

Skin structure variation in water frogs of the genus *Telmatobius* (Anura: Telmatobiidae)

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Abstract. Skin structure is studied in a broad sample of frog species of the genus *Telmatobius* and its relatives. These frogs exhibit different ecological habits and occupy different habitats. The results demonstrate that the coexistence of two types of serous glands, a rare feature among anurans, is widespread in *Telmatobius*. These types of serous glands, called Types I and II, are characterized by different sizes of their granules. However, some strictly aquatic species of the genus have only one type of serous glands (Type I); this feature might be interpreted, within *Telmatobius*, as the result of independent losses of serous glands of Type II. Another finding was the occurrence of the Eberth-Kastschenko (EK) layer in the dermis of almost all studied species of *Telmatobius*. This result was unexpected, because the EK layer is generally absent in aquatic anurans and was thought of as absent in *Telmatobius*. However, there are differences in its thickness that, combined with data of ecological habits and main habitats, reveal a complex pattern within *Telmatobius*, as well as within and between the other studied genera. Although we are far from understanding the significance of the presence of two types of serous glands in *Telmatobius* or the functions of the EK layer in general, these taxonomic and ecologic patterns could guide future research.

Key words. Skin glands, serous glands, Eberth-Kastschenko layer, calcified layer, aquatic frogs, semiaquatic frogs, terrestrial frogs, Andes.

Introduction

The occupation of terrestrial environments has been a major step in the evolution of tetrapods. A pluristratified integument with glands embedded in the dermis constitutes an important advancement from the integument of fishes (NOBLE 1931). Although of different embryological origins, in amphibians the epidermis (ectodermal) and dermis (mesodermal) co-form a unit with several functions. These functions include mechanical protection, chemical defence, respiration, osmoregulation, water balance, sensorial perception, and others more (e.g., DUELLMAN & TRUEB 1994, FOX 1994). The ectodermal glands are integrated in the dermis and classified as either mucous or serous glands, depending on intrinsic characteristics and their kinds of secretion. The gland's secretions play a central role in various functions of the skin (FOX 1986, TOLEDO & JARED 1995, CLARKE 1997). In the dermis of anurans, a calcified layer between the stratum compactum and stratum spongiosum may occur. The occurrence of this layer, also called the Eberth-Kastschenko (EK) layer, has been correlated to the mode of life (ELKAN 1968, 1976, TOLEDO & JARED 1993).

More than 6,400 known species of anurans (FROST 2015) have diversified to occupy extremely different environments, from hyper-wet rainforests to deserts, and from sea level to ponds of melting glaciers at more than 5,000 m a.s.l. (SEIMON et al. 2007). Several lineages have specialized in living in extremely arid conditions, and the modifications of their skin related to these habitats have received considerable attention (e.g., BENTLEY 1966, KOBELT & LINSSENMAIR 1986). By contrast, a strictly aquatic specialisation is less common amongst anurans. Strictly aquatic anurans normally spend the largest part of their lifetimes in water and are capable, for example, of feeding underwater. Additionally, these species may exhibit some of the morphological traits associated with an aquatic lifestyle such as a lateral line system, reduced tongue, reduced lungs, and bagginess of the skin. Globally, only a few species can be regarded as strictly aquatic (e.g., Pipidae, *Barbourula kalamantanensis*, *Lankanectes corrugatus*, and several species of *Telmatobius*).

Frogs of the genus *Telmatobius* live in Andean streams and lakes from Ecuador to Argentina. At present, 62 species have been described (FROST 2015). Although all species of the genus are usually considered aquatic in relation to oth-

er species of anurans, they actually show a more complex ecological pattern. The genus includes semiaquatic species living in streams in forested or semiarid habitats (VELLARD 1951, LAURENT 1970, 1973, WIENS 1993, DE LA RIVA 1994, BARRIONUEVO in press) to strictly aquatic species living in drier highland habitats (PARKER 1940, VELLARD 1951, DE LA RIVA 2005, FORMAS et al. 2005, BARRIONUEVO 2016). This diversity with regard to habitat specialisation in *Telmatobius* constitutes a promising scenario to evaluate characters associated with skin structure.

Several studies have dealt with the morphological variation of the skin in *Telmatobius*. Some characters of the skin, such as bagginess and vascularisation, have been described in relation to extreme aquatic habits in *T. culeus* (ALLEN 1922, VELLARD 1951, DE MACEDO 1960, ACCAME MURATORI et al. 1976, HUTCHISON 1976), *T. brachydactylus* and *T. macrostomus* (CZOPEK 1983). Histological skin character states for systematic studies of *Telmatobius* have been described for seven species from central Peru (SINSCH et al. 2005), and the intraspecific variation was explored in one of these species, *T. carrillae* (SINSCH & LEHR 2010). One of the most important findings of SINSCH et al. (2005) was the occurrence of two types of serous glands in the skin of some species of *Telmatobius*, which is a rare feature in anurans. Two types of serous glands have previously been described from a few species of different anuran clades [i.e., Bombinatoridae (DELFINO et al. 1982), Bufonidae (DELFINO et al. 1998, DELFINO et al. 1999), Leiopelmatidae (MELZER et al. 2011), and Hylidae (DELFINO et al. 1998, BRUNETTI et al. 2012, 2014)]. Several functions of specialized serous glands in anurans have been proposed (BRUNETTI et al. 2012, DELFINO et al. 1982, 1998, 1999), and in some cases, these functions have been related with the type of habitat (MELZER et al. 2011). In *Telmatobius*, the taxonomic distribution of the coexistence of two types of serous glands is still unknown as is its biological significance.

The presence of the EK layer in anurans has been interpreted as a feature that can reduce or prevent water loss (ELKAN 1968, 1976, TOLEDO & JARED 1993). Three lines of evidence have been taken into account for this association (ELKAN 1968, ELKAN & COOPER 1980, GARCÍA et al. 2011, TOLEDO & JARED 1993): (i) the chemical composition of the layer, which is constituted by hydrophilic glycosaminoglycans as the fundamental substance, (ii) it is more developed in the dorsal than in the ventral skin, and (iii) it is generally present in terrestrial species and absent in aquatic species. In the genus *Telmatobius*, the occurrence of the EK layer has been previously evaluated in *T. brachydactylus*, *T. culeus*, *T. jelskii*, *T. macrostomus*, and *T. marmoratus*. In these species, the EK layer was reported as absent and this condition was extrapolated to apply to the entire genus (ELKAN 1968, 1976).

The goals of the present contribution are (i) to study the taxonomic distribution of the serous glands types and the EK layer in a broad sample of species of *Telmatobius* and their relatives, and (ii) to analyse the results in the light of the available information regarding habits and habitats as well as the known phylogenetic relationships.

Materials and methods

Fifty-one skin samples of 17 species of *Telmatobius* from tree main types of habitat were included in this study as follows (number of specimens and habits in parenthesis): (i) highlands: *T. atacamensis* (4, aquatic), *T. culeus* (4, aquatic), *T. hauthali* (4, aquatic), *T. marmoratus* (1, aquatic), *T. platycephalus* (1, aquatic), *T. rubigo* (1, aquatic), (ii) dry inter-Andean valleys: *T. laticeps* (5, semiaquatic), *T. pinguius* (2, semiaquatic), *T. pisanoi* (4, semiaquatic), *T. scrochii* (5, semiaquatic), *T. simonsi* (2, semiaquatic); (iii) wet forested slopes: *T. bolivianus* (2, aquatic), *T. ceiorum* (2, semiaquatic), *T. schreiteri* (4, semiaquatic), *T. stephani* (3, semiaquatic), and *T. verrucosus* (2, semiaquatic). *Telmatobius oxycephalus* (5, semiaquatic) inhabit two types of habitat, dry inter-Andean valley and wet forested slopes.

Samples of 11 species of other genera were included. These genera have been found to be related to *Telmatobius* in previous phylogenetic hypothesis (LYNCH 1978, FROST et al. 2005, GRANT et al. 2006, PYRON & WIENS 2011) and live in various habitats. These are (number of specimens and habits in parenthesis): (i) wet and sub-wet temperate forest: *Alsodes neuquensis* (1, aquatic), *Atelognathus nitoi* (2, terrestrial), *Batrachyla taeniata* (2, terrestrial), *Eupsophus roseus* (1, terrestrial), *Hylorina sylvatica* (1, terrestrial), *Insuetophrynus acarpicus* (1, terrestrial); (ii) Patagonian steppe: *Atelognathus patagonicus* (2, aquatic), *Atelognathus reverberii* (2, terrestrial); (iii) Chacoan dry forest: *Ceratophrys cranwelli* (2, terrestrial), *Chacophrys pierottii* (2, terrestrial), and *Lepidobatrachus llanensis* (2, aquatic). All studied specimens with their collection numbers are detailed in Appendix 1. Museum acronyms are as follows: Fundación Miguel Lillo (FML), field number of S. BARRIONUEVO of specimens to be housed in FML (FML-SB), Museo de La Plata (MLP), Colección Boliviana de Fauna (CBF), Museo Argentino de Ciencias Naturales (MACN), Colección Herpetológica del Centro Nacional de Investigaciones Iológicas, currently housed in MACN (CENAI); field number of J. FAIVOVICH of specimens to be housed in MACN (MACN-JF), and field number of B. BLOTTO of specimens to be housed in MACN (MACN-BB).

A small strip of mid-dorsal skin (approx. 5–10 mm²) was removed from each previously fixed specimen. These skin samples were dehydrated in a graded ethanol series, cleared in butyl alcohol, paraffin-embedded, sectioned in transverse plane (10 µm thick), and mounted onto microscope slides. Sections were exposed to PAS and Alcian Blue (AB) 8GX to stain mucous secretion and detect glycosaminoglycans, and with Hematoxylin (H) to stain the basophilic granules of serous glands. Stained sections were examined using a Nikon Eclipse E200 microscope, and the micrographs were captured using a Nikon DS-U2 digital camera. Measurements from micrographs were taken with ImageJ v. 1.48. The thickness of the EK layer was measured at 611 sites, with a mean of 38.2 sites per species. The values of EK thickness are given throughout the text as mean ± standard error. To evaluate the variation in EK layer thickness, I conducted three one-way ANOVAs with three dif-

Table 1. Analysed skin features in 17 species of *Telmatobius* with their habits and main habitats. Thickness EK in μm (mean \pm standard error).

	Type-I serous glands	Type-II serous glands	EK layer	Thickness EK [μm]	Habits	Habitat
<i>T. atacamensis</i>	present	present	absent/present	3.4 \pm 0.22	aquatic, stream-dweller	highlands – dry Puna (Argentina)
<i>T. bolivianus</i>	present	absent	present	3.42 \pm 0.26	aquatic, stream-dweller	montane forest (Bolivia)
<i>T. ceiorum</i>	present	present	present	5.02 \pm 0.44	semiaquatic, stream-dweller	montane forest (Argentina)
<i>T. culeus</i>	present	absent	absent	–	aquatic, lake-dweller	highlands – wet Puna (Bolivia)
<i>T. hauthali</i>	present	present	present	2.35 \pm 0.13	aquatic, stream-dweller	highlands – dry Puna (Argentina)
<i>T. laticeps</i>	present	present	present	12.33 \pm 0.86	semiaquatic, stream-dweller	dry inter-Andean valley (Argentina)
<i>T. marmoratus</i>	present	present	present	1.88 \pm 0.17	aquatic, stream-dweller	highlands – wet Puna (Bolivia)
<i>T. oxycephalus</i> (Tilcara)	present	present	present	5.36 \pm 0.34	semiaquatic, stream-dweller	dry inter-Andean valley (Argentina)
<i>T. oxycephalus</i> (Calilegua)	present	present	present	1.97 \pm 0.13	semiaquatic, stream-dweller	montane forest (Argentina)
<i>T. pinguculus</i>	present	present	present	6.99 \pm 0.5	semiaquatic, stream-dweller	dry inter-Andean valley (Argentina)
<i>T. pisanoi</i>	present	present	present	8.33 \pm 0.58	semiaquatic, stream-dweller	dry inter-Andean valley (Argentina)
<i>T. platycephalus</i>	present	present	present	4.46 \pm 0.28	aquatic, stream-dweller	highlands – dry Puna (Argentina)
<i>T. rubigo</i>	present	present	present	3.05 \pm 0.41	aquatic, stream-dweller	highlands – dry Puna (Argentina)
<i>T. schreiteri</i>	present	present	present	2.88 \pm 0.18	semiaquatic, stream-dweller	montane forest (Argentina)
<i>T. scrocchii</i>	present	present	present	8.68 \pm 0.72	semiaquatic, stream-dweller	dry inter-Andean valley (Argentina)
<i>T. simonsi</i>	present	present	present	17.19 \pm 0.66	semiaquatic, stream-dweller	dry inter-Andean valley (Bolivia)
<i>T. stephani</i>	present	present	present	4.44 \pm 0.26	semiaquatic, stream-dweller	montane forest (Argentina)
<i>T. verrucosus</i>	present	present	present	1.64 \pm 0.15	semiaquatic, stream-dweller	montane forest (Bolivia)

ferent factors: (i) species, (ii) habitats, and (iii) habits. Post hoc LSD tests were used for multiple comparisons. The habits were classified in two categories (aquatic and semiaquatic) whereas the habitats were classified in three (highlands, dry inter-Andean valleys, and forests). I employed the SPSS software package, version 19.0 (2010, SPSS Inc., Chicago, Illinois, USA).

Results

Serous glands

The dorsal integument of all studied species has the typical structure of anurans and consists of a multi-stratified epidermis and a dermis composed of two layers: the more superficial stratum spongiosum and the stratum compactum. The serous and mucous glands are embedded in the stratum spongiosum. The mucous glands are smaller and more superficially distributed in the stratum spongiosum than the serous glands. The content of the mucous glands can react with PAS, with AB or both. In 15 of the 17 studied species of *Telmatobius*, two types of serous glands are present (Fig. 1, Table 1): one type containing small homogeneous granules (Type I), and other type containing large heterogeneous granules (Type II). These two types of glands do not represent two maturation stages of the same type of

gland, because no intermediate serous gland morphology between the two types was found. Both the small and the large granules can be stained with hematoxylin. The small granules have a mean diameter of 1.5 μm (0.8–2.5 μm), whereas the large granules have a mean diameter of 4.2 μm (2.8–5.9 μm). In two species (*T. culeus* and *T. bolivianus*), only Type-I glands are present (Table 1).

Among the studied species of other genera, only serous glands of Type II were found (Fig. 1) with the exception of *Hylorina sylvatica* that has only Type-I serous glands (Fig. 1; Table 2). In *Alsodes neuquensis*, the granules of the serous glands have a diameter that is larger than in all other species (mean 13.2 μm [9.5–17.3 μm]).

EK layer

In *Telmatobius*, a calcified or EK layer is present in all species studied herein with the exception of *T. culeus* (Fig. 2, Table 1). In *T. atacamensis*, the presence of an EK layer appears to be intraspecifically variable, because it is absent in two specimens (FML-SB 156, 225). In all species where it is present, the EK layer could be stained with AB and is restricted to the boundary between the stratum spongiosum and stratum compactum. The EK layer occurs in traces in *T. atacamensis*, *T. bolivianus*, *T. marmoratus*, *T. platycephalus*,

lus, *T. rubigo*, and *T. verrucosus* and in some specimens of *T. oxycephalus* (FML 12070, 12079). In these cases, the EK layer is normally absent from areas with well-developed serous glands (Fig. 2A). The thickness of the EK layer is notably variable within the genus, ranging between 1 and 20 μm (Fig. 3, Table 1). The thinner layer (less than 2 μm of mean thickness) is found in *T. verrucosus*, *T. marmoratus*, and some specimens of *T. oxycephalus* (FML 12070, 12079, Fig. 2C), whereas a mean between 2 and 5 μm is found in *T. atacamensis*, *T. bolivianus*, *T. ceiorum*, *T. hauthali*, *T. platycephalus*, *T. rubigo*, *T. schreiteri*, and *T. stephani*. An EK layer with a mean thickness > 5 μm is found in *T. laticeps*, *T. pinguiculus*, *T. pisanoi*, *T. scrocchii*, *T. simonsi*, and

in some specimens of *T. oxycephalus* (FML-SB 016, 019, 021). Figure 3 shows that species with the thicker EK layer are distributed in dry inter-Andean valleys, whereas a thinner layer is found in species from highlands and forests.

Due to unequal sample sizes, the results of ANOVA should be taken as an exploratory approach. A one-way ANOVA with species as a factor indicates significant differences in EK layer thickness between the 16 species where the layer is present ($F_{15,17} = 7.042$, $P = 0.000$). A post hoc test of multiple comparisons cannot be performed because four species are represented only by one specimen. For this reason, another ANOVA was conducted on a subsample comprising the 12 species represented by more than one individual (*T. atacamensis*, *T. bolivianus*, *T. ceiorum*, *T. hauthali*, *T. laticeps*, *T. oxycephalus*, *T. pinguiculus*, *T. pisanoi*, *T. schreiteri*, *T. scrocchii*, *T. simonsi*, and *T. stephani*). This analysis revealed that *T. laticeps*, *T. pisanoi*, *T. scrocchii*, and *T. simonsi* have a significantly thicker layer ($F_{11,17} = 8.631$, $P = 0.000$) than the other species.

The results of a one-way ANOVA on the entire dataset with the ecological habits as a factor (aquatic vs semiaquatic) indicated that semiaquatic species have thicker layers than aquatic species ($F_{1,27} = 5.401$, $P = 0.028$), whereas an

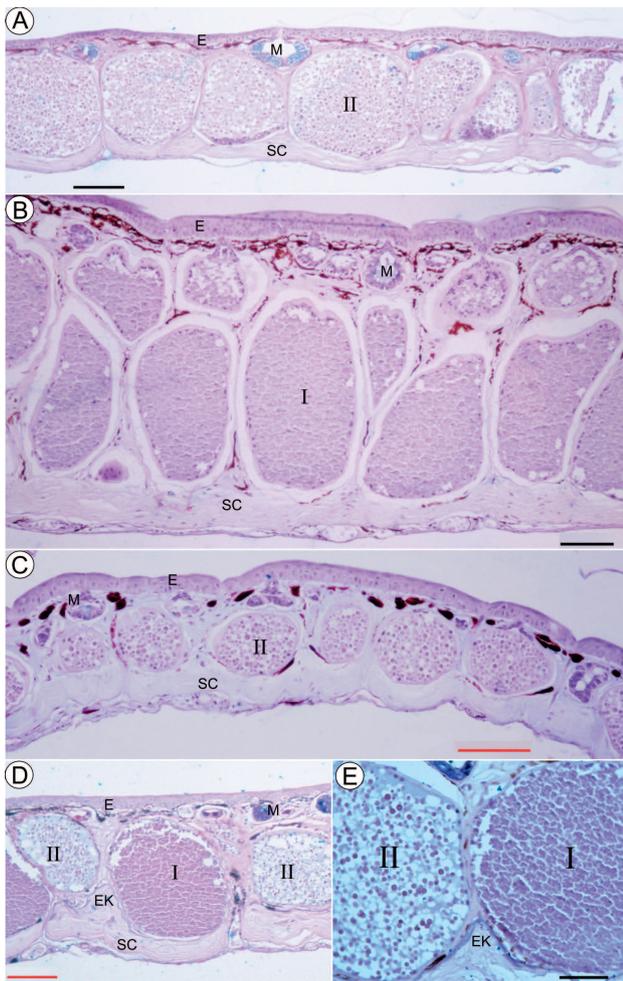


Figure 1. Light micrographs of cross sections of the dorsal skin region of: (A) *Eupsophus roseus*, (B) *Hylorina sylvatica*, (C) *Batrachyla taeniata*, and (D, E) *Telmatobius pinguiculus*. Type-II serous glands are evidently present in *Eupsophus* and *Batrachyla* (A, C) and those of Type I in *Hylorina* (B) whereas an EK layer is absent in these three species. In *Telmatobius pinguiculus*, both types of serous glands and the EK layer are evident at different magnifications (D, E). Abbreviations: E – epidermis; EK – Eberth-Kastschenko layer; M – mucous gland; I – serous gland Type I; II – serous glands Type II; SC – stratum compactum. Bar = 100 μm (A, B, C, D), bar = 50 μm (E).

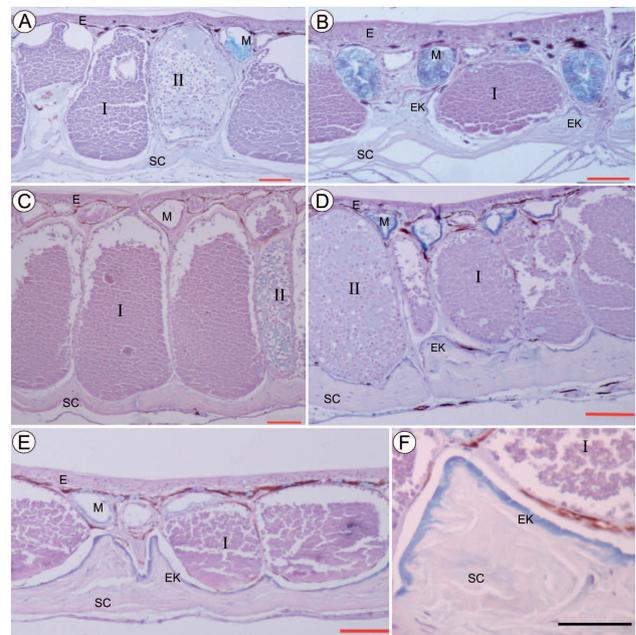


Figure 2. Light micrographs of cross sections of the dorsal skin region of some species of *Telmatobius*: (A, B) *Telmatobius atacamensis*; (C, D) *T. oxycephalus*; and (E, F) *T. pisanoi*. The EK layer is completely absent (A) or present in isolated traces (B) in specimens of the strictly aquatic *T. atacamensis* from the same locality. In specimens of *T. oxycephalus* from a wet forest (C) the EK is absent or present but very thin, although the EK layer is well developed in specimens from a dry valley (D). The EK layer is present and well developed in all studied specimens of *T. pisanoi* (E, F). Abbreviations: E – epidermis; EK – Eberth-Kastschenko layer; M – mucous gland; I – serous gland Type I; II – serous glands Type II; SC – stratum compactum. Bar = 100 μm (A, B, C, D, E), bar = 50 μm (F).

Table 2. Analysed skin features in selected species of *Atelognathus*, *Alsodes*, *Batrachyla*, *Eupsophus*, *Hylorina*, *Insuetophrinus*, *Ceratophrys*, *Chacophrys* and *Lepidobatrachus* with their habits and main habitats. * data from MANGIONE et al. (2011) and QUINZIO & FABREZI (2012). Thickness EK in μm (mean \pm standard error)

	Type I serous glands	Type II serous glands	EK layer	Thickness EK [μm]	Habits	Habitat
<i>Atelognathus nitoi</i>	absent	present	present	0.59 \pm 0.04	terrestrial	Ecotone forest
<i>Atelognathus patagonicus</i>	absent	present	absent	–	aquatic	Patagonian lagoons
<i>Atelognathus reverberii</i>	absent	present	present	1.47 \pm 0.10	terrestrial	Patagonian steppe
<i>Alsodes neuquensis</i>	absent	present	absent	–	aquatic	Ecotone forest
<i>Batrachyla taeniata</i>	absent	present	absent	–	terrestrial	Valdivian forest
<i>Eupsophus roseus</i>	absent	present	absent	–	terrestrial	Valdivian forest
<i>Hylorina sylvatica</i>	present	absent	absent	–	terrestrial	Valdivian forest
<i>Insuetophrinus acarpicus</i>	absent	present	absent	–	terrestrial	Valdivian forest
<i>Ceratophrys cranwelli</i>	absent	present	present	15*	terrestrial	Chaco
<i>Chacophrys pierottii</i>	absent	present	present	10–25*	terrestrial	Chaco
<i>Lepidobatrachus llanensis</i>	absent	absent	present	15–30*	aquatic	Chaco

ANOVA with the habitat as a factor revealed that species living in dry inter-Andean valleys have significant thicker layers ($F_{2,26} = 11.905$, $P = 0.000$) than the species living in highlands and forests.

To evaluate separately the influence of habits (aquatic vs semiaquatic) on a subsample distributed only in dry habi-

tats (highlands and dry inter-Andean valleys), an ANOVA with habits as a factor was performed. This analysis shows that semiaquatic species living in dry habitats have significant thicker layers than the aquatic species ($F_{1,270} = 14.203$, $P = 0.001$). An ANOVA with the habits as a factor on a subsample distributed only in wet habitats (montane for-

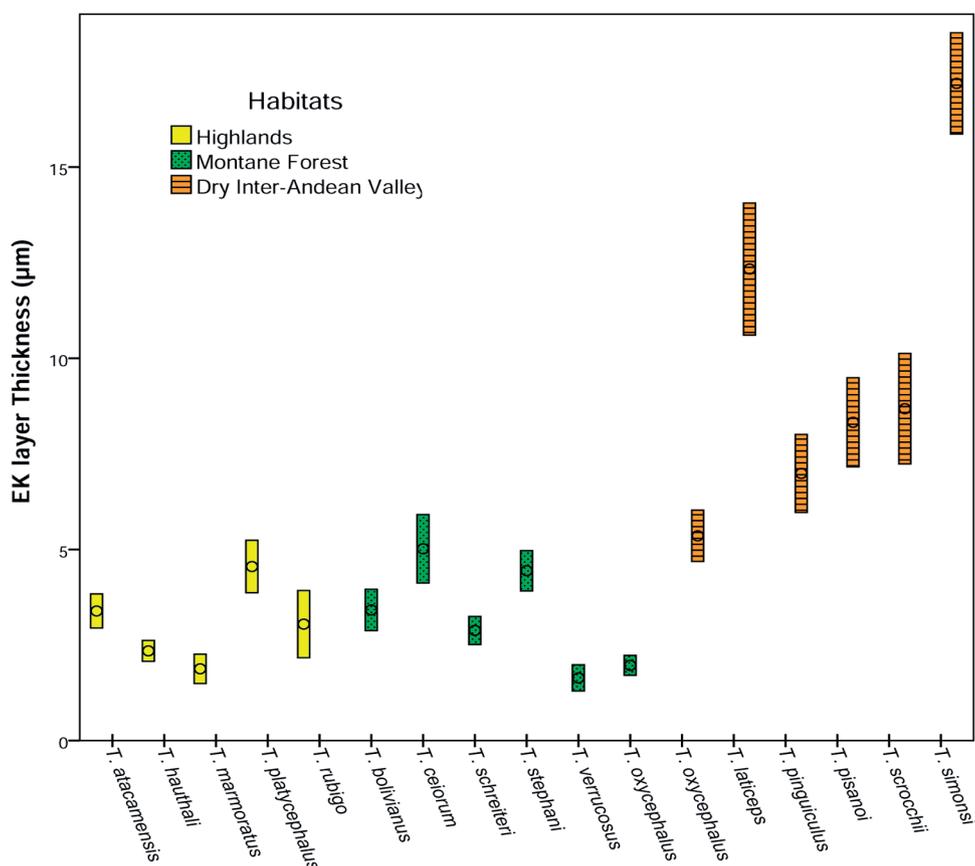


Figure 3. EK layer thickness of the studied *Telmatobius* species depicted as an error bar plot. Each bar represents the mean \pm standard error in μm .

ests) shows no significant differences between aquatic and semiaquatic species ($F_{1,9} = 0.002$, $P = 0.964$). Similarly, to evaluate the influence of the habitat on species of similar ecologic habits, an ANOVA with habitats as a factor on a subsample including only the semiaquatic species shows that specimens distributed in dry inter-Andean valleys have significant thicker layers than those distributed in the wet montane forests ($F_{1,22} = 15.172$, $P = 0.001$). An ANOVA with the habitat as a factor on a subsample including only aquatic species shows no significant differences between habitats ($F_{1,6} = 1.326$, $P = 0.293$).

Among the other genera, the EK layer is absent in *Eupsophus roseus*, *Batrachyla taeniata*, *Hylorina sylvatica*, *Atelognathus patagonicus*, *Insuetophrynus acarpicus*, and *Alsodes neuquensis* (Figs 1A, B, C, Tab. 2), whereas it is present in *Atelognathus nitoi* and *A. reverberii*. The EK layer is discontinuous in both species of *Atelognathus*, but thicker in *A. reverberii* than in *A. nitoi* (Table 2). Amongst Ceratophryidae, as is well known, the EK layer is present, and it is thick in the three species analysed (*Ceratophrys cranwelli*, *Chacophrys pierottii*, and *Lepidobatrachus llanensis*).

Discussion
Serous glands

SINSCH et al. (2005) have previously described the two types of serous glands from four species of *Telmatobius* (*T. hockingi*, *T. jelskii*, *T. mayoloi*, and *T. rimac*) and reported the absence of Type-II glands in three species (*T. macrostomus*, *T. brachydactylus*, and *T. carrillae*). The results presented here provide a wider picture in which it is evident that the presence of two types of serous glands is widespread in *Telmatobius*. This is confirmed for 15 of the 17 studied species of *Telmatobius*. Type II is absent in *T. culeus* and *T. bolivianus*. The coexistence of two types of serous glands is not observed in the other genera included in this study. The published phylogenetic hypotheses of the genus *Telmatobius* are not comprehensive (AGUILAR & VALENCIA 2009, DE LA RIVA et al. 2010, SÁEZ et al. 2014), and only a few species with known phylogenetic positions are represented in the current taxon sampling. Despite this limitation, the mapping of characters describing the presence of gland types on the available phylogenies (Figs 4+5) shows that the co-occurrence of the two types of serous glands would be the basal condition in *Telmatobius*, whereas the

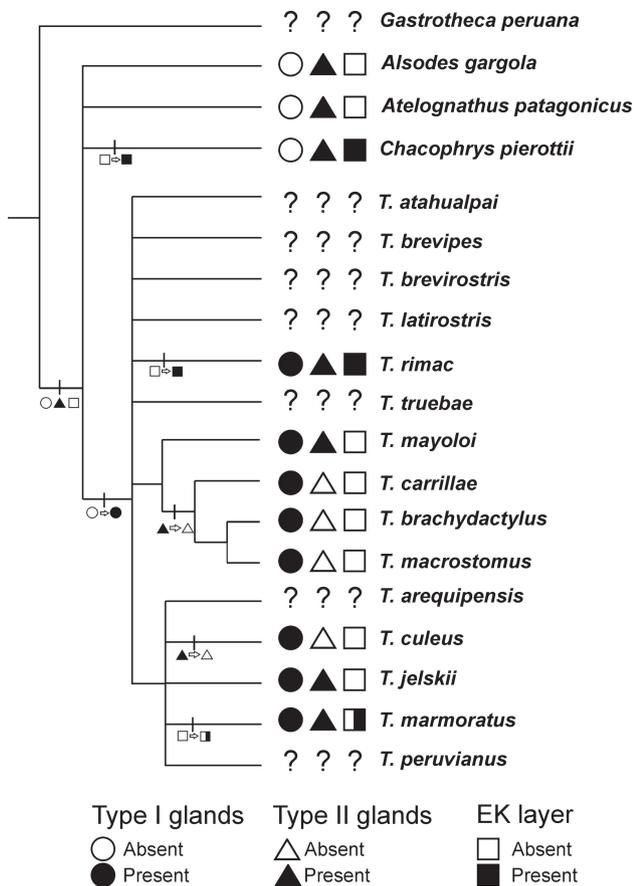


Figure 4. Optimisation of serous glands and EK layer characters in the phylogenetic hypothesis proposed by AGUILAR & VALENCIA (2009).

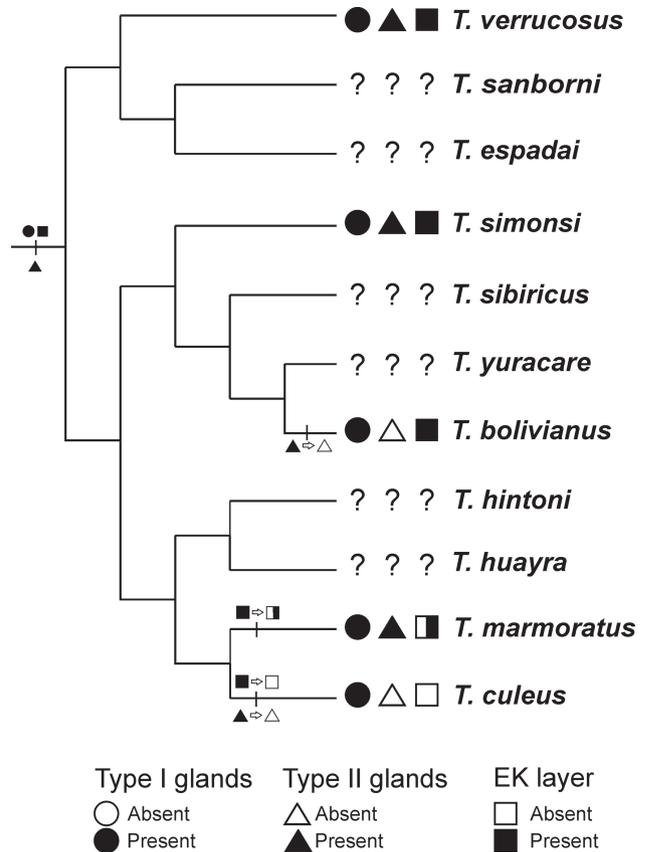


Figure 5. Optimisation of serous glands and EK layer characters in the phylogenetic hypothesis proposed by DE LA RIVA et al. (2010).

loss of serous glands of Type II would have occurred three times within *Telmatobius*. The losses occur independently in *T. culeus*, in *T. bolivianus*, as well as in the clade containing *T. macrostomus*, *T. brachydactylus*, and *T. carrillae*. Due to the limitations mentioned above these conclusions must be confirmed in the context of a comprehensive phylogenetic hypothesis.

SINSCH & LEHR (2010) related the absence of glands of Type II in *T. macrostomus*, *T. brachydactylus*, and *T. carrillae* to the ecological demands of high altitud habitats (4,000–4,600 m a.s.l.) and added that *T. mayoloi*, which is also distributed at similar altitudes, possesses a low frequency of Type-II glands. Although they did not mention such demands, a more aquatic lifestyle has been interpreted as a response to living at high altitudes (VELLARD 1951). *Telmatobius culeus* also lacks serous glands type II, live at lower altitudes (3,800 m), but is one of the most aquatic species of the genus. The other species, that lacks type II glands, *Telmatobius bolivianus*, lives at even lower altitudes (2,000 m) but is one of the most aquatic members of the *T. bolivianus* group (DE LA RIVA 2010, SAEZ 2014).

The widespread occurrence of two types of serous glands in *Telmatobius* is intriguing. This is a rare condition in anurans and it has been associated with several functions such as reproduction, communication, and a more specialised defence against predators (DELFINO et al. 1982, 1998a, b, MELZER et al. 2011, BRUNETTI et al. 2012, 2014). The available evidence in *Telmatobius* is still too patchy to associate this feature with a possible function.

EK layer

Even though ELKAN (1968, 1976) regarded the EK layer as absent in *Telmatobius*, the broad sample of taxa analysed here reveals that the EK layer is in fact present in most species of *Telmatobius*. The sample of taxa analysed by ELKAN (1968, 1976) included *T. brachydactylus* (as *Batrachophrynus brachydactylus*), *T. culeus*, *T. jelskii*, *T. macrostomus*, and *T. marmoratus*. Although the absence of an EK layer was confirmed here for *T. culeus*, the condition of *T. marmoratus* seems to be variable because the EK layer is present in the specimen analysed here. ELKAN (1968) stated that the EK layer is absent in *T. marmoratus* although he reported weak traces in one specimen (as *T. marmoratus angustipes*). This variation was not included in his discussion, and the EK layer considered absent in this species.

SINSCH et al. (2005), in their histological analysis of skin variation within *Telmatobius*, did not evaluate the occurrence of the EK layer. However, from the published figures (SINSCH et al. 2005, p. 247, Figs 9B–G), it is evident that the layer is absent in *T. jelskii*, *T. macrostomus* (as *Batrachophrynus macrostomus*), and *T. brachydactylus* (as *B. brachydactylus*), confirming ELKAN's observations in these species. Additionally, it is evident from the figures presented by SINSCH et al. (2005) that the EK layer is absent in *T. carrillae*, *T. hockingi* and *T. mayoloi*, whereas it is present in *T. rimac* (SINSCH et al. 2005, p. 247, Fig. 9H).

Considering published and new data, it is possible to evaluate the occurrence of the EK layer in 25 species of the genus. Within this sample, the EK layer is present in 18 species. The optimisation of this character in available phylogenetic hypothesis (AGUILAR & VALENCIA 2009, DE LA RIVA et al. 2010) shows a high level of homoplasy (Figs 4+5). However, as was mentioned above, the low taxon overlapping between phylogenetic and histological studies result in numerous missing entries. For this reason, it is not possible to conclude if a phylogenetic pattern exists.

The evaluation of the condition of the EK layer with the available information on the ecologies and distribution ranges of the species shows that the layer is absent in the two larger lacustrine species of the genus, *T. culeus* and *T. macrostomus*. These species spend their entire life cycles exclusively in large Andean lakes (GARMAN 1876, BARBOUR & NOBLE 1920, VELLARD 1951) such as Lakes Titicaca (*T. culeus*) and Junín (*T. macrostomus*). The EK layer is also absent in the species living in the highland streams of the Peruvian Wet Puna, *T. brachydactylus*, *T. carrillae*, *T. hockingi*, *T. jelskii*, and *T. mayoloi* (SINSCH et al. 2005). As far as some highland species are concerned (*T. atacamensis* and *T. marmoratus*), the EK layer is absent in some individuals and present in others.

ELKAN (1968, 1976) mentioned that the thickness of the layer might vary between 5 and 30 μm . In *Telmatobius*, its thickness varies considerably, being thinner than the range mentioned by ELKAN (Fig. 3) in several instances. The species with a layer of less than 5 μm thick live in highland streams (*T. atacamensis*, *T. hauthali*, *T. marmoratus*, *T. platycephalus*, and *T. rubigo*) and montane forest streams (*T. bolivianus*, *T. ceiorum*, *T. oxycephalus*, *T. schreiteri*, *T. stephani*, *T. verrucosus*). A thicker EK layer, with a mean $> 5 \mu\text{m}$, is found in the species living in the streams of dry inter-Andean valleys (*T. laticeps*, *T. pinguiculus*, *T. pisanoi*, *T. scrocchii*, and *T. simonsi*). The published information suggests that the highland species are highly aquatic (PARKER 1940, VELLARD 1951, DE LA RIVA 2005). They are normally found underwater, and evidence from their stomach contents indicates that they feed mostly on aquatic prey (LAVILLA 1984, FORMAS et al. 2005, BARRIONUEVO 2016). On the other hand, the species distributed at lower altitudes and inhabiting streams in montane forest and inter-Andean valleys are, in general, semiaquatic. They can be found both in the water and on land (VELLARD 1951, LAURENT 1970, 1973, WIENS 1993, DE LA RIVA 1994), and their diet consists both of terrestrial and aquatic prey (LAVILLA 1984, WIENS 1993, BARRIONUEVO 2016).

The condition of the EK layer in the semiaquatic *T. oxycephalus* deserves some commenting. This species is widely distributed and occupies different habitats (arid inter-Andean valley and wet forested slopes). Specimens from both habitats have been analysed in the present study. The EK layer of the specimens from Tilcara, situated in an arid inter-Andean valley, has a mean thickness of 5.8 μm (4.3–7.4 μm). By contrast, the specimens from the streams of the montane forests of Calilegua exhibit a poorly devel-

oped and discontinuous EK layer with a mean thickness of 1.96 μm (1.87–2.08 μm). The wet forested slopes of Calilegua are situated 50 straight-line km to the east of Tilcara, but separated by an orographic barrier. Curiously, a similar pattern of intraspecific variation has been described for *Leptodactylus fuscus* and *L. latinasus*. The samples of these two species collected in Calilegua have a thinner EK layer than the samples from Salta in the dry valley of Lerma (GARCÍA et al. 2011). Seasonality has been proposed as a factor influencing the intraspecific variation of the thickness of the EK layer (PORTO 1936, ELKAN 1968), however, GARCÍA et al. (2011) emphasized that in the case of *L. fuscus*, all specimens were collected in summer, suggesting the influence of additional or other factors. The analysis of seasonality requires samples of the same species from the same locality collected at different times of the year. This kind of analysis is not possible with the sample currently available, and it is far beyond the scope of this contribution. However, the only species where such a comparison can be done is *T. oxycephalus* from Tilcara, located in a dry inter-Andean valley. Two specimens collected in spring (FML-SB 019, 021) have a mean thickness of 5.4 and 4.3 μm respectively, whereas the specimen collected in summer (FML-SB 016) has a mean thickness of 7.39 μm . Although the sample size is too small to come to any conclusion, these results do not match the observations by PORTO (1936) on *Rhinella arenarum*. This author stated that the thickness of EK layer in this species acquired its maximum thickness during spring.

The results regarding the presence of the EK layer in other genera show that it is absent in the terrestrial species living in the hyper-wet Valdivian forest (*Eupsophus*, *Batrachyla*, *Hylorina*, *Insuetophrynus*). The Valdivian temperate forest is one of the wettest regions of South America, with more than 3,000 mm of annual precipitation (DIMITRI 1977). It is known that other terrestrial or semiaquatic species living in hyper-wet environments lack an EK layer, as is the case with *Rhinella margaritifera* (as *Bufo typhonius*) from the Amazon basin (TOLEDO & JARED 1993) or *Ascaphus truei* from the temperate rainforest of the Pacific Northwest (ELKAN 1968, 1976). Although ELKAN regarded *A. truei* as an aquatic species, but a more detailed analysis of the biology of *A. truei* shows that this is a semiaquatic species that lives in streams, but forages mainly on land, as it revealed by its stomach contents (METTER 1964).

The EK layer is also absent in *Alsodes neuquensis*, distributed in a semi-wet ecotone area between the Valdivian forest and the Patagonian steppe. However, with regard to its habits, *A. neuquensis* is an aquatic species with some characters associated with an aquatic mode of life such as skin bagginess. In the genus *Atelognathus*, three conditions of the EK layer are found: (i) it is absent in *A. patagonicus*, a lake-dwelling species of Laguna Blanca in the Patagonian steppe, (ii) it is reduced (traces) and thin in *A. nitoi*, a terrestrial species living in semi-wet forest between the Valdivian forest and the Patagonian steppe, and (iii) it is well developed and thicker in *A. reverberii*, a terrestrial inhabitant

of the Patagonian steppe. Although a phylogenetic framework for Patagonian species is available (e.g., BLOTTO et al. 2013), the lack of data on skin histology from a denser sample of taxa precludes evaluating the existence of phylogenetic patterns.

The occurrence of a thick EK layer in Ceratophryinae is well known (ELKAN 1968, 1976, MANGIONE et al. 2011, QUINZIO & FABREZI 2012). Although the three species studied here occur in semi-arid to semi-wet habitats, *Ceratophrys cranwelli* and *Chacophrys pierottii* are terrestrial species whereas *Lepidobatrachus llanensis* is aquatic. Although we cannot discard the effect of phylogenetic inertia in the retention of the EK layer in the genus *Lepidobatrachus* (FAIVOVICH et al. 2014), the species of this genus live in temporary ponds and undergo aestivation when conditions are unfavourable (McCLANAHAN et al. 1976, 1983).

The taxonomic distribution of EK layers in the studied species shows that this layer is (i) absent in strictly lacustrine species, in aquatic species living in wet environments, and in terrestrial species living in hyper-wet environments such as the Andean Valdivian forest, (ii) it is present but thin in aquatic species (e.g., stream-dwellers) living in arid environments and in semiaquatic species inhabiting wet environments, and (iii) it is thick and well developed in semiaquatic and terrestrial species living in semi-wet to arid environments.

The present evidence on *Telmatobius* suggests that both ecological habits and the types of habitat could be related to the development of the EK layer. The influence of the type of habitat seems to be stronger in semiaquatic species, because there are marked differences between specimens living in wet (thinner layer) and those living in dry conditions (thicker layer). On the other hand, the influence of the ecological habits seems to be strong in species living in dry environments, because there are marked differences between aquatic (thinner layer) and semiaquatic species (thicker layer). Although, considering the published phylogenetic hypotheses, there seems to be no phylogenetic influence on the occurrence and development of the EK layer, the inclusion of a comprehensive phylogenetic framework is essential to evaluate this aspect more seriously. On the other hand, ontogeny seems to be a factor with an influence on the EK layer (ELKAN 1968, FABREZI et al. 2010). Although the pattern shown here is consistent with the current data, phylogenetic and ontogenetic factors need to be evaluated in the future in the light of new evidence.

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Appendix

Voucher specimens of the species included in this study

Telmatobius atacamensis: FML-SB 156, 224, 225, 226; *T. bolivianus*: CBF 2063, 5379; *T. ceiorum* FML 2629/5, 2629/16; *T. culeus*: CBF 741,1084, 4050, 4057; *T. hauthali* FML 3264/1, 3264/9, 3264/27, 3264/29; *T. laticeps* FML 3957/3, 3957/4, 03960/1, 3960/5, 3960/9; *T. marmoratus* CBF 3622; *T. oxycephalus* FML 12070, 12079, FML-SB 016, 019, 021; *T. pinguiculus* FML-SB 197, 208; *T. pisanoi* FML 3269/2, 3269/7, 2963/1, 2963/3; *T. platycephalus* FML-SB 082; *T. rubigo* FML 20829; *T. schreiteri* FML 1977/6, 1977/10, 1976/19, 1976/26; *T. scrocchii* FML 1515, 1515/62, 5772/97, 5772/99, 5772/103; *T. simonsi* CBF 3081, 3082; *T. stephani* FML 1594/2, 1594/2, 1594/7; *T. verrucosus* CBF 02765, 5372; *Alsodes neuquensis* MACN 37951; *Atelognathus nitoi* CENAI 6882, 7263; *A. patagonicus* CENAI 1070 (two specimens); *A. reverberii* MACN 33937, 33938; *Batrachyla tainiata* CENAI 6865, 6866; *Ceratophrys cranwellii* MACN-JF 924, 926; *Chacophrys pierottii* MACN-BB 1899, 1972; *Eupsophus roseus* MLP 4014; *Hylorina sylvatica* MACN-BB 2258; *Insuetophrynus acarpicus* CENAI 6896; *Lepidobatrachus llanensis* (2 specimens from Salta, Argentina, numbers not assigned).