour figure can be viewed at wileyonlinelibrary.com].

Table 2 Morphological characteristics of the skin from different parts of the body of *Argenteohyla siemersi*

Region of the body	Bone spines	Type of dermal gland	Pigmentary cell
Dorsum of cranium at region of cranial vault	Present	Mucous gland, Type I and II granular glands	Melanophores
Loreal	Present	Mucous gland, Type I and II granular glands	Melanophores
Perimandibular	Present	Mucous gland, Type I and II granular glands	Melanophores
Immediate postcranial	Absent	Mucous gland, Type I and II granular glands	Melanophores
Middle region of dorsum	Absent	Mucous gland, Type I granular gland	Melanophores
Hindlimb with coral colouration	Absent	Mucous gland, Type I granular gland	Xantophores
Hindlimb with dark colouration	Absent	Mucous gland, Type I granular gland	Melanophores

replaced with dermal bone in its more inner portion, forming a basal layer of bone on which there is a layer of collagen fibres (Fig. 8a). The connective tissue that separates the partially ossified taenia tecti marginalis from the dermal frontoparietal (Fig. 8a–c) has abundant collagen fibres and chondrocyte-like cells with eccentric nuclei (Fig. 8b). This binding tissue stains positive for acidic glycoconjugates (Fig. 8c) and occupies a considerable space between the endo- and exocranium. Posteriorly, in the occipital region, muscles

lie between the dermal casque and the endocranium (Fig. 8d). In the frontal region, bony protuberances are intermingled with enlarged Type I and II granular glands, but the most abundant glands are Type I (Fig. 8). A similar pattern occurs in the loreal region of the nasal, where the stratum compactum is completely or partially ossified (Fig. 9a,b). Some of the spines extend into the epidermis and nearly pierce the skin in areas where the epidermis is thinner and composed only of the keratin layer (Fig. 9c). Histological sections of the region of the

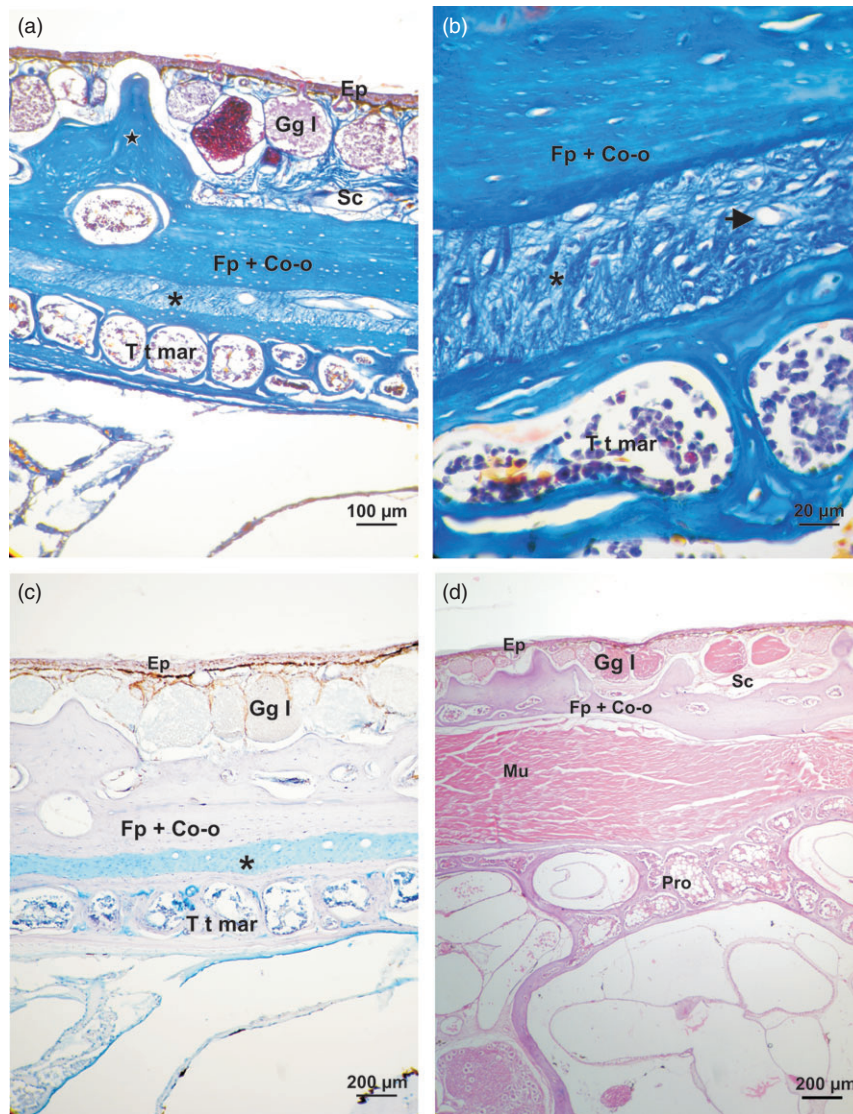


Figure 8 Skin co-ossification (Co-o) in the frontal region of *Argenteohyla siemersi* from histological sections number IV, V, and VI (Fig. 1). (a, b) anterior zone, sections stained with MT. a, note that some parts of the stratum compactum (Sc) are completely replaced with dermal bone and the bony spines are present (star; Co-o). In contrast to other parts of the dermis, only the basal portion of the Sc is ossified, and dense collagen fibres remain in the upper Sc. The (*) indicates the tissue that binds the partially ossified taenia tecti marginalis (T t mar; neurocranium) with the frontoparietal (Fp; dermal bone). b, detail of the binding tissue in A. Notice the presence of abundant collagen fibres and chondrocyte-like cells (arrow), with eccentric nucleus. c, anterior zone, section stained with AB shows that the binding tissue between endochondral and dermal bone is positive for detection of acidic glycoconjugates. d, posterior zone, section stained with H-E. Note the presence of muscle (Mu) between the Fp and prootic (Pro) bones. Abbreviations: Co-o; Ep (epidermis); Fp; Gg I (type I granular gland); Mu; Pro; Sc; T t mar. [Colour figure can be viewed at wileyonlinelibrary.com].

cranium that lacks dermal sculpturing (endochondral sphenethmoid, Fig. 4a) show that the dermis has two rows of enlarged Type II granular glands and that the stratum compactum is not ossified (Fig. 9d).

The hindlimbs have different types of pigment cells in the upper portion of the dermis (Fig. 10). The dark ground colour of the skin is formed by melanophores (Fig. 10a,b). The coral spots are rich in xanthophores (Fig. 10c,d), which are empty cells with eccentric nuclei in the upper portion of the stratum

spongiosum (Fig. 10d). Only Type I granular glands occur in the skin of the hindlimbs, and these glands are distributed in both colours (Fig. 10).

Otoccipital region of the skull, occipital-atlas junction and related muscles

The bony morphology of the occipital region of the three species is similar except for subtle differences in the occipital

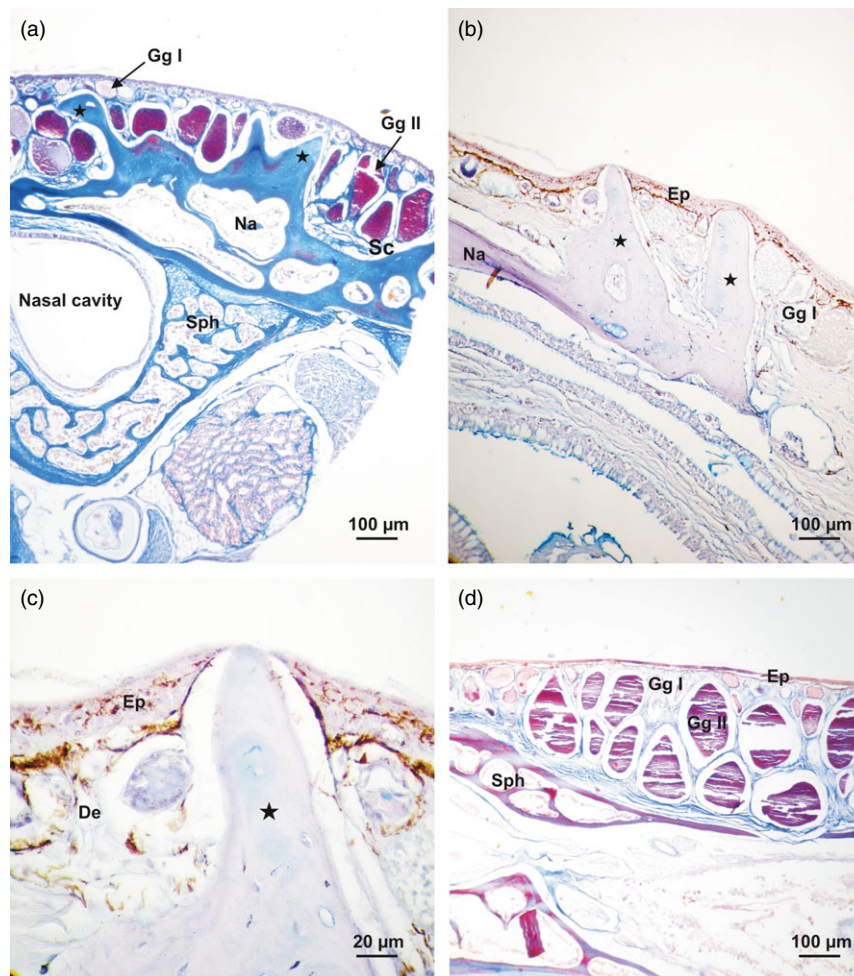


Figure 9 Skin co-ossification (Co-o) in the loreal region of the nasal *Argenteohyla siemersi* from histological sections number II and III (Fig. 1). (a) Section stained with MT, showing the skin co-ossification with the nasal (Na), and the presence of the bony spines (star). In other parts of the dermis, only the basal portion of the stratum compactum (Sc) is ossified, and dense collagen fibres remain in the upper Sc. The big glands between bony spines are Type II granular glands (Gg II). (b) Section stained with AB, showing bony spines (stars) that almost pierce the epidermis. (c) Detail of the bony spine (star) in b. (d) Section stained with MT of the head region without dermal sculpturing, the endochondral sphenethmoid (Fig. 3a). There is an accumulation of Gg II forming two rows of glands in the dermis. Abbreviations: De (dermis); Ep (epidermis); Gg I (Type I granular gland); Gg II; Na; Sc; Sph (sphenethmoid). [Colour figure can be viewed at wileyonlinelibrary.com].

condyles (Fig. 11). The condyles of *Argenteohyla siemersi* are more rounded and located in lower position respect to the foramen magnum than in the two non-phragmotic species. The dorsal margins of the condyles of *Trachycephalus typhonius* and *Hypsiboas raniceps* are lower than the mid-height of the foramen. In fact, the condyles of these non-phragmotic species are oval instead of round and, in both cases, the long axes of the condyles and the angle of the long axis to the ventral/horizontal plane are more acute than those in *A. siemersi* (Fig. 12). The shape of the articular facets of the first presacral vertebra (atlas) reflects the differences in the occipital condyles of *A. siemersi* in contrast to the non-phragmotic *T. typhonius* and *H. raniceps* (Fig. 12).

We examined the axial, appendicular and hyobranchial muscles that insert on or originate from the occipital region

of the skull, in addition to the muscles that connect the hyoid and the mandible, and the pectoral girdle and the hyoid. The origins, insertions and general observations about these muscles in *Argenteohyla siemersi* are summarized in Table 3. In contrast to *A. siemersi*, the *m. rhomboideus anterior* of *Trachycephalus typhonius* inserts anterior to the insertion of the *m. longissimus dorsalis*, and in both *T. typhonius* and *Hypsiboas raniceps* some fibres of the *m. rhomboideus anterior* reach a fascia on the *m. levatorae mandibulae internus*.

The relationship between the myological architecture of *Argenteohyla siemersi* and the ability of the frog to flex the head at a 90° angle to the body is not apparent. The contraction of the *intertransversarius capitis superior*, *levator scapularis inferior*, *levator scapularis superior*, *opercularis* and

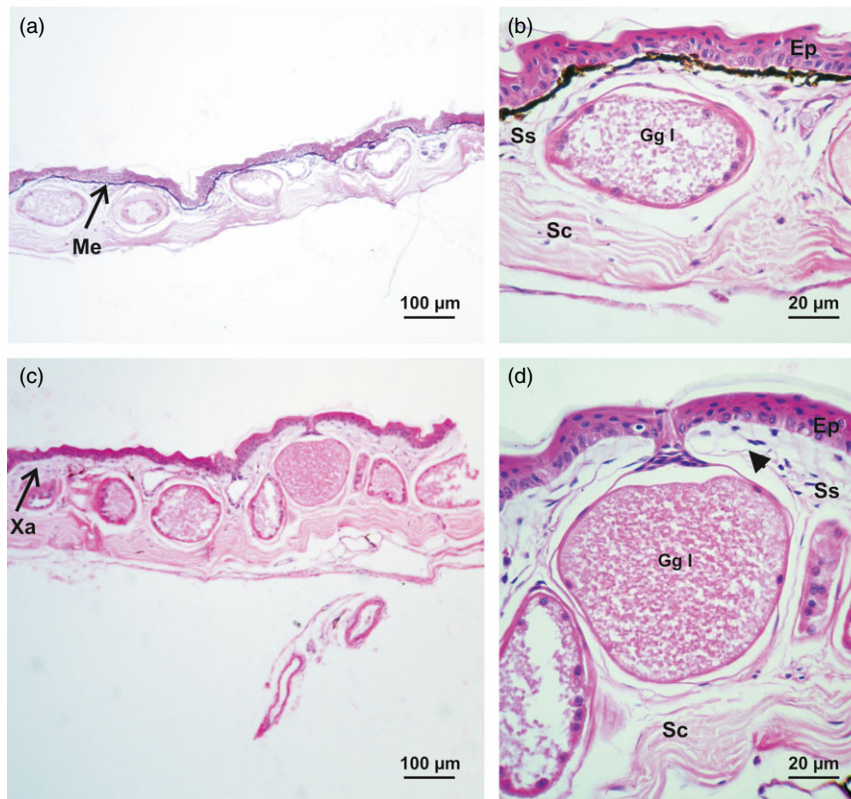


Figure 10 Dermal glands in skin of the hindlimbs of *Argenteohyla siemersi*. (a, b) Section of the dark-coloured skin stained with H&E. Melanophores (Me) lie deep to the epidermis and the only type of granular glands present are Type I (Gg I). (c, d) Sections of the skin of coral spots. Xantophores (Xa) lie deep to the epidermis (arrowhead) and Gg I. Abbreviations: Ep (epidermis); Gg I; Me; Sc (stratum compactum); Ss (stratum spongiosum); Xa. [Colour figure can be viewed at wileyonlinelibrary.com].

Petrohyoidei I–IV surely produce the downward movement of the skull. The group of muscles that contract and lift the head seems to be the *rhomboideus anterior*, *longissimus dorsi*, *Intercruralis I* and *intertransversarius capitis superior*. These muscles connect the axial, appendicular and hyobranchial skeletons with the occipital region. There are no significant differences among them in the three species examined (Fig. 11).

Envenomation symptoms and skin secretion lethality

The individuals manipulated to extract skin secretions produced a milky substance, principally on the head (Fig. 3). The estimated LD₅₀ for Balb/C mice was 4.75 (3.64–6.19) (Table 4). In large doses (10 and 15 µg), the secretion quickly killed the mice, with the death usually being preceded by signs of prostration and neurological disorder evidenced by disrupted movements.

Discussion

Phragmotic behaviour was first described in insects that blockade the entrance of a burrow or a hole using a part of the

body (Wheeler, 1927). Anurans practice phragmosis when they block the entrance of burrows and refuges using their heads (Barbour, 1926; Trueb, 1970b). Within hylids, phragmosis usually is considered a striking anti-predator mechanism, as was observed for *Triprrion petasatus* (Cope, 1865) and *Corythomantis greeningi* (Stuart, 1935; Jared *et al.*, 2005). As a complement, the exposed head offers a dense net of spiny projections usually associated with toxic gland secretions (Jared *et al.*, 2005, 2015). Because most casque-headed hylids inhabit arid environments, some authors have hypothesized that phragmosis evolved as a mechanism to prevent water loss in extreme arid conditions (Duellman & Klaas, 1964; Trueb, 1970b; Seibert, Lillywhite & Wassersug, 1974). However, *Argenteohyla siemersi* inhabits humid environments that are rich in umbellifers and bromeliads within which this frog takes refuge; consequently, previous authors supposed that *A. siemersi* lacks phragmotic behaviour (Trueb, 1970a,b; Cajade *et al.*, 2010). Phragmosis should be most effective if the frog uses a tunnel-like structure to shelter itself (Toledo *et al.*, 2011). However, *A. siemersi* never has been seen in a phragmotic posture in the decidedly untunnel-like refuges it frequents (Ceï & Pierotti, 1955; Lutz & Barrio, 1966; Williams & Bosso, 1994; present work). Nonetheless, the frog can flex its head nearly perpendicular to its trunk when it is handled (phragmotic posture),

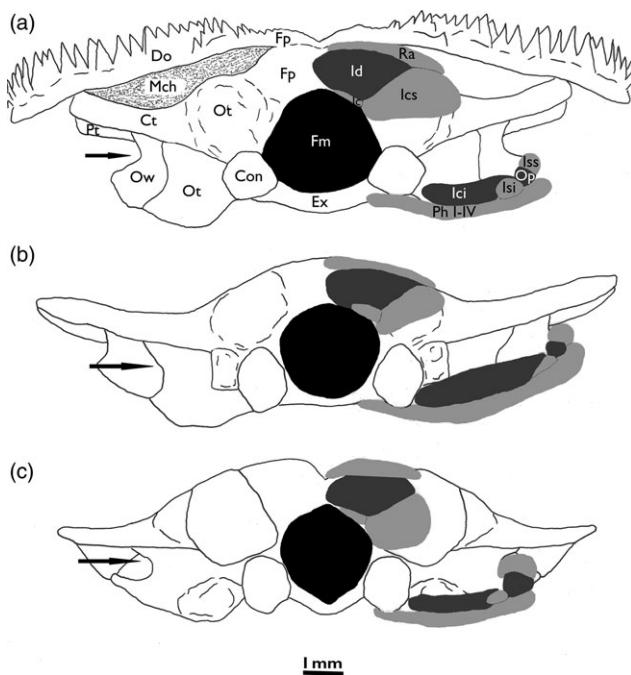


Figure 11 - Posterior view of the skull of *Argenteohyla siemersi* (a), *Trachycephalus typhonius* (b), and *Hysiboas raniceps* (c). Left side of each figure illustrates the arrangement of posterior cranial bones, whereas the right side illustrates the hyobranchial, appendicular and axial muscles that insert in this region. Muscles were immediately adjacent to their points of insertion. Abbreviations: Con (condyle); Do (dermal ossification of frontoparietal); Ct (crista parotica); Ex (exoccipital); Fm (foramen magnum); Fp (frontoparietal); Lci (*m. intertransversarius capitis inferior*); lcs (*m. intertransversarius capitis superior*); Ld (*m. longissimus dorsii*); Lsi (*m. levator scapularis inferior*); Lss (*m. levator scapularis superior*); Mch (muscular channel); Op (*m. opercularis*); Ot (prootic); Ow (oval window); Ph I-IV (*m. petrohyoideus I-IV*); Ra (*m. rhomboideus anterior*). Arrows indicate the middle ear opening.

thereby suggesting that the phragmotic posture in *A. siemersi* is primarily a mechanism to deflect predators. Some authors consider head flexion as a part of phragmotic behaviour and have termed it as ‘chin-tucking behaviour’ employed by the frog to avoid capture and to direct the glands toward the predator (Toledo *et al.*, 2011). Like Lutz & Barrio (1966), we consider *A. siemersi* to be a semi-phragmotic, being a species capable of assuming a phragmotic posture, but incapable of displaying phragmotic behaviour owing to the architecture of their refuges.

Trueb (1973) defined three modes of hyperossification of the anuran skull. (1) Casquing involves the marginal extension of dermal roof bones to form a ‘casqued’ appearance. (2) Co-ossification integrates the skin with underlying dermal bones by ossification of the *stratum compactum* of the dermis, which fuses to underlying dermal bones. (3) Exostosis occurs when additional membrane bone is laid down over dermal cranial elements to form ridges, crests and spines. Like Trueb (1970a), we observed partial co-ossification in the cranium of

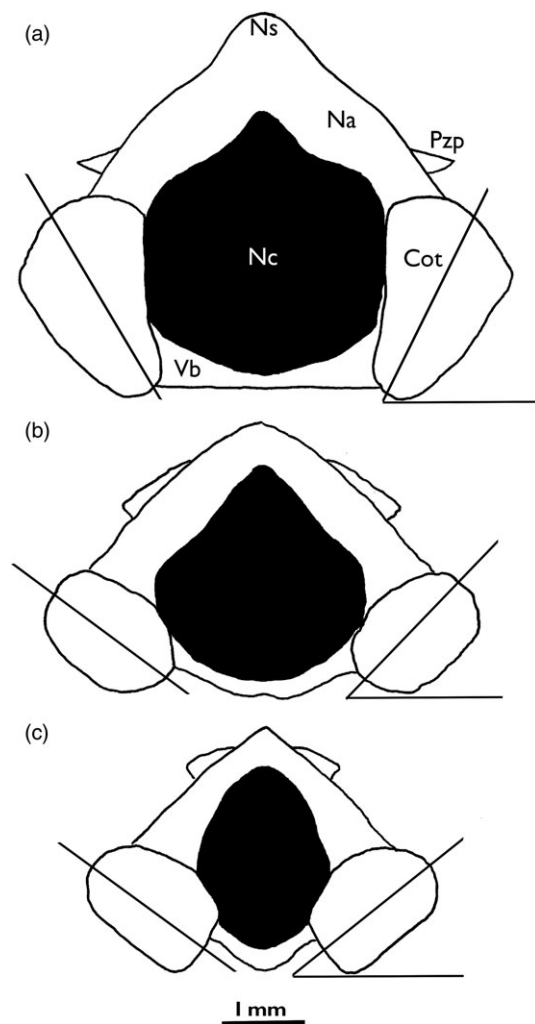


Figure 12 Frontal view of the first vertebra (atlas) of *Argenteohyla siemersi* (a), *Trachycephalus typhonius* (b) and *Hysiboas raniceps* (c). Abbreviations: Cot (cotyle); Na (neural arch); Nc (neural channel); Ns (neural spine); Pzp (postzygapophysis); Vb (vertebral body). Black lines indicate the angle of the long axis of the ovoid cotyle.

Argenteohyla siemersi; thus, the *stratum compactum* is entirely replaced by dermal bone in certain areas of the skull, mainly at sites where spines and protuberances are present (Fig. 7a). Histology reveals that spines, ridges, protuberances, pits and pores of *A. siemersi* principally comply with Trueb’s definition of exostosis, rather than co-ossification, as was proposed by Jared *et al.* (2005, 2015). Such structures may be inferred from plates published by Jared *et al.* (2005, 2015), who studied the dermal histology of the skulls of *Corythomanthis greeningi* and *Aparasphenodon brunoi*. Comparing the three species, *A. siemersi* and *C. greeningi* share sculptural ridges with sinuate edges and spiny projections on roofing bones. *Argenteohyla siemersi* differs from *Aparasphenodon brunoi* in lacking denticulate ridges on the terminal portion of the snout and a radial pattern of ridges on the skull table. All three species have granular glands (Table 2; but not predominantly mucous glands

Table 3 Origin, insertion and comments on the muscles that relate the occipital region of the skull with the axial and scapular skeleton of *Argenteohyla siemersi*

Muscle	Origin	Insertion	Comments
Hyoid muscles			
Depressor mandibulae	Pars scapularis on a fascia upon m. levator scapularis. Pars tympanica on the caudal margin of the tympanic ring and on a fascia upon the m. l. mandibulae longus	Both insert on retroarticular process of the mandible	Pars scapularis clearly wider and higher than pars tympanica. There are variations among species
Branchial muscles			
Omohyoideus	Scapula	Lateral face of the hioideis between the anterolateral and posterolateral processes	Obscured by M. interhyoideus. No variation among species
Hipobranchial muscles			
Sternohyoideus	M. rectus abdominis (lateral fibres) and visceral face of the sternum (medial fibres)	Lateral face of the hioideis between the anterolateral and posterolateral processes	Obscured by m. interhyoideus. No variation among species
Axial muscles			
Longissimus dorsi	Dorsal from first three vertebrae	Occipital region dorsal to the foramen magnum	No variation among species.
Intertransversarius capitis superior	Anterior and distal on first transverse process	Occipital region lateral and dorsal to the foramen magnum tapered by m. longissimus dorsi	Tapered by m. longissimus dorsi. No variation among species
Intertransversarius capitis inferior	Anterior and distal on first transverse process	Occipital region lateral to the foramen magnum	Tapered by both mm. longissimus dorsi and intertransversarius capitis superior. No variation among species
Interspinalis I	Anterior on dorsal face of first vertebra	Occipital region dorsal to foramen magnum	Tapered by m. longissimus dorsi. No variation among species
Appendicular muscles			
Rhomboideus anterior	Anterodorsal margin of the suprascapula	Caudal margin of the frontoparietal and slightly on a fascia upon the m. levator mandibulae longus	The point of insertion is the same that the point at which inserts the m. longissimus dorsi. It varies among species
Cucularis	Medial face of the anteroventral margin of the scapula	Medial and behind the crista parotica and tympanic ring	Composed by two or three muscular layers. No variations among species
Levator scapularis inferior	Medial face of the suprascapula at level of posterior arm of the cleitrum	Bony floor of the posterior part of the capsula auditiva	No variation among species
Levator scapularis superior	Medial face of suprascapula at level of the anterior arm of the cleitrum	Ventral and posterior face of the bony capsula auditiva surrounding the oval window	There are variations among species
Opercularis	Indistinguishable from the m. l. s. superior	Operculum	No variation among species

Table 4 Comparison of LD50 values and details of protocols in *Argenteohyla siemersi*, frogs, and other animals

Animals	Species	LD50 ($\mu\text{g}/\text{mouse}$)	Mice CEPA	Test time (HS)	Confidence limits	Source
Frogs	<i>Argenteohyla siemersi</i>	4.75	Balb/C	48	(3.64–6.19)	This study ^a
	<i>Corythomantis greeningi</i>	69.75	Balb/C	72	(68.42–71.08)	Jared <i>et al.</i> (2005) ^a
	<i>Aparasphenodon brunoi</i>	3.12 ^c	Swiss	Not specified	–	Jared <i>et al.</i> (2015) ^b
	<i>Aparasphenodon brunoi</i>	4.36 ^d	Swiss	Not specified	–	
	<i>Corythomantis greeningi</i>	51.94 ^c	Swiss	Not specified	–	
	<i>Corythomantis greeningi</i>	49.34 ^d	Swiss	Not specified	–	
	<i>Ameerega flavopicta</i>	75.0	–	24	–	Mortari <i>et al.</i> (2004) ^a
	<i>Phyllobates terribilis</i>	0.1	–	–	–	Daly, Spande & Garraffo (2005) ^b
Vipers	<i>Bothrops alternatus</i>	230.7	CF ₂	48	(200–258.3)	Sanchez <i>et al.</i> (1992) ^a
	<i>Bothrops diporus</i>	128.2	CF ₂	48	(111.0–162.8)	
Scorpions	<i>Tityus confluens</i>	14.0	NIH	24	(9.0–21.0)	De Roodt <i>et al.</i> (2009) ^a

Values of LD50 calculated from: $\mu\text{g}/\text{mouse}$ of full secretion (^a) and $\mu\text{g}/\text{mouse}$ of chemical compose concentrated (^b).

(^c) Skin secretion extracted from the head or from the body (^d).

as reported by Trueb (1970a)). *Argenteohyla siemersi* has two types of granular glands, whereas studies on *A. brunoi* and *C. greeningi* have not reported whether these species have different types of granular glands. Granular glands in *Argenteohyla siemersi* are interspersed among the dermal ornaments and are intimately associated with the spines from which the venom secretions could be delivered as it is in *A. brunoi* and *C. greeningi* (Jared *et al.*, 2015). *Argenteohyla siemersi* differs from the other two species in having fewer spiny projections, and projections that primarily are restricted to the loreal region of the nasal; in addition, the maxilla is not expanded. Experimental evidence suggests that the expanded, spiny maxilla of *C. greeningi* and *Aparasphenodon brunoi* (Jared *et al.*, 1999; Navas *et al.*, 2002) provide an efficient mechanism for keeping the animal safe inside a refuge (Jared *et al.*, 2005). *Argenteohyla siemersi* lacks such a modification and does not take refuge in tunnel-like refuges.

Early studies of casque-headed hyloid frogs by Trueb (1970b) suggest that phragmosis is enabled by the contraction of the particularly enlarged *m. rhomboideus anterior*, which seems particularly well developed in phragmotic species. With reference to *Pternohyla* Boulenger 1882, and *Tripurion spatulatus* Günther, 1882, Trueb (1970b:702) said that ‘In most of these frogs this muscle is moderately to greatly enlarged, which enables the frogs to flex the head up to nearly a 90-degree angle to the body.’ The horizontal orientation of this muscle dorsal to the point of flexure (occipital-vertebral juncture) of the skull suggests that contraction of the muscle should move the head up instead of down, as should occur when the frog practices phragmosis. In addition, the *m. rhomboideus anterior* of the species we studied differs from Trueb’s observations (1970b). In fact, *A. siemersi* (phragmotic) has a less-developed *m. rhomboideus anterior* than the two non-phragmotic species that we studied. The single morphological feature that might be related to phragmotic behaviour of *A. siemersi* is the configuration of the occipital condyles and the atlantal cotyles, which are smaller, relatively lower, and more rounded than in the two non-phragmotic species. Conversely, the atlantal cotyles on which the condyles pivot, are dorsoventrally enlarged, taller

and more ovoid in *A. siemersi* than in the non-phragmotic study species.

The skin glands over the entire body and head of *Argenteohyla siemersi* produce a highly lethal secretion that is several times more potent than the venoms of some other animals, such as snakes and scorpions (Table 4). The secretions of *A. siemersi* are more powerful than that of some dendrobatid anurans but less powerful than that of other dendrobatids (Table 4). In comparison to casque-headed frogs for which there are data, the lethality of the secretion of *A. siemersi* is similar to that of *A. brunoi*, but about 10 times more potent than that of *C. greeningi* (Jared *et al.*, 2005, 2015) (Table 4). These values correlate with the most toxic species being the more colourful and the less toxic being less colourful, as has been demonstrated in dendrobatids (Summers & Clough, 2001). *Aparasphenodon brunoi* seems to be an exception to this observation; it has a similar toxicity to *Argenteohyla siemersi*, yet it is a plain brownish colour. However, the LD50 values of these two taxa may not be exactly comparable because that of *Aparasphenodon brunoi* is based on a concentrated chemical compound (protein), whereas that of *Argenteohyla siemersi* is based on full skin secretions. Among the other factors that may be involved, a fewer number of spiny projections could be offset by a high toxicity of the secretions, as were argued for *Aparasphenodon brunoi* and *Corythomantis greeningi* (Jared *et al.*, 2015), and which is consistent for *A. siemersi*. The link between aposematic colouration and diurnal activity is associated with predators such birds, mammals, and fishes that locate their prey visually (Toledo & Haddad, 2009). The bright colouration, together with the high toxicity in the red-spotted Argentina frog is compatible with an aposematic mechanism to protect the frogs during explosive reproductive events (Cajade *et al.*, 2010).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Material examined.

Video S1. Brief phragmosis.

Video S2. Persistent phragmosis.

Video S3. Without phragmosis.