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DIPLOMARBEIT

Structure and function of the skin of *Tylototriton verrucosus* (Anderson, 1971) with special emphasis on cutaneous glands.

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ABSTRACT

T. verrucosus shows at least three different multicellular cutaneous gland types, embedded in the dermis with ducts that open through the epidermis to the exterior. Mucous glands (MG) are the smallest glands, homogenously distributed throughout the body. They are responsible for producing an assortment of mainly neutral mucopolysaccharids. MGs are build up by a layer of secretory cells, encircling a central lumen and provide the secretions of the slippery mucus film that covers the body, generally participated in several crucial functions. The latter two gland types are serous in nature and differ in several morphological and histostaining properties from MGs. They are big and especially enlarged in areas of glandular accumulations, most conspicuous in the parotoids and the tail dorsum. Notably these areas with enlarged granular gland concentrations are used against aggressor in defensive postures and can be regarded as antipredator adaptations.

In the skin of *T. verrucosus* two different types of GGs were identified. GG1s are present throughout the skin whereas GG2s are restricted to the tail, reaching giant diameters up to 1550 µm. Based on the findings of this study, added to the findings from the literature, both granular gland types are supposed to produce noxious to toxic chemicals deployed during encounter behavior to repel predators.

Morphological and histostaining findings of both mucous and granular glands indicated their particular functionalities. Mucous glands mainly release their secretion in a merocrine way, due to the constant necessity of a surface covering mucus film. MGs are similar in juveniles and adults and probably have a regular regeneration process. In this process, cells continuously proliferate from apical to basal and replace dying cells. In contrast to MGs, the appearance of granular glands differs between juveniles and adults. Their maturation is characterized by long biosynthetic and maturational processes during the growth of granular glands. Mature granular glands are huge organelle-poor structures pointing to a state of suspended activity. They are build up by several huge cells, filled with secretion and the glandular contents are stored ready for expulsion. A fast expulsion, for effective defense, is provided by muscular contraction of a well-developed myoepithelial sheath, encircling the gland, a conspicuous characteristic of granular glands in *T. verrucosus*.

ABSTRACT

T. verrucosus besitzt mindestens drei unterschiedliche Typen von multizellulären Hautdrüsen. Diese Drüsen liegen eingebettet in die Dermis und besitzen Ausführgängen, die durch die Epidermis reichen und sich nach außen hin öffnen. Schleimdrüsen sind die kleinsten aller Hautdrüsen und homogen über den Körper verteilt. Sie sind verantwortlich für die Produktion von einem Gemisch aus vor allem neutralen Mukopolysacchariden. Das zentrale Lumen ist von einem einschichtigen sekretorischen Epithel umgeben, welches die Sekrete für den schlüpfrigen Schleimfilm bereitstellt, der den Amphibienkörper bedeckt und mehrere wichtige Funktionen übernimmt.

Die übrigen zwei Hautdrüsentypen zählen zu den seröse Drüsen und werden auch als Giftdrüsen bezeichnet. Sie unterscheiden sich von Schleimdrüsen in mehreren morphologischen Kriterien und in ihren histologischen Färbungseigenschaften. An manchen Körperstellen, besonders in den Parotoiden und im dorsalen Schwanzbereich treten solche Giftdrüsen sehr konzentriert und stark vergrößert auf. Als Anpassung im Zuge der Feindabwehr werden durch spezielle Körperhaltungen gerade jene Bereiche Feinden entgegengestellt.

In der Haut von *T. verrucosus* konnten zwei unterschiedliche Typen von Giftdrüsen identifiziert werden. Typ 1 (GG1) ist am gesamten Körper zu finden, während Typ 2 (GG2) auf den Schwanzbereich begrenzt ist und gigantische Drüsendurchmesser von 1550 µm erreichen kann. Basierend auf Ergebnisse dieser Studie in Kombination mit Erkenntnissen aus der Literatur kann man davon ausgehen, dass beide Typen gesundheitsschädliche bis giftige Chemikalien produzieren, die bei einem Zusammentreffen mit Feinden eingesetzt werden um diese abzustoßen.

Morphologische Erkenntnisse von Schleim- und Giftdrüsen und Ergebnisse histologischer Färbungen gaben Aufschluss über ihre möglichen Funktionsweisen. In Folge der konstanten Notwendigkeit eines oberflächendeckenden Schleimfilms entlassen Schleimdrüsen ihre Sekrete fortwährend auf eine vor allem merokrine Art und Weise. Sie sind bei Juvenilen und Adulten gleich ausgeprägt und besitzen vermutlich einen regelmäßigen und ständig ablaufenden Regenerationszyklus. In diesem Prozess vermehren sich Zellen von apical nach basal und ersetzen dabei absterbende Zellen. Im Gegensatz zu Schleimdrüsen, ist die Ausprägung von Giftdrüsen bei Juvenilen und Adulten unterschiedlich. Deren Reifung ist durch lang dauernde biochemische Prozesse charakterisiert. Reife Giftdrüsen sind generell aus sehr vielen, großen Zellen aufgebaut, die gefüllt sind mit Sekret. Ausgereifte Giftdrüsen sind

organellenarme Strukturen, die auf einen Zustand eingestellter Aktivität hinweisen. Folglich stehen in reifen Drüsen die Sekrete fertig bereit um bei Bedarf ausgestoßen zu werden. Ein deutlich ausgeprägtes und gut entwickeltes Myoepithel, welches die gesamte Drüse umgibt ist bei *T. verrucosus* vorhanden. Dessen Muskelkontraktionen gewährleisten eine schnelle Ausscheidung der Drüseninhalte, als effektiven Schutz gegen Fressfeinde.

INTRODUCTION

One of the most conspicuous features of living amphibians involves their skin. As interface between organism and environment, this organ is a complex, elaborate, dynamic and highly organized structure involved in a great variety of functions (Fox, 1994; Stebbins and Cohen, 1997). The multiple roles performed by the skin in amphibians include amongst others larval adhesion, chemo- electro- and mechanoreception, ion transfer, excretion, respiration, water retention, protection against abrasion, pathogens or predators (e.g. Fox, 1994; Brizzi et al., 2002). One of the main components of amphibian skin is the glandular tissue, whose products are involved in a variety of functions (Duellman and Trueb, 1994; Fox, 1994). Two types of dermal glands – granular and mucous – are believed to be present in all adult extant amphibians (Dawson, 1920; Noble and Noble, 1944; Le Quang Trong, 1967; Le Quang Trong, 1973; Duellman and Trueb, 1994; Houck and Sever, 1994; Fontana et al, 2006). They are responsible for producing skin secretions released onto the body surface (Delfino, 1976; Hoffman and Dent, 1978; Alvarez et al., 2005; Arifulova et al., 2007 and others). Skin secretions can be used for protective purposes: to make the surface slippery to facilitate escape from aggressors (Stebbins and Cohen, 1997) or to make it sticky to completely immobilize a predator (Duellman and Trueb, 1994) or be unpleasant tasting, irritating or even toxic, making the amphibian unpalatable to predators (Brandon et al., 1979; Brodie, 1983; Heiss et al., 2009). Accordingly, amphibians produce a remarkable diversity of bioactive substances in their skin glands: more than 100 bioactive peptides, 30 bioactive amines (Erspamer, 1994) and over 800 alkaloids (Daly et al., 2005) have been isolated from amphibian skin secretions to date.

Mucous and granular glands can be distinguished by morphological and histostaining properties. Mucous glands are smaller than granular glands and widely distributed throughout the integument. They are thought to secrete their contents in a merocrine manner continuously onto the skin (Hoffman and Dent, 1978). The mucous glands have a central lumen and the secretory cells are positioned around it. Mucous glands are mainly responsible for producing the slimy and slippery amphibian mucus. There has been disagreement concerning the mucous gland surrounding myoepithelium, as in some urodeles and anurans, such a contractile sheath was reported to be absent (Fox, 1994; Duellman and Trueb, 1994). However, the mucous secretion plays an important role as it regulates water loss, acts as barrier against pathogens, is an important lubricant, reduces friction under water and minimizes mechanical damage to the skin out of the water (Fontana et al, 2006).

Granular glands, the second type of amphibian skin glands, are large in size and produce a mainly proteinaceous product (Le Quang Trong, 1973; Reyer et al., 1992; Fontana et al., 2006; Heiss et al., 2009). They are found throughout the skin but in some areas concentrations of enlarged granular glands can emerge (Brodie, 1977; Brodie et al., 1984; Duellman and Trueb, 1994). The granular glands are built up by giant cells containing granular material and often fuse to syncytia. These glands are capable of synthesizing and secreting mainly bioactive substances such as amines, peptides and alkaloids (Erspamer, 1994) and are thought to be the site of skin toxin production (Noble, 1931; Stuhr, 1936; Quay, 1972; Delfino, 1976; Nowak and Brodie, 1978; Neuwirth et al., 1979; Daly, 1995; Toledo and Jared, 1995; Alvarez et al., 2005; Daly et al., 2005; Arifulova et al., 2007.)

The granular glands have a distinct myoepithelium, used for fast expulsion of glandular contents onto the body surface (Duellman and Trueb, 1994). The material is thought to be discharged through an apical pore in a holocrine manner (Quay, 1972; Reyer et al., 1992). The nature of granular gland secretions can vary significantly among amphibian species. In most amphibians, the granular gland products are - if not poisonous - at least harsh and irritating to mucous membranes of potential predators (Hoffman and Dent, 1977). The granular gland secretions of most salamandrids in fact are noxious to toxic, able to truly harm or even kill a would-be predator (Brodie, 1977; Brodie, 1983; Brodie and Smatresk, 1990; Erspamer, 1994; Daly, 1995; Delfino et al., 1995; Tsuruda et al., 2002; Heiss et al., 2009). Noxious skin secretions have been considered to be the most important “tools” to repel predators in terrestrial salamanders and most other antipredator adaptations are dependent upon these secretions. Antipredator posturings, where concentrations of granular glands are positioned toward a threatening stimulus, increase the likelihood that a predator contact will occur with the most noxious part of the salamander (Nowak and Brodie, 1978). This causes the predator to learn to avoid the salamander after initial contact. Recent work by Heiss et al. (2009) on the salamandrid *Pleurodeles waltl* show not only enormous glandular concentrations but also two kinds of granular glands. Both granular gland types are presumably involved in defensive behavior and their occurrence diverges regionally. Similarly, Brodie et al. (1984) reported distinct antipredator posturings after threatening stimulation in *T. verrucosus*, a closely related species to *P. waltl*. Even if *T. verrucosus* has been reported to be one of the most poisonous salamandrids (for overview see Brodie et al., 1984), little is known on the structure of its skin glands and their functional role.

Therefore, a detailed analysis on the integument in *T. verrucosus* is provided in the present study - with special emphasis on the cutaneous glands. This study provides new data on

morphology and function of the different gland types. The findings are then discussed in the light of antipredator adaptations with the aim of providing a possible interpretation of the adaptive and functional role of the different gland types. As this study includes two age groups - postmetamorphic juveniles and sexually mature adults - additionally developmental relevant aspects of the different gland types are generated.

MATERIAL AND METHODS

Systematics

Salamanders of the family Salamandridae are informally subdivided into two major subgroups, the "true salamanders" and the newts. Analysis of the combined morphological and molecular characters gives strong support to the monophyly of the true salamanders (includes genera *Chioglossa*, *Lyciasalamandra*, *Mertensiella* and *Salamandra*) and to monophyly of a group containing all remaining extant newts except *Salamandrina terdigitata* (Steinfartz et al., 2006; Weisrock et al., 2006). Within the newt clade the genera *Tylototriton* and *Echinotriton* are monophyletic and together form a clade with the genus *Pleurodeles* (Fig. 1.), sister to the remaining newts, except *Salamandrina* (Steinfartz et al., 2006; Weisrock et al., 2006).

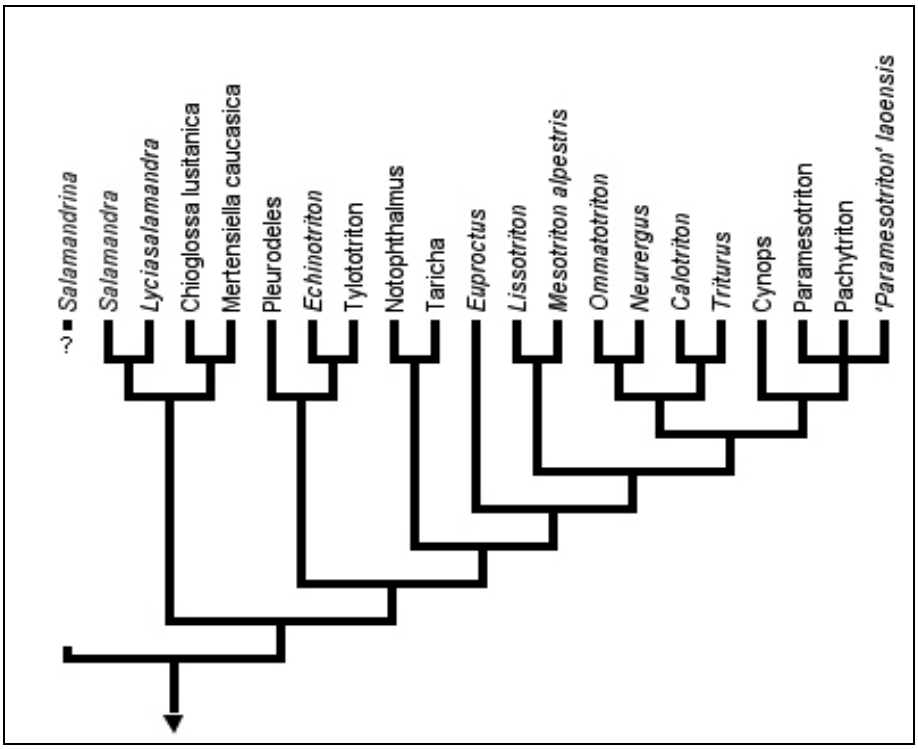

Fig. 1. Phylogenetic relationships of extant salamandrids. Modified after Steinfartz et al., 2006 and Weisrock et al., 2006.

Natural distribution

The family Salamandridae has a holarctic distribution and is well represented in Europe, Asia, and North America. *T. verrucosus* itself has a large distribution area, ranging from India and eastern Nepal through the Kachin and Shan Hills of Myanmar to southwestern Yunnan, China and scattered mountains in northern Thailand (Fig. 2). The terrestrial habitats are largely moist forests or sites where mountain forests previously existed. The animals live in high altitudes up to 2250 m and are closer confined to water than allied species and generally remain in or close to water (Seglie et al, 2003).

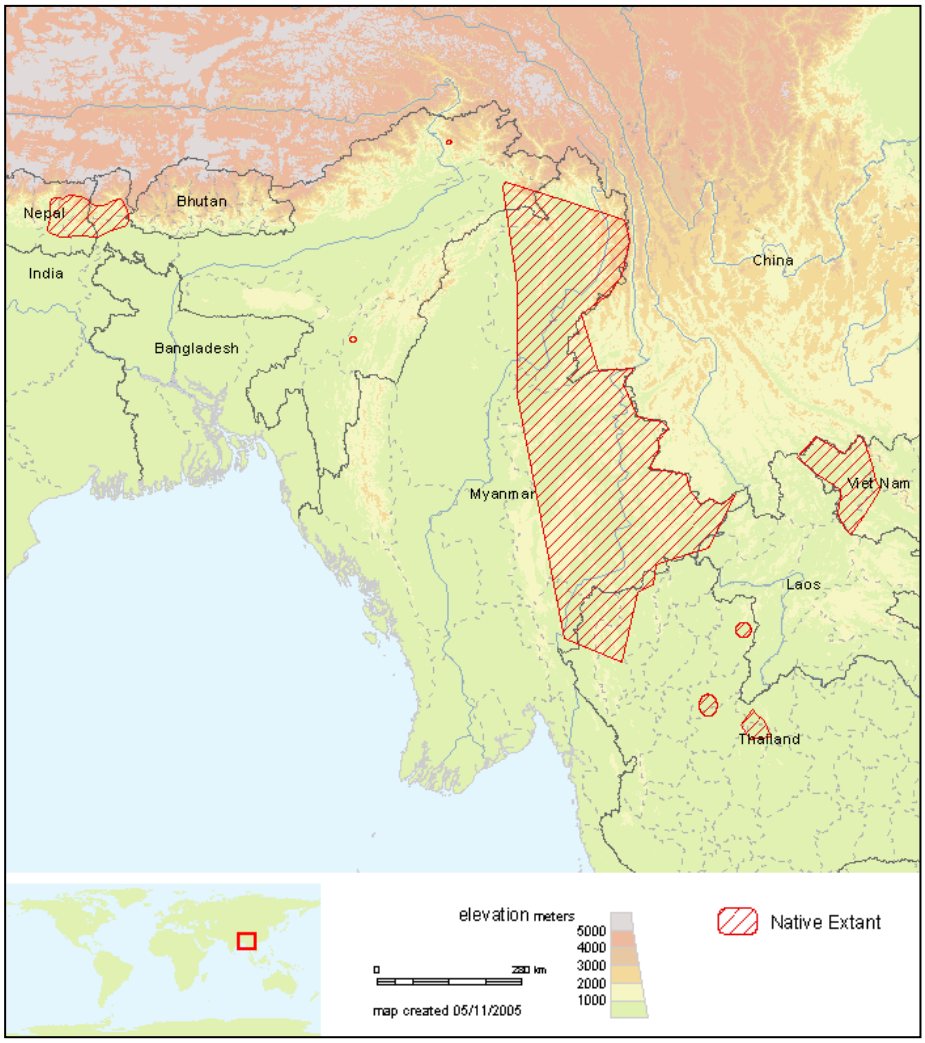

Fig. 2. Natural distribution area of *T. verrucosus*. Modified after © 2006 IUCN, Conservation International and NatureServe.

Experimental animals

Four female and two male *T. verrucosus* were examined for the present study. Three metamorphosed juvenile individuals (two female, one male) were 6 months old. Total body-length ranging from 78 mm to 93 mm and bodyweight ranging from 3.2 g to 5.8 g. Three adult individuals (two female, one male), having already reached sexual maturity were 12 months old. Total body-length ranging from 115 mm to 130 mm and bodyweight ranging from 12.4 g to 18.2 g.

The examined individuals were colored uniformly dark-brown to black. Head, tail and soles of the extremities were lighter brown than the body. Especially parts of the parotoids, the rows of dorso-lateral warts and the underside of the tail were colored contrasting to the body base color in different shades of light brown to orange (Fig. 21).

The newts were obtained commercially and kept in a 250-liter tank with 12h light / 12h dark frequency. They were fed twice a week with earthworms, bloodworms and fish pieces. For investigations the newts were anesthetized with dissolved MS222 and decapitated. Skin samples from the head, the parotoid region, the back, the lateral trunk, the belly region as well as the dorsal and ventral part of the tail were removed and immersed in fixation solution.

Scanning electron microscopy (SEM)

For scanning electron microscopic investigations samples were fixed in Bouin's-solution (Romeis, 1989) for 4 weeks, rinsed in 70% ethanol and dehydrated in a graded ethanol series. Subsequently the samples were transferred into acetone and dried in a critical point drying machine (Polaron: Watford, UK). The dried samples were then coated with gold in an AGAR B7340 Sputtercoater and observed in a Philips XL-20 scanning electron microscope. (for more details please see Tab. 4 and Tab.10 in appendix).

Light microscopy (LM)

Light microscopy analysis included paraffin based histological investigations as well as investigations of semi thin sections.

For paraffin-based histological investigations samples were fixed in Bouin's solution (Romeis, 1989) for 4 weeks. After fixation, samples were rinsed in 70% ethanol, dehydrated in a graded ethanol series, immersed in Isopropanol and embedded in Paraffin (for more details please see Tab. 1 and Tab. 10 in the appendix). After polymerization, 7 µm-thick cross sections were cut on a Reichert-Jung 2030 rotary microtome (Reichert-Jung, Bensheim, GER) and mounted on glass slides and, after removing the paraffin, stained with Haematoxylin -

Eosin (HE), Azan, alcian blue (AB) at pH 2.5, periodic acid Schiff (PAS) and Coomassie Brilliant blue (all standard staining procedures after Romeis, 1989; Kiernan, 2003). Detailed staining reports are listed in the appendix (Tab. 5-8). Observations and photographic documentations were made using a Nikon Eclipse 800 light microscope.

For semi thin sectioning, two fixation and embedding methods were used. For the first procedure, samples were fixed in Bouin‘s solution (Romeis, 1989), rinsed in 70% ethanol, dehydrated in a graded ethanol series and embedded in LR white resin (TED PELLA, INC.) which was polymerized at 60°C. More detailed reports are listed in appendix (Tab. 2).
For the second procedure, samples were fixed in modified Karnovsky-solution (Karnovsky, 1965) (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer). After rinsing in 0.5% cacodylate buffer, samples were postfixed for 2 h in buffered 1% osmium tetroxide at room temperature. This was followed by dehydration in a graded ethanol and acetone series, embedding in Agar 100 Resin (Agar Scientific) and polymerization at 65°C for 15 h. (for more details please see Tab. 3, Tab. 9 and Tab.11 in the appendix)

The embedded and polymerized samples from both methods were then cut into 1 µm thin sections on a Reichert Ultracut S microtome (Leica) using diamond histo-knives (Diatome). The semithin sections were mounted on glass slides, stained with Toluidine blue and documented as described above for histological sections.

Transmission electron microscopy (TEM)

For ultra thin sectioning samples were fixed in modified Karnovsky-solution (Karnovsky, 1965) (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer) and adjacent treated like described above in the second procedure for semithin sectioning. After polymerization at 65°C for 15 h, ultrathin (60–70 nm) sections were made on a Reichert Ultracut S microtome (Leica) using histo- and ultra diamond knives (Diatome). Ultrathin sections were collected with copper grids 3.05mm. Observations and digital photographic documents were made using a Philips EM 208

Behavioral experiments

For behavioral experiments, the reactions of 'predator-like stimulations' were tested and documented using a Canon EOS 350D digital camera. The animals were prodded repeatedly but gently with dull probes or pinched lightly with forceps, to simulate a predator attack, until they showed defensive behavior. The animals recovered rapidly after the experiments and all showed natural behavior such as feeding immediately after the experiments.

RESULTS

1. Scanning electron microscopic (SEM) findings

Scanning electron microscopic investigations included the examination of the skin surface as well as observations of skin cross sections. In connection with light- and transmission electron microscopic findings, scanning electron microscopy enabled greater comprehension of the construction of the skin and its glandular tissue.

The epidermis itself was stratified and consisted of several layers whereof a sheath of irregularly shaped keratinocytes coated the skin surface (Fig. 3). Numerous pores of cutaneous glands were clearly visible at the body surface (Fig. 4). Higher magnification revealed that the gland ducts, traversing the epidermis and connecting the secretory unit and the exterior, were lined by flattened keratinocytes (Fig. 4).

Examining cross sections it became apparent that high densities of large cutaneous glands were present in the dermis. All cutaneous glands consisted of an excretory duct traversing the epidermis and a secretory unit embedded in the dermis. The secretory cells itself were indicated by the multiplicity of vesicles (Fig. 3).

Besides cutaneous glands of ordinary size, distributed throughout the skin, high concentrations of enlarged glands could be detected in certain regions. The most impressive concentrations were found in the parotoid-area and the tail dorsum (Fig. 5).

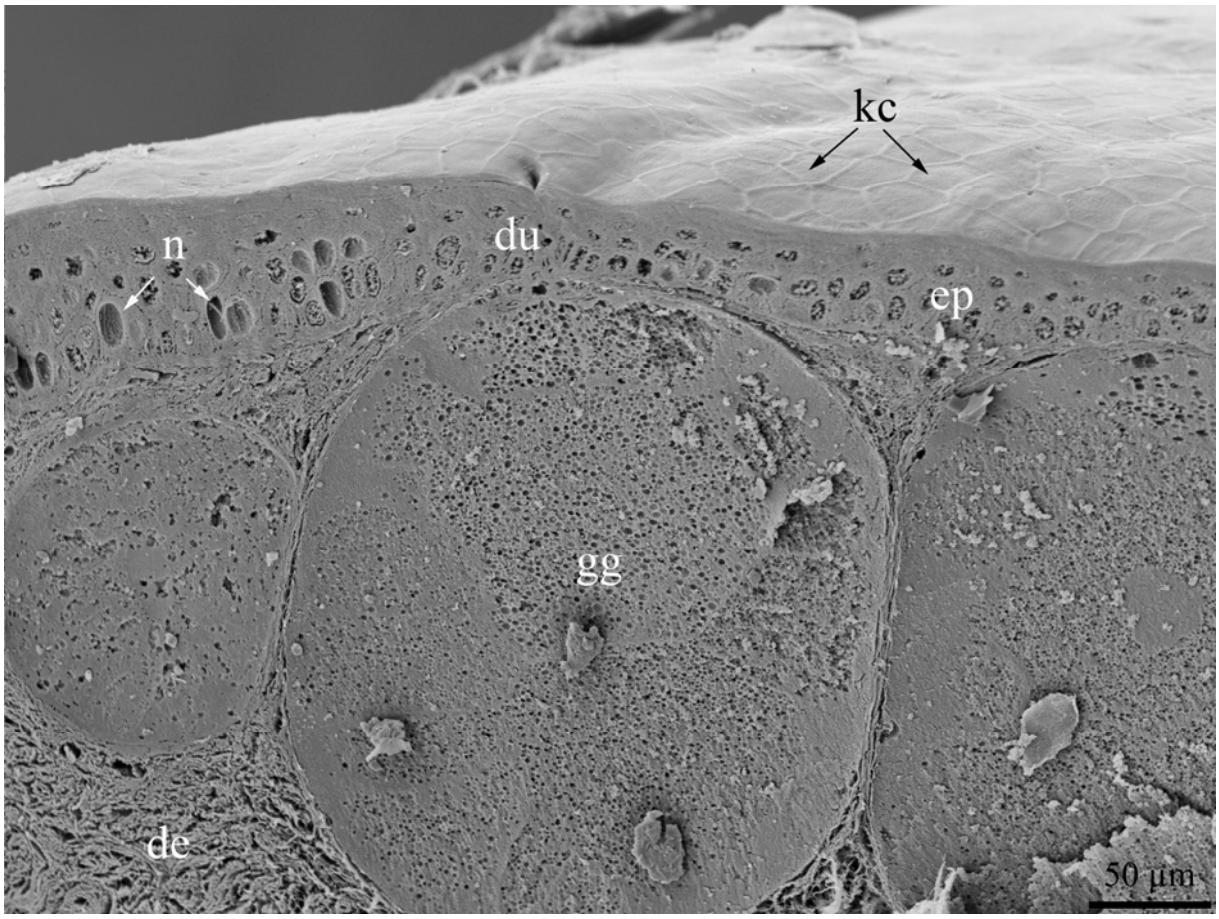

Fig. 3. Scanning electron micrograph showing a cross section through the skin of the dorsal trunk of a juvenile *T. verrucosus*. The entire skin is densely interspersed with cutaneous glands embedded in the dermis, with ducts that open onto the body surface. The stratified epidermis becomes clearly apparent by the multitude of big cell nuclei. The outermost epidermal layer consists of irregularly shaped keratinocytes. de, dermis; du, duct; ep, epidermis; gg, granular gland; kc, keratinocyte; n, nucleus.

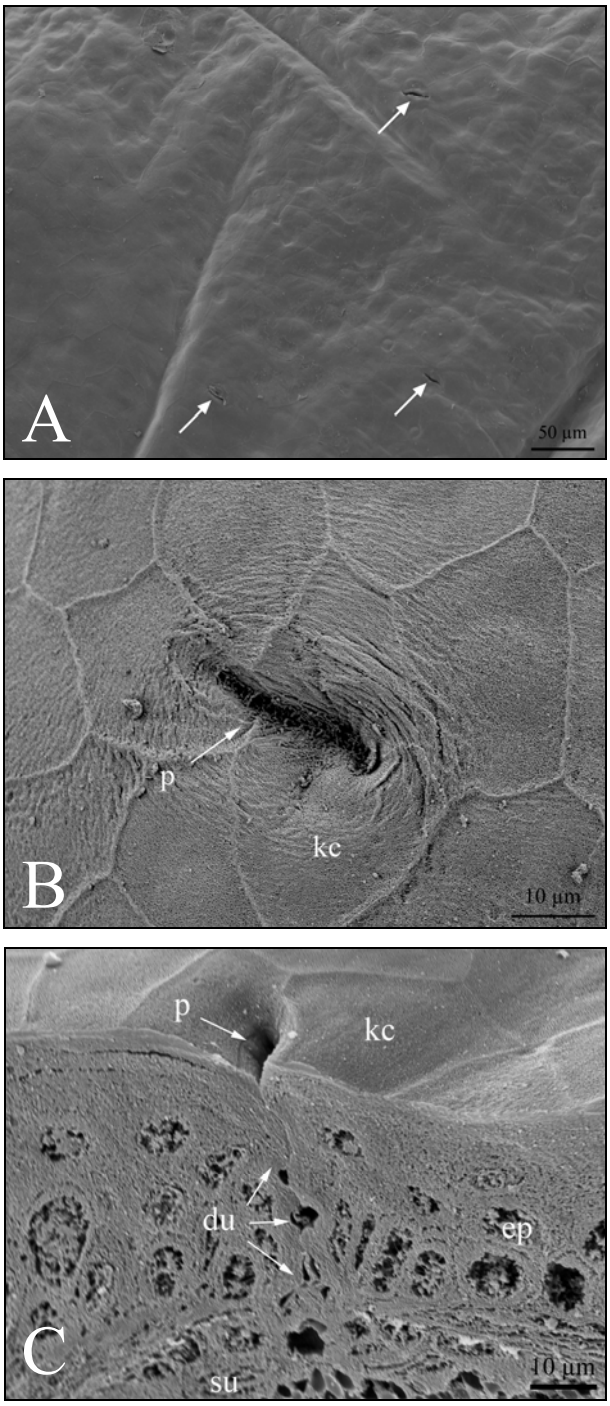

Fig. 4. Scanning electron micrographs showing the excretory ducts in the integument of *T. verrucosus*. A: Overview of the skin surface illustrates the presence of numerous gland pores. B: Higher magnification reveals that the ducts are lined by keratinocytes. C: A cross section of the skin shows a truncated duct. The ducts traverse the epidermis and hence allow the transport of synthesized secretions onto the body surface. du, duct; ep, epidermis, kc, keratinocyte; p, pore; su, secretory unit.

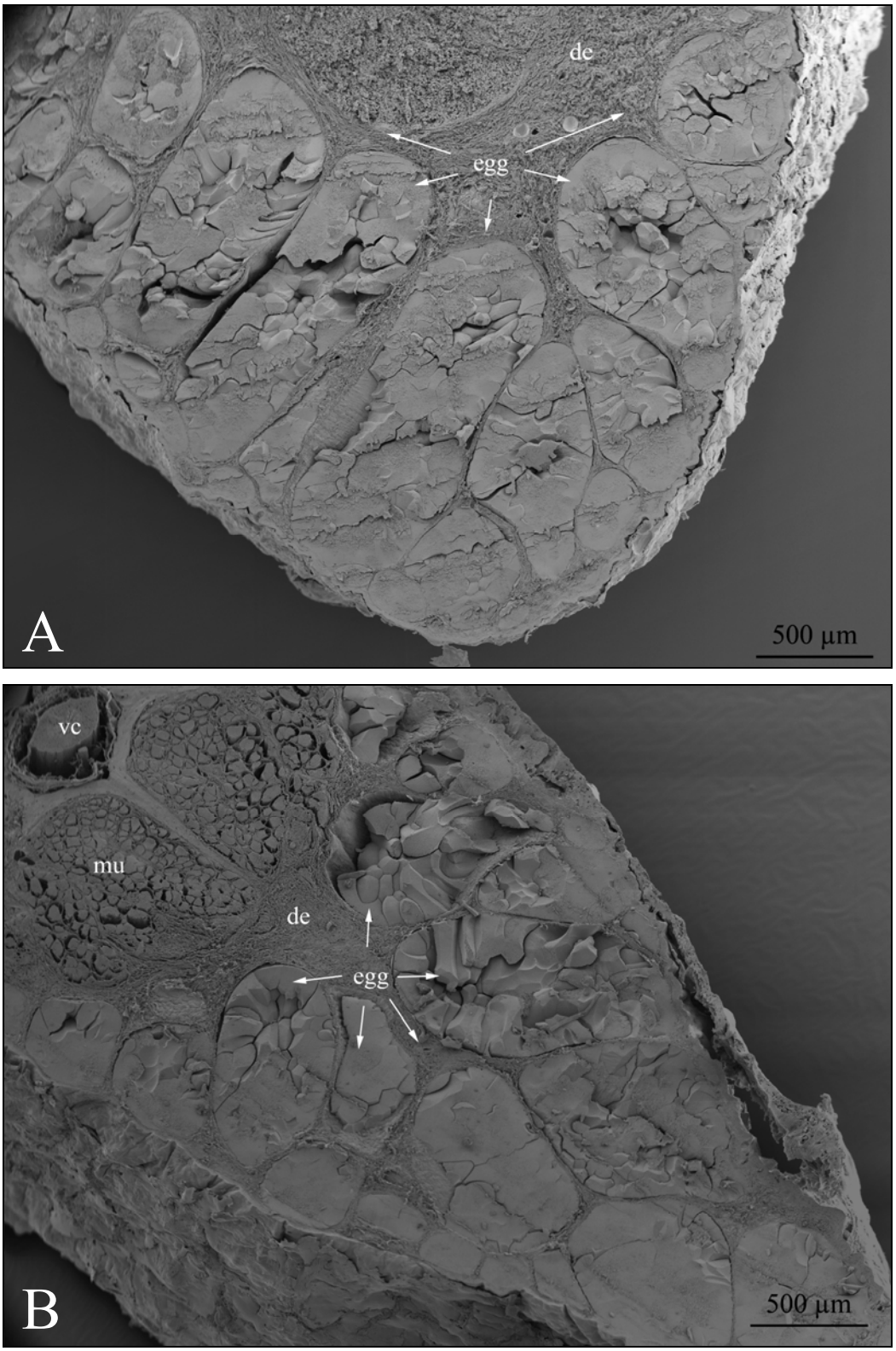

Fig. 5. Scanning electron micrographs showing accumulations of enlarged granular glands in adult *T. verrucosus.* A: Cross section through the parotoid region and (B) cross section through the tail dorsum. Note the concentrations of enormous glands in special areas of the body that almost fill in the entire dermis. Both the parotoids and the tail dorsum contain the largest glands in highest concentration in *T. verrucosus*. de, dermis; egg, enlarged granular gland; m, muscularity; vc, vertebral column

2. Light microscopic (LM) findings

2.1 General structure of the skin in T. verrucosus

Like all vertebrate integument, the skin of *T. verrucosus* consisted of two distinct components, an outer stratified layer, the epidermis and an underlying connective tissue layer, the dermis (Fig. 6). In the interface between epithelium and connective tissue layed the basement membrane: a thin extracellular layer separating the epithelium and the loose connective tissue (Fig. 7, Fig. 8).

The epidermis was stratified and in adult individuals mostly composed of 5 to 7 epithelial cell layers, which could be assigned, from proximal to distal to four strata: the innermost stratum germinativum or stratum basale, the stratum spinosum, the stratum granulosum and the outermost stratum corneum (Fig. 7). The innermost cell layer, called the stratum germinativum, lied above the basement membrane and was arranged by tall, cuboidal to columnar cells with round to oval, central located nuclei. The cells of this basal layer proliferated to the overlying layer, the stratum spinosum. During distal cell migration, cell plasma percentage increased while the nuclei size was reduced. In the stratum granulosum the cells appeared rather flattened and their nuclei became pycnotic. The overlying, outermost stratum corneum was build up by a single layer of flattened keratinocytes which lacked their nuclei.

The epidermis also included further different types of cells like the characteristic flask cells, which were expanded vertically and reached beneath the layer of keratinized cells. Light microscopic investigations revealed the presence of neuromast organs, the epithelial sensory receptors of the lateral-line system. Each neuromast consisted of a pear-shaped group of cells, comprising mantle cells and sensory cells, embedded in the epidermis and resting on the basement membrane, innervated by nerve fibers (Fig. 9). Chromatophores stuffed with pigment granules, as well as scattered pigment granules, were located either in the epidermis and the superficial layers of the dermis.

As mentioned above, the epidermis was separated from the dermis by the basement membrane. The basal cells of the epidermis showed connections to the underlying lamina propria by periplasmatic protrusions (Fig. 8). The dermis itself consisted of two layers, the outer stratum spongiosum, build up by loose connective tissue and the underlying stratum compactum, composed of compactly arranged tight collagenous fibers (Fig. 6). The dermis showed regional variation in the proportions and extensions of these layers. Embedded into the dermis there were prominent acinar glands with ducts opening to the skin surface. While

the smaller mucous glands were completely enclosed by the stratum spinosum the bigger granular glands often reached into the stratum compactum.

The dermis was well supplied with blood vessels and showed a huge amount of capillaries forming an extensive network especially in the superficial dermal layers immediately subjacent to the epidermis. Furthermore, the dermis contained structures such as nerve fibers and muscle fibers as well as various types of cells, including pigment-bearing chromatophores.

2.2 Skin glands in T. verrucosus

The skin of *T. verrucosus* showed a great amount of cutaneous glands, which were scattered throughout the dermis (Fig. 14, Fig. 15). All cutaneous gland types shared some common features as they were multicellular and acinar in shape. They were located in the dermis and opened to the skin surface with an excretory duct, lined by keratinocytes.

Three clearly distinguishable multicellular gland types were identified and characterized by structural and histostaining properties. They could be divided into the main cutaneous glands: mucous and granular.

The first glandular type described is the mucous gland (MG): small acinar glands with wide lumina and homogeneously distributed throughout the body surface. MGs were the smallest cutaneous glands in *T. verrucosus*, with maximal diameters up to 130 µm. They were completely enclosed by the stratum spongiosum. Mucous glands were roundish in shape and had a wide lumen, which was continuous with the excretory duct of the gland (Fig. 10, Fig. 11). The wide lumen, which was a characteristic feature for this gland type, was encircled by low to moderately tall epithelial cells filled with granular contents. Apical located cells were smallest, increased in size in the lateral walls and were biggest in the basal part of the gland (Fig. 11). Basally located secretory cells were tall pyramidal to cuboidal shaped with round nuclei that were regularly arranged in the basal cell pole. These cells contained large granules of secretory products distributed throughout their cytoplasm. Within a given gland the staining of sectretory granules often varied from cell to cell (Fig. 11). Myoepithel cells, surrounding the gland entity, were present. The ducts of mucous glands were lined by keratinocytes (Fig. 10).

In general most of the cells within the mucous gland stained strongly PAS positive: pink to purple (Fig. 11). Some cells within the mucous glands reacted slightly positive to the alcian blue test at pH 2.5 (Fig. 15). The glandular lumen usually contained secretions staining slightly PAS positive. All mucous glands reacted negatively to the Coomassie blue test for proteins.

The second cutaneous gland type in *T. verrucosus* was the granular gland. Almost the entire skin was densely interspersed with this second type of gland. They were roundish, large in size and could extend through the stratum spinosum into the stratum compactum of the dermis. Within granular glands, two subtypes could be distinguished based on structural differences. The first subtype, termed here granular gland type 1 (GG1), was found in all skin samples examined, whereas the second subtype was restricted to the dorsal and ventral tail

edges. This second subtype is named here granular gland type 2 (GG2) and treated separately in this study.

GG1s were present throughout the skin (Fig. 14, Fig. 15). In general these glands appeared roundish and big but in regions with concentrations of granular glands where available vertical space in the stratum spinosum of the dermis was scarce, they could become oval-shaped. GG1s lacked a distinct lumen in the common sense but an exiguous cavity could sometimes be visible beneath the neck region. The neck region represented the intercalated tract between secretory unit and duct and had a thick wall of undifferentiated cells. The duct itself was lined by keratinocytes and opened to the exterior. GG1 were build up by densely packed, big secretory cells that filled in the entire gland with remarkable amounts of dense granules (Fig. 12, Fig. 13). The secretory unit of GG1 was transformed during the secretory cycle and hence the appearance of this gland type varied, depending on the different stages of the secretory cycle. During maturational processes the secretory cells within the same gland could differ dramatically, mainly due to the different appearance of their granules. The granules could differ in size, shape, density and staining properties (Fig. 12). The mature glands were composed of single secretory cells with homogenous appearance and with flat nuclei positioned in the gland periphery. The secretory cells were filled with similarly sized and shaped granules. No cellular fusions to syncytia could be observed (Fig. 13). GG1 possessed a distinct myoepithelial sheath that encircled the gland. In general, the GG1 were eosinophilic, reacted negatively to the alcian blue test at pH 2.5 and stained slightly positive for PAS (Fig. 12, Fig. 14, Fig. 15).

Whereas the entire skin was interspersed with relatively ordinary sized GG1, some areas in *T. verrucosus* exhibited concentrations of much enlarged GG1. These areas with enlarged GG1 included the bony ridge of the head, starting at the tip of the snout over the eyes passing on to the distinct parotoid region. Enlarged GG1 were also found arranged in longitudinal rows lateral to the mid-dorsal ridge, starting at the posteriormost part of the head and extending to the tail base, as well as in the lateral warts (Fig. 21). These enlarged GG1 had an increased number of secretory cells, but the general design did not differ to that of ordinary GG1. However, the highest density and the largest diameters of GG1 were measured in the parotoids. Toward the areas of enlarged granular gland accumulations, the granular glands in general featured a gradual increase of size.

GG2 were only found in samples taken from the dorsal and ventral tail edges and were biggest and most abundant in the dorsal edge of the tail. In general, they appeared oval and in relation to the skin surface they were horizontally oriented (Fig. 16). Mature GG2s lacked a

distinct lumen but a small cavity could sometimes be visible beneath the neck region. This exiguous cavity was continuous with the excretory duct of the gland, the connection between the glandular unit and the exterior. The secretory unit was arranged by a great amount of densely packed single secretory cells that filled in the entire glandular volume, separated by clearly visible cell membranes. The secretory cells of mature glands did not differ morphologically from each other. Their secretory granules were all similar in shape and size but were not distributed homogenously throughout the cell. They were rather restricted to certain areas that formed granular patches (Fig. 13). Furthermore it was noticeable, that the granules of GG2 were significantly smaller than those of GG1s of ordinary size.

GG2, like all other granular glands, possessed a distinct myoepithelial sheath that encircled the gland, followed by a tight layer of collagen fibers. In general GG2s were eosinophilic, reacted negatively for the alcian blue test at pH 2.5 and slightly positive for PAS. The positive Coomassie brilliant blue staining further evidenced the presence of proteinaceous secretory content.

2.2.1 Particulars of skin glands in juveniles

The general structural design of mucous glands in juveniles was similar to the MGs described for *T. verrucosus* in general but they featured particular histostaining properties. Most of the secretory cells within this gland type were positive for PAS but none of the mucous glands of juveniles was positive for alcian blue at pH 2.5 (Fig. 11).

The general structural design of granular glands in juveniles was for the same as described above. A conspicuous attribute was that predominantly immature granular glands of several maturational stages could be found in the skin of juveniles. During maturational processes the gland size itself was differing and the appearance of the secretory cells within one gland could differ due to varying size, shape, density and staining properties of their granules (Fig. 12).

The granular glands of juveniles could be divided into the two described subtypes: GG1s and GG2s. The two types were distributed like mentioned above and concentrations of enlarged glands were present in similar areas. Interestingly, the biggest glands at all in juveniles were measured in the parotoid area among enlarged GG1s, reaching maximal diameters of up to 1020 µm.

The biggest glands among GG2s were found in the dorsal tail edges and reached maximal diameters of up to 478 µm. GG2s in general were different in shape compared to GG1s and grew in a horizontally elongated manner, in relation to the skin surface (Fig. 16). GG2s were

the only glands in juvenile *T. verrucosus* with an increased positive staining for the Coomassie blue reaction.

2.2.2 Particulars of skin glands in adults

In adult individuals granular glands during maturational processes could be found but predominantly mature granular glands were present. Mature granular glands were big and composed of a high amplitude of single secretory cells with homogenous appearance. All granular glands observed in adults were bigger than granular glands observed in juveniles of equivalent body areas.

Interestingly not GG1s but GG2s represented the biggest cutaneous glands in adult *T. verrucosus*, extending to maximal diameters of up to 1550 µm. The giant GG2s of adult individuals were densely packed, leading to a situation where almost the entire tail was stocked with these glands. The biggest GG1s in adults were measured in the parotoids reaching maximal diameters of up to 1140 µm.

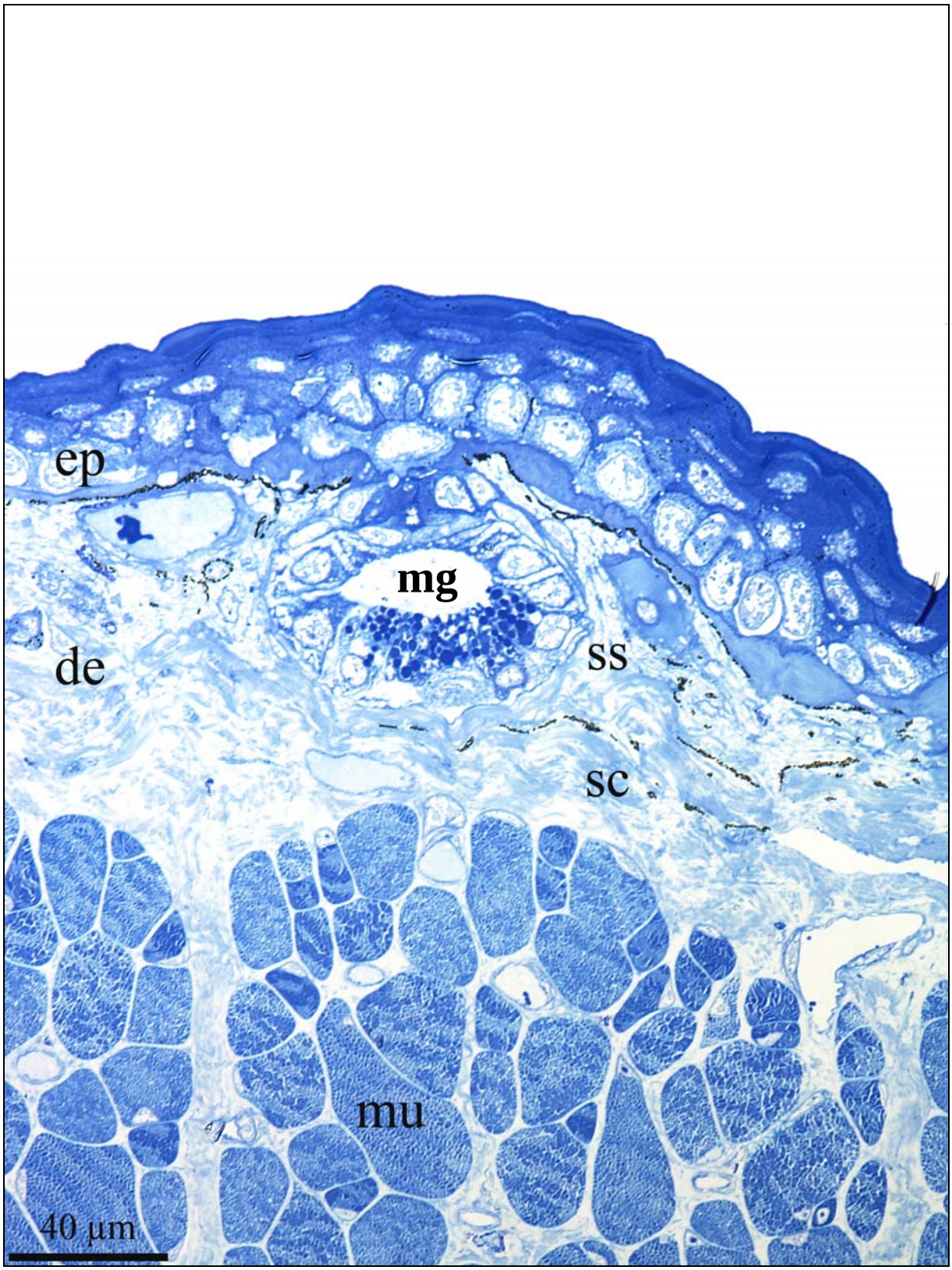

Fig. 6. Light micrograph showing a semi thin cross section of the skin of a lateral region of the tail of juvenile *T. verrucosus*. The skin is composed of a stratified epidermis and an underlying dermis, separated into a stratum spongiosum and a stratum compactum. The proportions and extensions of epidermis, dermis and the underlying muscles exhibit regional variations. Note the mucous gland, representative for cutaneous glands embedded in the dermis. ep, epidermis; de, dermis; mg, mucous gland; mu, muscles; sc, stratum compactum; ss, stratum spongiosum. Toluidine blue staining.

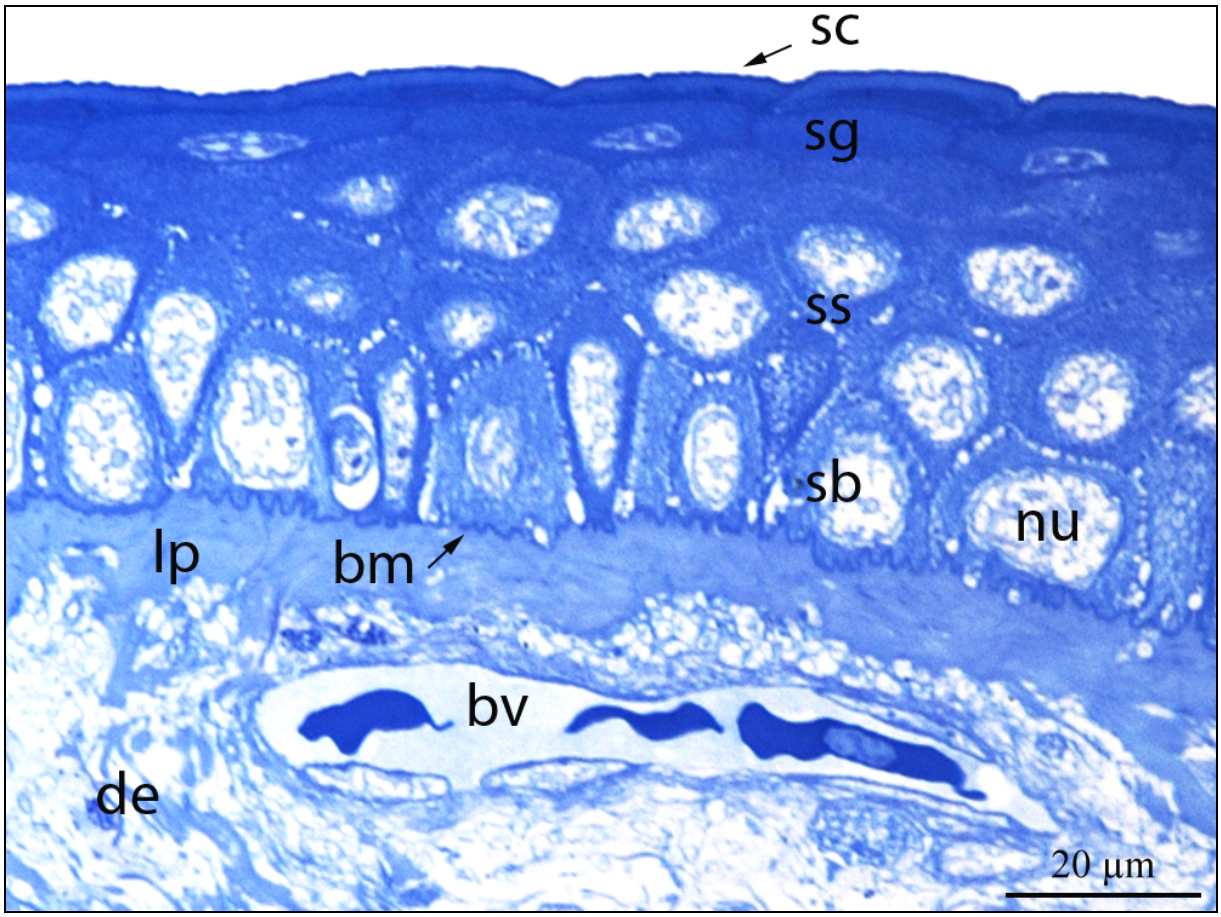

Fig. 7. Light micrograph showing in detail the epidermis of a lateral region of the tail of juvenile *T. verrucosus.* The epidermis rests on the lamina propria and consists in most of its parts of 5-7 cell layers. The proximal stratum basale is build up by tall, columnar cells; the following stratum spinosum can be composed of several cell layers. The stratum granulosum exhibits flattened cells with pycnotic nucleus and the outermost single-layered stratum corneum is arranged by much flattened, keratinocytes which have lost their nucleus. Note the flattening of the cells and the reduction of the nuclei during proximal to distal cell migration. bm, basement membrane; bv, blood vessel; de, dermis; lp, lamina propria; nu, nucleus; sb, stratum basale; sc, stratum corneum; sg, stratum granulosum; ss, stratum spinosum. Toluidine blue staining.

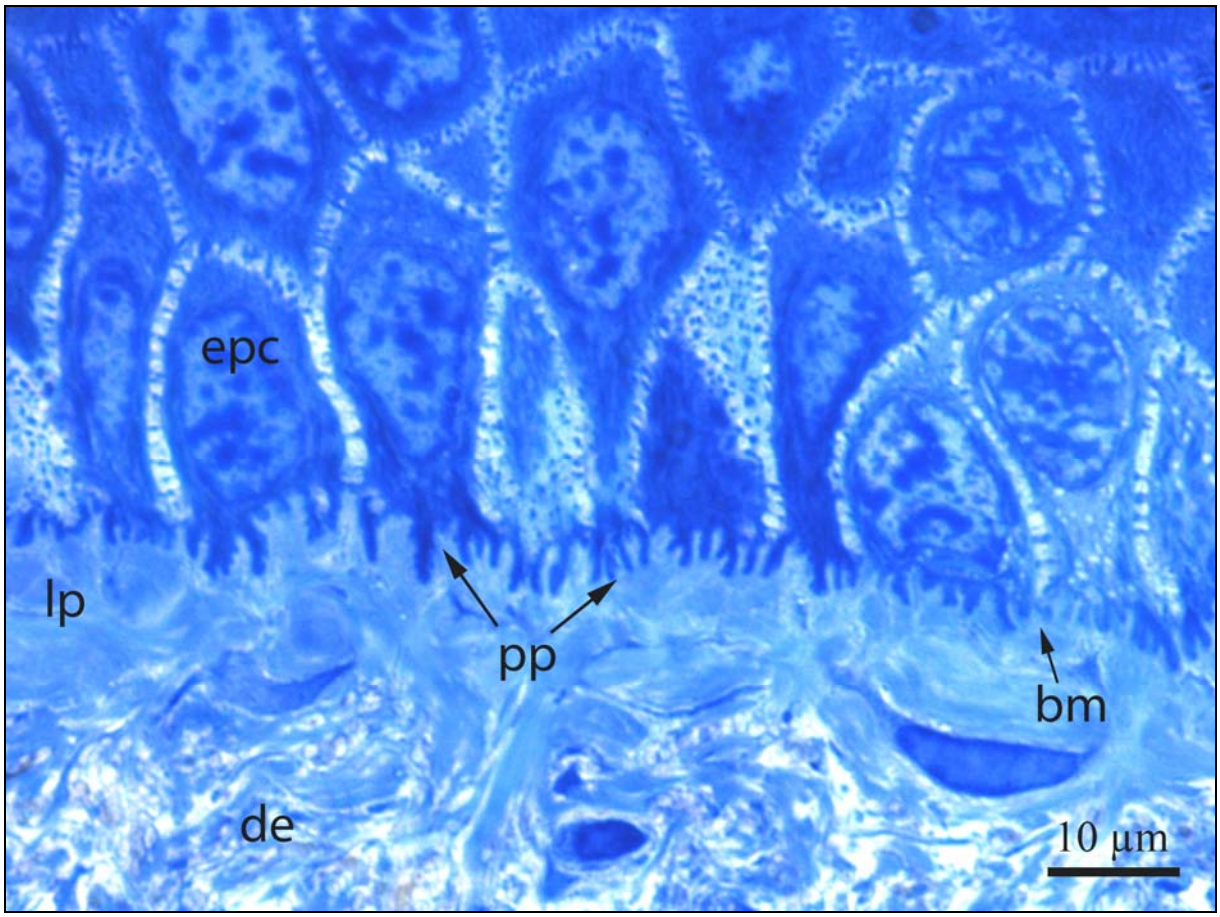

Fig. 8. Light micrograph showing in detail the basal epidermal layers of a dorsal region of the tail of juvenile *T. verrucosus*. Note that the epithelium is connected basally to the lamina propria through numerous periplasmatic protrusions (pp). These periplasmatic protrusions are especially prominent in the tail. bm, basement membrane; epc, epidermal cell; de, dermis; lp, lamina propria; pp, periplasmatic protrusions. Toluidine blue staining.

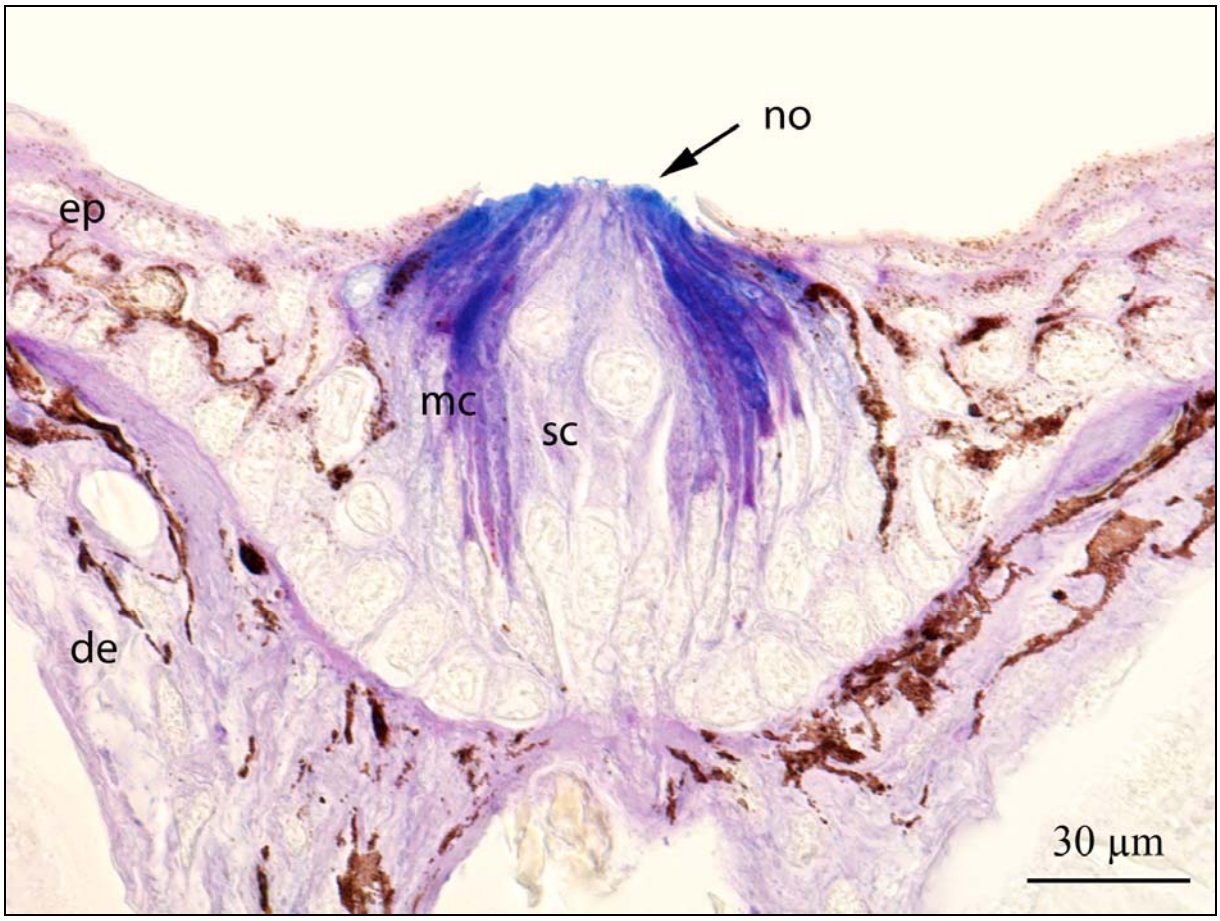

Fig. 9. Light micrograph from a lateral part of the head of juvenile *T. verrucosus* showing a neuromast organ, the sensory receptors of the lateral-line system. The pear-shaped mantle cells and sensory cells of the organ are clearly visible. Neuromast organs are present in both juvenile and adult individuals and underline the predominantly aquatic mode of life of *T. verrucosus.* ep, epidermis; de, dermis; mc, mantle cells; no, neuromast organ; sc, sensory cells; AB-PAS staining

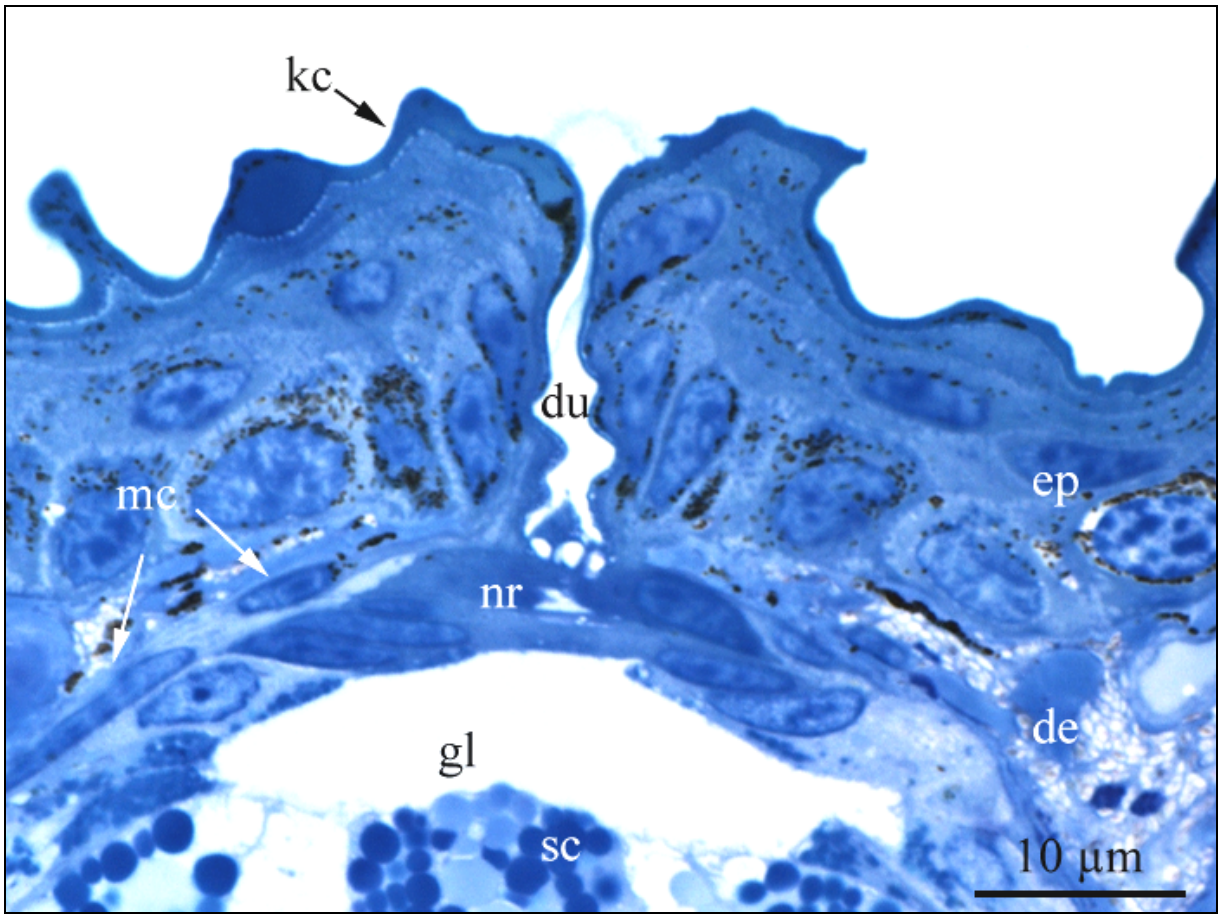

Fig. 10. Light micrograph showing a cross-section of the apical area of a mucous gland. All cutaneous glands exhibit an excretory duct, lined by keratinocytes. Note the neck region (nr) that provides regenerative stem cells, which develop both secretory and myoepithelial cells. ep, epidermis; de, dermis; du, duct; gl, gland lumen; kc, keratinocytes; mc, myoepithelial cell; nr, neck region; sc, secretory cell. Toluidine blue staining.

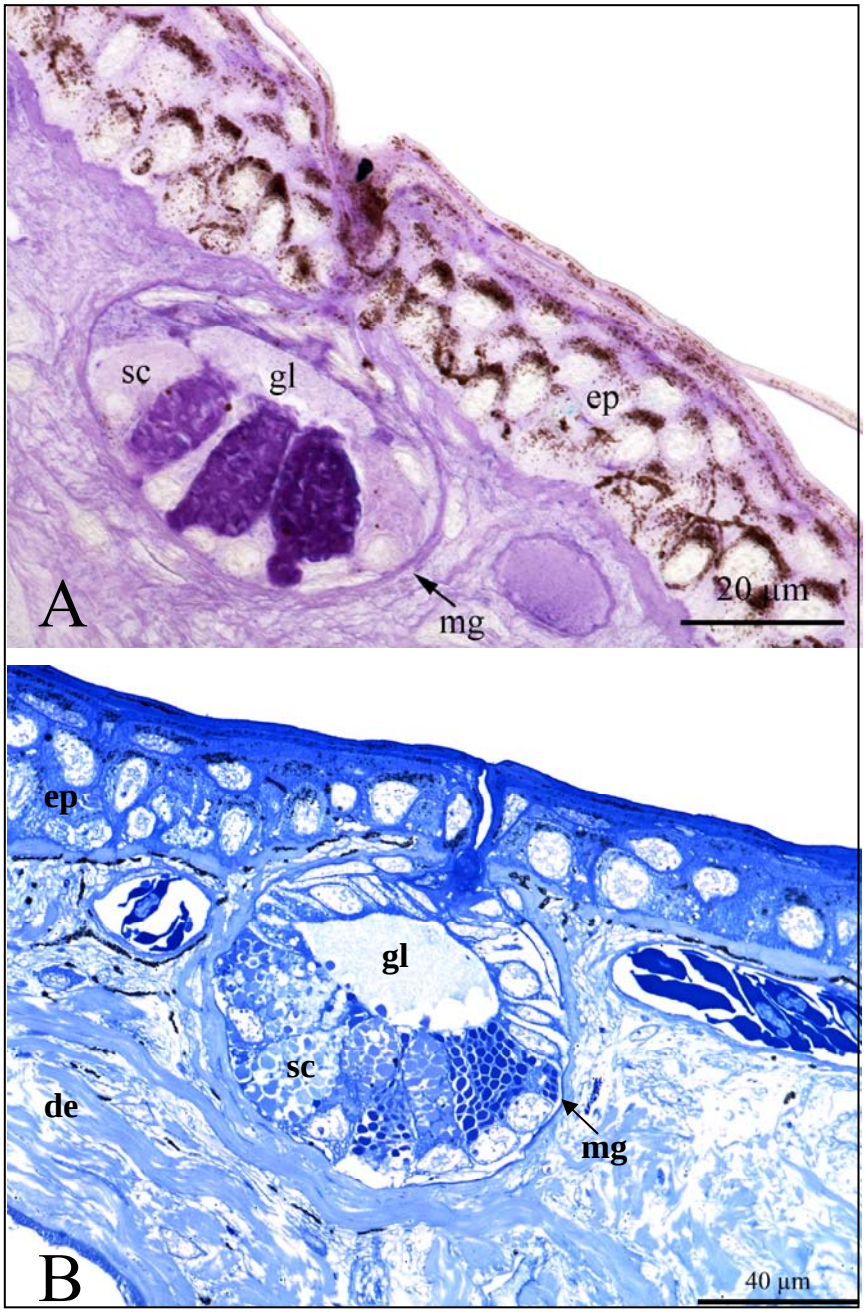

Fig. 11. Light micrographs showing mucous glands in juveniles. A: Most of the secretory cells of mucous glands react strongly positive to PAS and none of the cells in juveniles stain with alciane blue. Note that within the same gland the staining of secretory granules often varies from cell to cell, pointing to the content of different components within one gland. B: Mucous glands are small, roundish glands with wide lumen, encircled by vesicle-filled secretory cells. These secretory cells proliferate from apical to basal, where they increase in size and synthesize a great amount of secretions. Note the content in the glandular lumen, visible in both sections. de, dermis; ep, epidermis; gl, gland lumen; mg, mucous gland; sc, secretory cell. A: PAS-AB staining; B: Toluidine blue staining

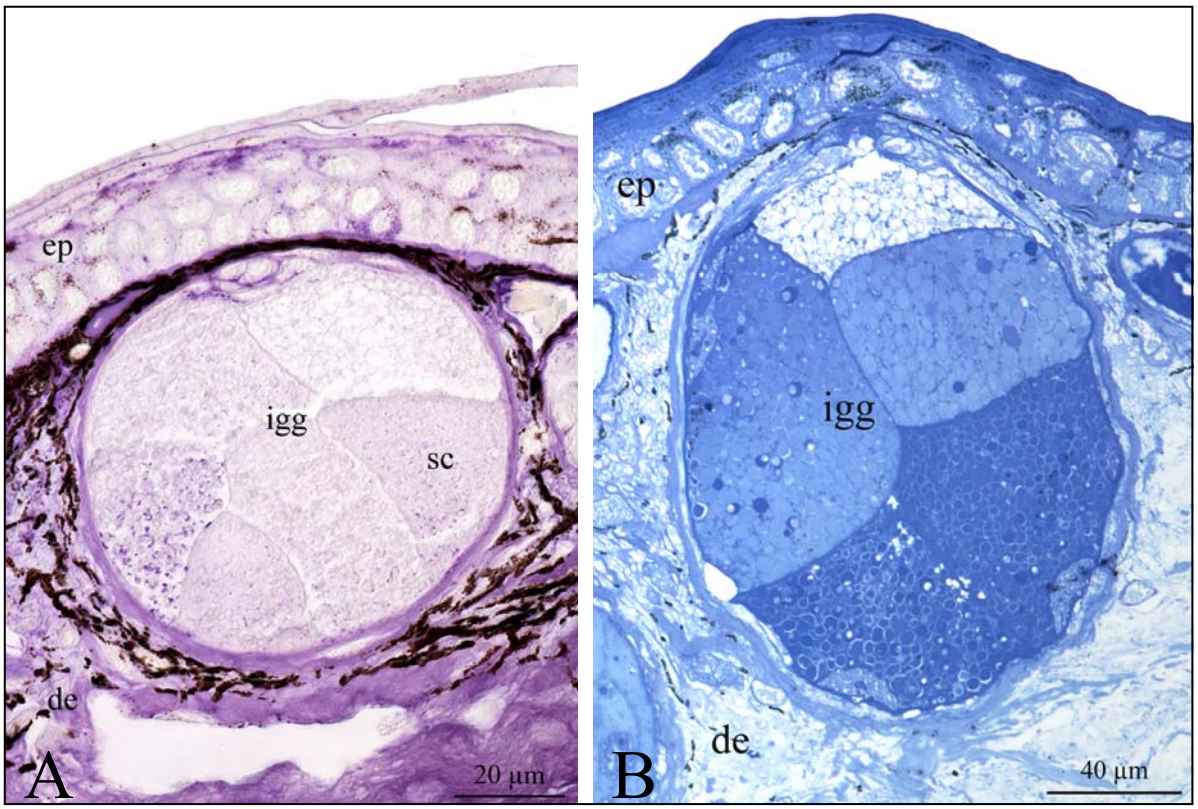

Fig. 12. Light micrographs showing cross-sections of GG1s in juveniles. Note that the appearance of GG1s is dependent upon the stage of maturation. Juveniles possess predominantly immature granular glands of several maturational stages. A: GG1s react slightly positive to PAS and negative to alciane blue. Note the diverse staining properties of the single secretory cells of this immature gland. B: Note the large size of secretory cells in GG1s. During maturation, the single secretory cells within the same gland differ from one another due to size, shape and density of their granules. ep, epidermis; de, dermis; igg, immature granular gland; sc, secretory cell. A: AB-PAS staining; B: Toluidine blue staining.

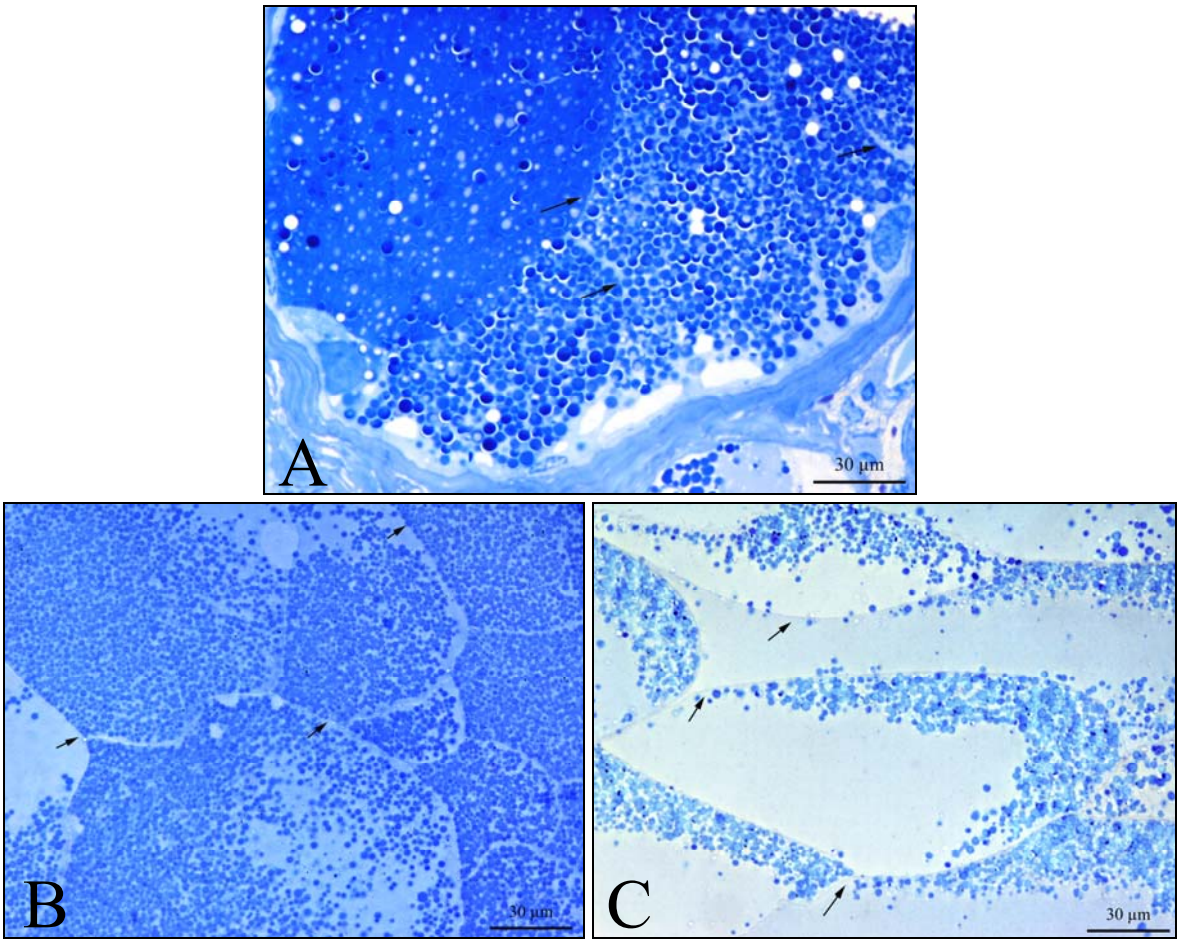

Fig. 13. Light micrographs showing the distribution of secretory granules within the different multi-cellular granular glands in an adult *T. verrucosus*. A: Ordinary GG1 from the trunk. The secretory cells contain a great amount of large, densely arranged granules which almost fill in the entire glandular volume. B: Enlarged GG1 from the parotoids. The secretory cells of enlarged GG1s exhibit smaller granules which are loosely distributed (compare with A). C: GG2 from the tail dorsum. The single secretory cells of this maximum gland type contain very small granules that characteristically form granular patches and are restricted to certain areas. All granular glands consist of single secretory cells, separated from each other by clearly visible cell membranes (arrows). Toluidine blue staining.

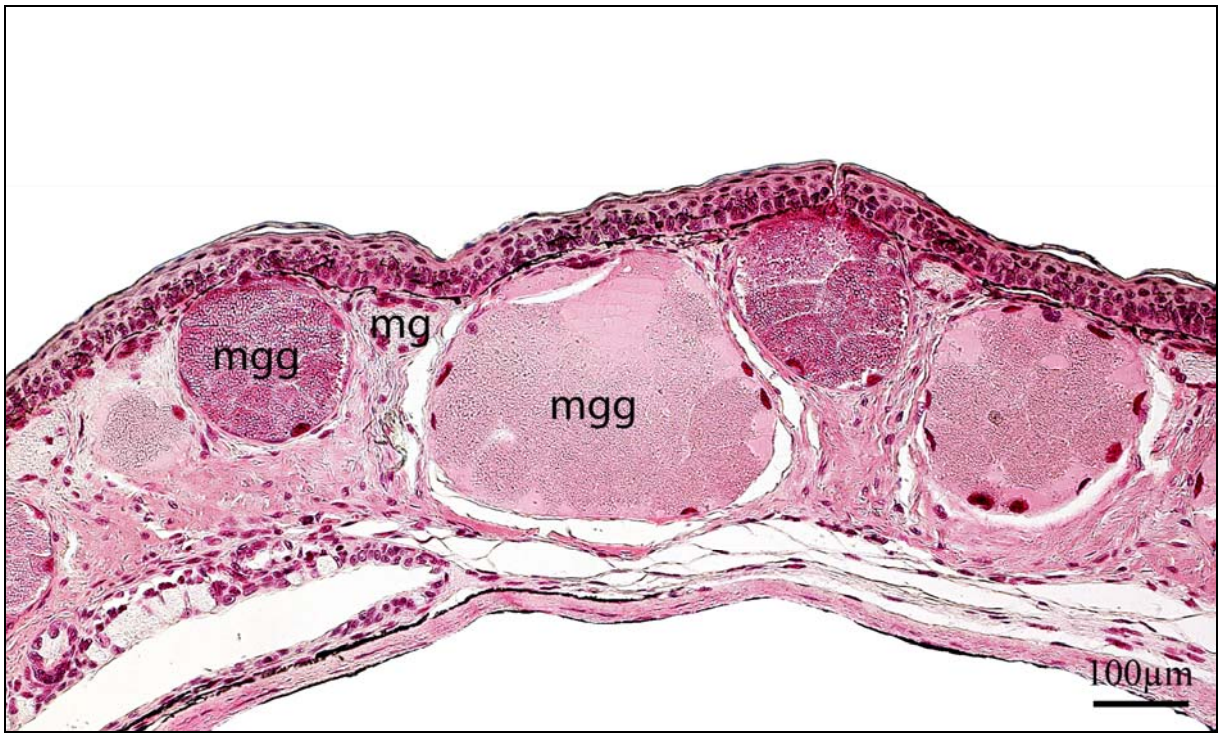

Fig. 14. Light micrograph showing the glandular distribution representative for the skin of a juvenile *T. verrucosus*. Predominantly immature granular glands (GG1s) of several maturational stages can be found in the skin of juvenile individuals. During maturation the granular glands expand and histostaining properties of their secretory cells change. Note the difference in size and different histostaining properties of the two main cutaneous gland types: mucous and granular glands. Granular glands show a positive reaction to eosin whereas mucous glands don't. mg, mucous gland; mgg, maturing granular gland. HE staining.

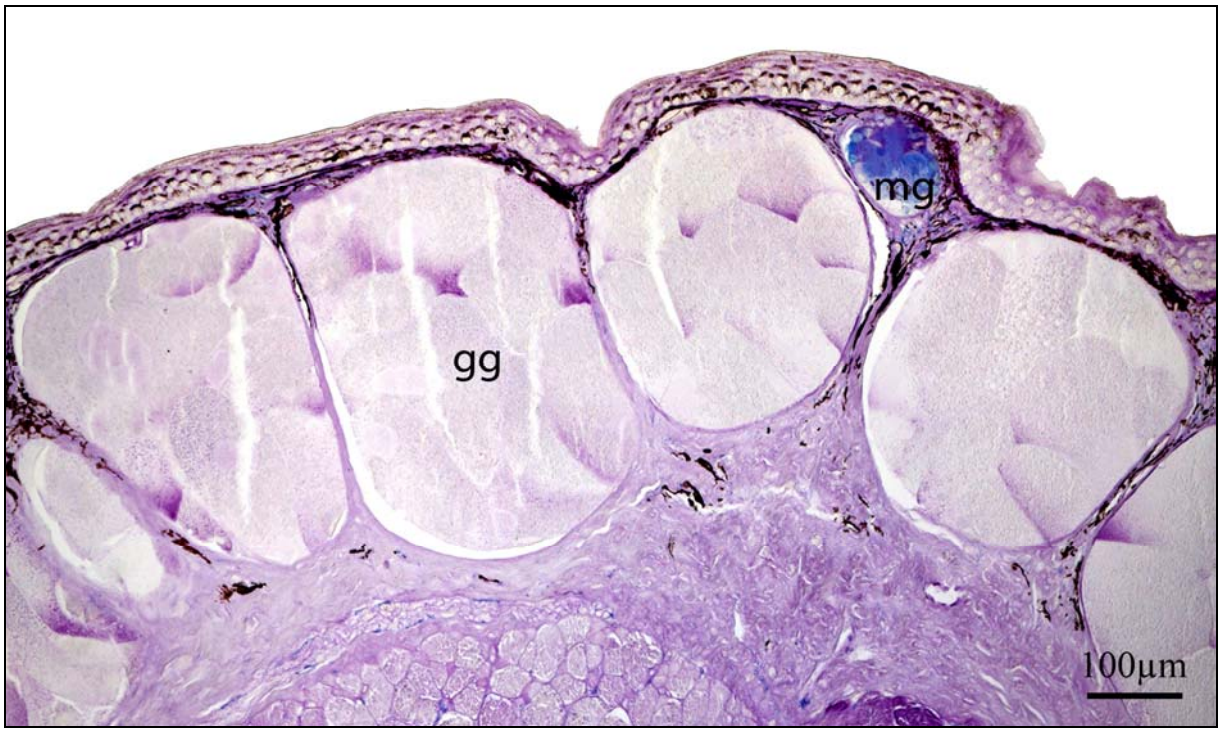

Fig. 15. Light micrographs showing the general glandular distribution representative for the skin of an adult *T. verrucosus*. Predominantly mature granular glands (GG1s) can be found in the skin of adult individuals. Note the different histostaining properties of the two main cutaneous gland types: mucous and granular glands. Mucous glands show a strong positive reaction to PAS and slightly to alciane blue. Granular glands (GG1s) slightly react positively to PAS and do not stain with alcian blue. gg, granular gland; mg, mucous gland. AB-PAS staining.

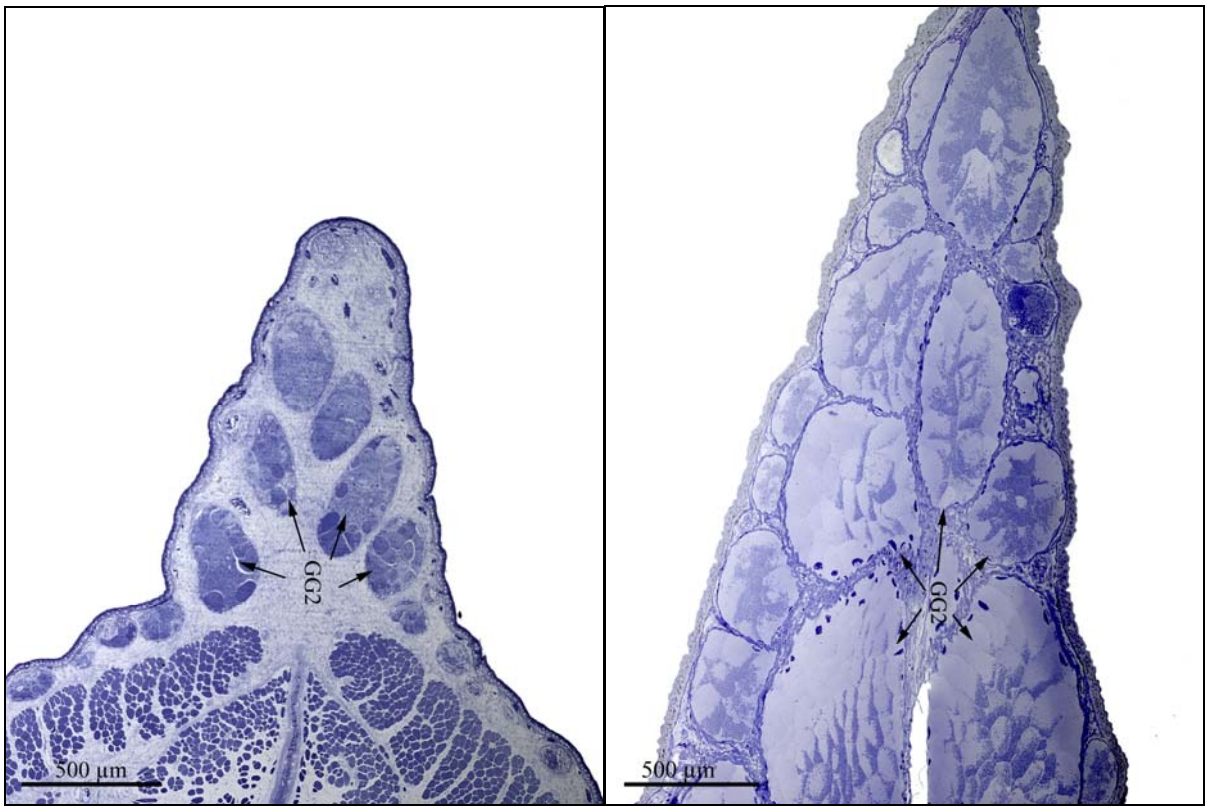

Fig. 16. Light micrographs showing cross-sections of the tail dorsum of (A) juvenile and (B) adult *T. verrucosus*. Note the distinct characteristics of granular glands type 2 (GG2) in juvenile and adult *T. verrucosus*. A: GG2s in juveniles are much smaller (maximal diameters up to 478 µm) than in adults (maximal diameters up to 1550 µm). They have an oval appearance and are horizontally elongated relative to the skin surface. B: GG2s in adults are huge glands that fill in the entire dermal layer. The picture only shows the dorsalmost third of the adult tail edge. Note the patchy distribution of the granules in the GG2s, a characteristic attribute for mature GG2s. Toluidine blue staining.

3. Transmission electron microscopic (TEM) findings

In the course of this research skin samples from adult individuals were used for ultrastructural investigations to provide additional information on the cellular organization of the two main cutaneous gland types – mucous and granular glands.

Mucous glands consisted of secretory cells encircling a central lumen. The cells from the apical pole were smallest and increased in size toward the basal gland pole. The apical cells contained a large number of organelles, mainly mitochondria, rough endoplasmatic reticula and Golgi apparati (Fig. 19) as well as densely packed vesicles: rather small in size compared with the vesicles from the basally located secretory cells. The secretory cells from the basal gland pole were the biggest cells within the mucous glands. They exhibited round-oval shaped, basally located nuclei and were filled with big, round vesicles. Organelles, including endoplasmatic reticulum and Golgi apparatus appeared very frequently and were especially concentrated around the nucleus. Mitochondria were scattered throughout the few vesicle-free areas and appeared most frequently in the apical areas close to the cell boarders. The surfaces of the secretory cells toward the lumen bore microvilli what provided an enlarged surface area (Fig. 20). The mucous glands were enclosed by a network of myoepithelial cells, followed by a tight layer of dermal collagen fibers. Secretory cells and myoepithelial cells were connected to each other by intervening periplasmatic protrusions.

Mature GG1s and GG2s consisted of large cells with a great amount of abundant round vesicles, distributed through their cytoplasm (Fig. 17). The vesicles were densely arranged in the central parts of the gland and less frequently in the periphery. The cell nuclei were positioned basally where most of the organelles were present. Rough endoplasmatic reticula and Golgi apparati could be found in close proximity to the nucleus, whereas mitochondria were found everywhere in the cytoplasm. In general mature GG1s and GG2s in *T. verrucosus* were organelle-poor structures but densely crowded with vesicles. All secretory cells were separated from each other by clearly visible cell membranes (Fig. 17) and were connected together by meshing periplasmatic protrusions. A distinct myoepithelial sheath enclosed the gland (Fig. 18). The myoepithelial cells possessed elongated flattened nuclei and a large number of mitochondria. The myoepithelial cells were connected to each other and to the secretory cells by meshing periplasmatic protrusions of their cell membranes. Adjacent to the myoepithelial sheath, a thick wall of tight collagen fibers encircled the glandular unit (Fig. 18).

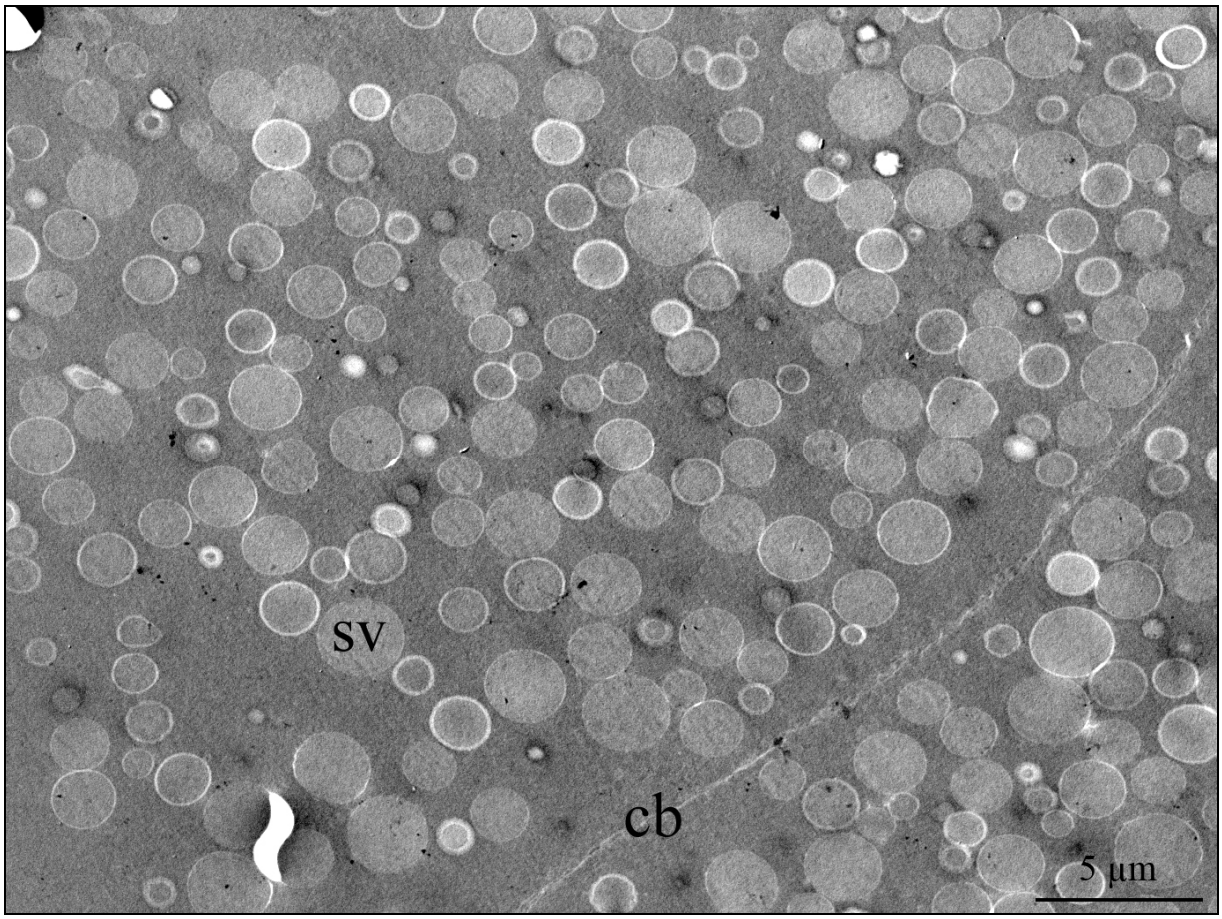

Fig. 17. Transmission electron micrograph showing parts of the secretory unit of a mature GG1. Mature granular glands are build up by huge secretory cells filled with vesicles. Apart from vesicles that are stored ready for expulsion, mature granular glands are organelle-poor structures, pointing to a state of suspended activity. Note the distinct cell border, indicating the absence of syncycial fusion in *T. verrucosus*. cb, cell boarder; sv, secretory vesicle.

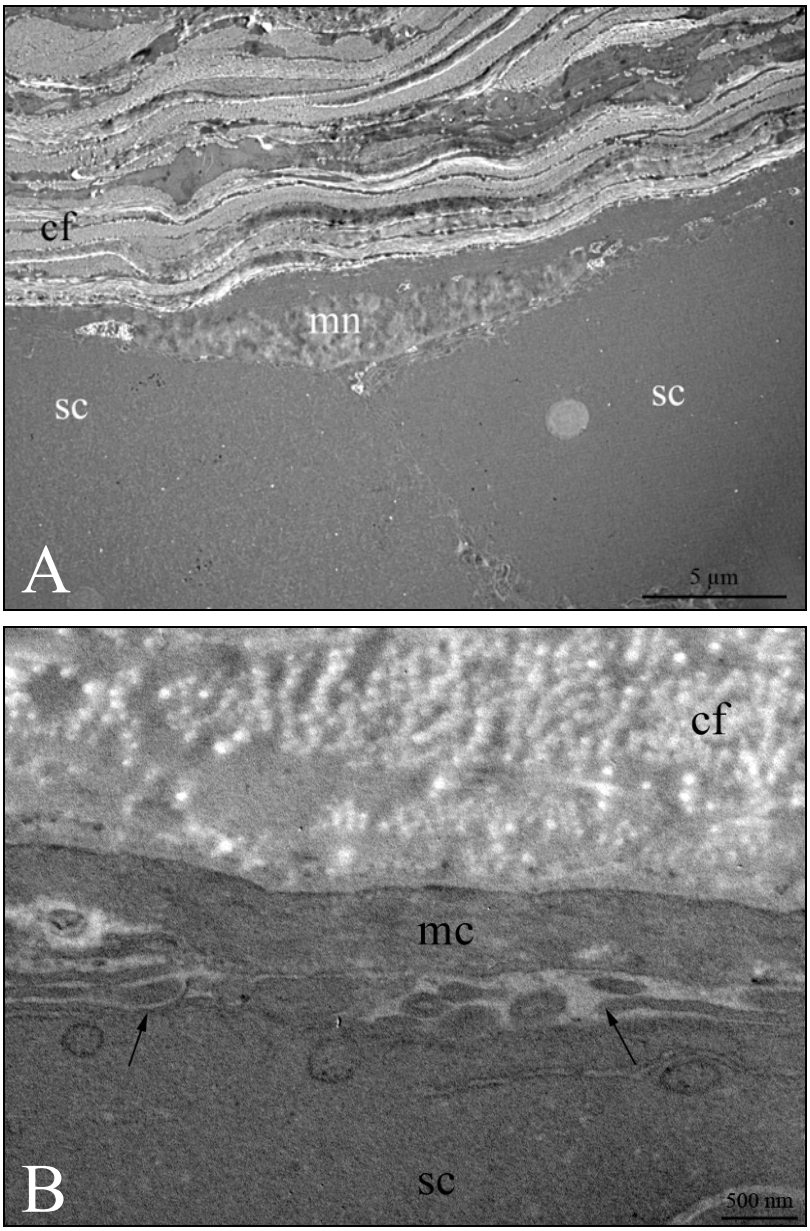

Fig. 18. Transmission electron micrographs showing the myoepithelial sheath of a GG2. The granular gland possesses a contractile myoepithelial sheath, responsible for fast expulsion of glandular contents. A: The nuclei of myoepithelial cells are flattened. The myoepithelial sheath encircling the granular gland is distinct visible and followed by a tight layer of dermal collagen fibers B: The interdigitating periplasmatic protrusions (arrows) from the myoepithelial- and the secretory cells provide adhesion. cf, collagen fibrils; mc, myoepithelial cell; mn, myoepithelial cell nucleus; sc, secretory cell;

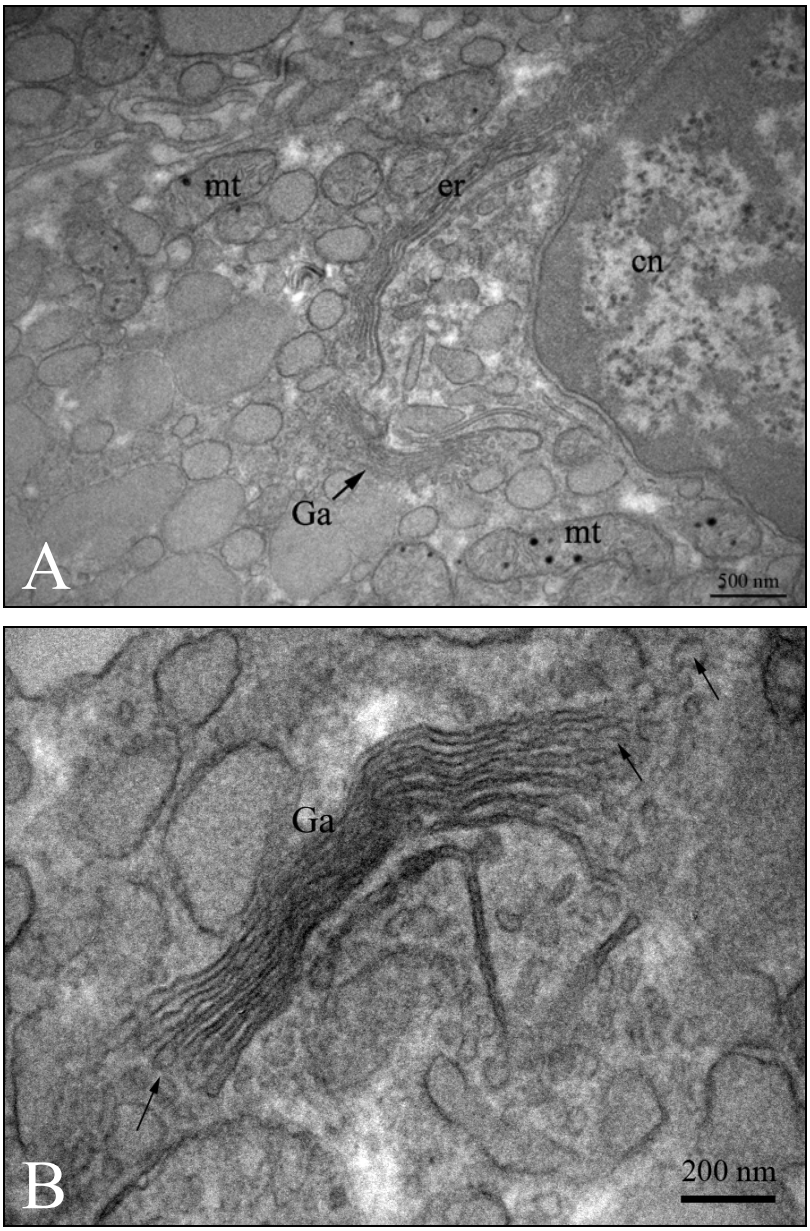

Fig. 19. Transmission electron micrographs showing the organelle-rich facilities of mucous glands. A: Note the presence of Golgi apparati, endoplasmatic reticula, mitochondria and secretory granules in the highly active, apically located mucous gland cells. B: Detail of a Golgi apparatus with delivering vesicles (indicated by arrows). cn, cell nucleus; er, endoplasmatic reticulum; Ga, Golgi apparatus; mt, mitochondrium;

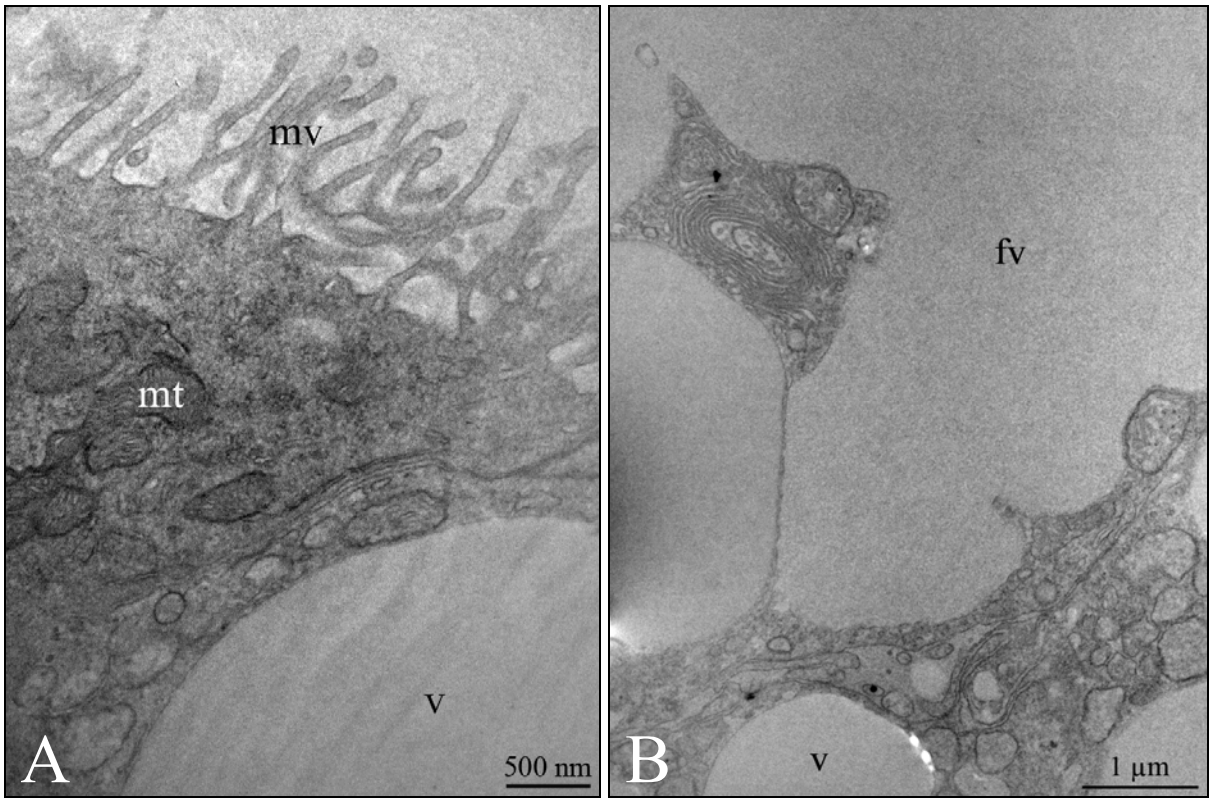

Fig. 20. Transmission electron micrographs showing the apical regions of basal located secretory mucus gland cells. A: The surface of a secretory cell to the lumen bears microvilli. Note the high number of mitochondria in the apical portion of the cells. B. Single vesicles can merge together before fusing with the cell membrane and discharging their contents into the lumen. fv, fusing vesicles; mt, mitochondria; mv, microvilli; v, vesicle.

4. Anti-predator behavioral findings

When animals were prodded repeatedly, they displayed several defensive behavior patterns. Most of the defensive postures in *T. verrucosus* could be related to the presence of skin secretions. Dependent upon the site of the stimulus, the body was always orientated in the way to present concentrations of enlarged granular glands towards the stimulus (Fig. 21; Fig. 22). Within seconds, secretions of glandular contents could be visible, as white, milky fluids covering the surface of the body (Fig. 22).

T. verrucosus exhibited a great diversity of antipredator postures when being stressed and the characteristics are listed.

At the beginning of predator simulation, most of the animals tried to escape by moving away from the threatened stimulus. If stimulation continued and an escape was prohibited, the response was immobility. In the following, several postures were displayed during defensive behavior. Sometimes the body was arched, the legs were extended and the midbody was elevated off the ground. The ribs could be erected and elevated the lateral warts. As the mid-dorsal ridge as well as the lateral warts exhibited concentrations of enlarged granular glands (see Fig. 21), this would increase the likelihood of predator contact with these glandular concentrations.

Especially the tail, which was stocked with giant granular glands, was used intensively during defensive behavior. The tail was either undulated and moved in a sinuous manner vertically or wagged, whereby the tail was raised off the substrate and swung from side to side. This leads the attention to the most dispensable part of the body, the glandular tail.

During posturing, the head and tail in general were often coiled toward the attack. Especially juveniles exerted the head in antipredator postures, as the glandular parotoid region was swung into the attacking stimulus, accompanied by body coiling and tail positioning over the body.

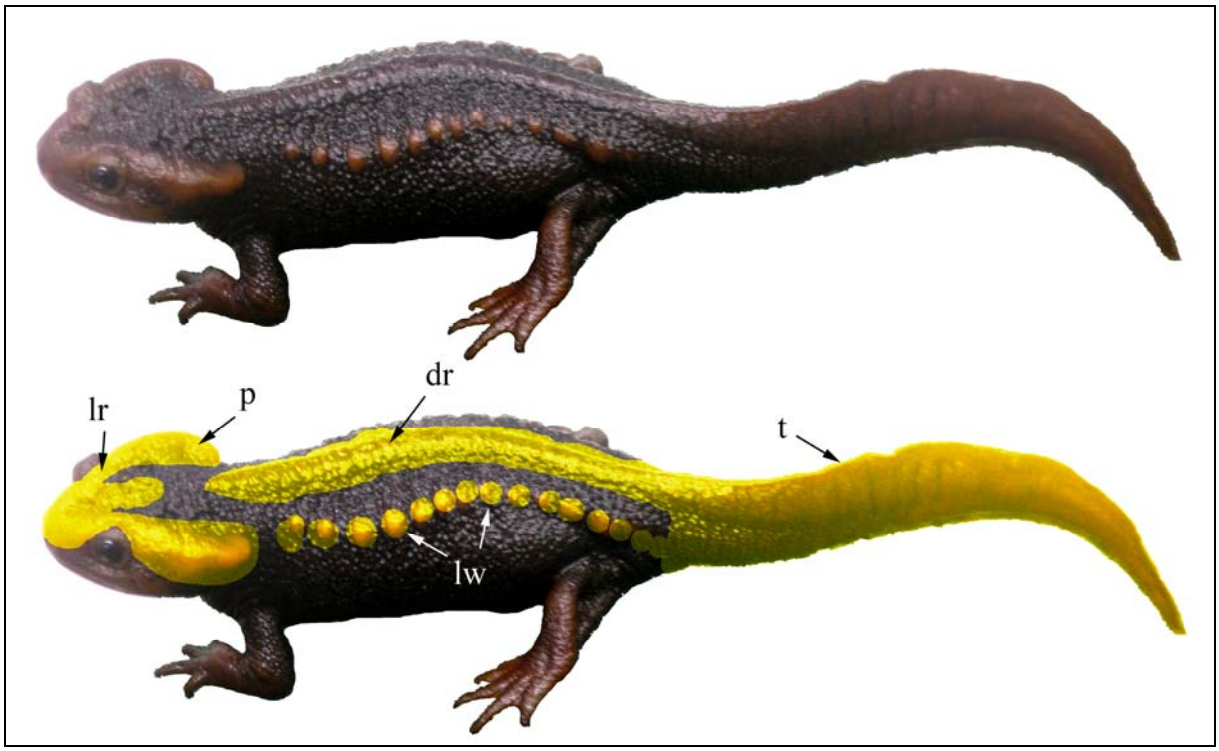

Fig. 21. Photographs of *T. verrucosus* and schematic demonstration of enlarged granular gland accumulations of examined individuals. Several body areas exhibit concentrations of enlarged granular glands, marked yellow in this figure. These areas include the dorso-lateral bony ridges of the head, the parotoids, the mid-dorsal ridge, the dorso-lateral rows of warts and almost the entire tail. Head and tail exhibit the greatest accumulation of enormous, enlarged granular glands and especially these areas are deployed during defensive behavior. dr, mid-dorsal ridge; lr, dorso-lateral ridge of the head; lw, lateral wart; p, parotoid; t, tail.

Fig. 22. Anti-predator behavior of *T. verrucosus*. Various antipredator postures were displayed in response to mild threatening stimuli. Depending on location and intensity of stimuli, various patterns were performed. A: The tail was coiled if animal was touched from lateral. B: White, milky excretions became visible in close proximity to threatened stimuli. Note the single beads of secretion released from single glands. C: Especially head and tail (see also A) were involved in defensive behavior. Both regions contained concentrations of enlarged granular glands providing a great amount of noxious secretion.

DISCUSSION

Amphibians may appear to be rather defenseless animals. Many are small in size, move slowly or tire after rather short bursts of activity. Most lack protective armors like claws, and powerful teeth and jaws are generally unsuited for defense against animals larger than themselves. As the skin is involved in respiration in most species, it lacks thickenings that might provide a mechanical protection against predators (Stebbins and Cohen, 1997). However, terrestrial salamanders from the families Hynobiidae, Ambystomatidae, Salamandridae and Plethodontidae have independently evolved elaborate combinations of antipredator mechanisms in response to the predatory pressure on land (Brodie, 1977). Morphological, physiological, and behavioral features have evolved, which alone or in combination provide protection from potential predators. *T. verrucosu*s is a member of the salamandrids, the family showing the greatest diversity of antipredator mechanisms of any urodele family (Brodie, 1983). Most salamanders have skin secretions that can repulse predators. Others, such as *Notophthalmus viridescens*, *Taricha granulosa*, *Salamandra salamandra* and *Tylototriton verrucosus* not only have repulsive secretions, but noxious and toxic ones as well to truly harm any would-be predator (Brodie, 1983; Brodie et al., 1990). Noxious skin secretions, warning coloration and behavior are the primary defense of many amphibians (Stebbins and Cohen, 1997). The antipredator arsenal of *T. verrucosus* is based on such noxious skin secretions (in accordance to Brodie et al., 1984), which is considered to be the most important means of repelling predators within terrestrial salamanders. Most other antipredator adaptations, such as color and posture are dependent upon the presence of these secretions (Brodie, 1977; Nowak and Brodie, 1978).

The class Amphibia is the phylogenetic oldest tetrapod class, and in many ways amphibians are transitional between fishes and reptiles (Porter, 1972; Westheide and Rieger, 2004). The integument of modern amphibians performs several functions and the main evolutionary advance of the amphibian skin over the ones of fishes is the presence of large numbers of multicellular alveolar skin glands. Single secretory cells, embedded in the epidermis, are still widely distributed in bony fishes, but became arranged into complex multicellular organs in amphibians. In amphibians, they considerably increased secretory activity and gained several new important functions (Delfino et al, 2002; Porter, 1972). Originally engaged in regulative functions, serous/granular glands were later employed in defensive strategies, playing a role in repellent or toxin production (Delfino et al, 2002; Duellman and Trueb, 1994). Toads and

salamanders have been considered noxious creatures for centuries and indeed many amphibians produce a remarkable diversity of antipredator substances in their skin glands.

Tylototriton verrucosus, is supposed to be one of the most toxic salamanders; more toxic than *Cynops pyrrhogaster, Paramesotriton honkongensis*, *Notophthalmus viridescens* (eft and adult) or *Pleurodeles waltl* (Brodie et al., 1974; Nowak and Brodie, 1978) – and more or less as toxic as *Taricha rivularis* and *Taricha torosa* (Brodie et al., 1984). When placed together with shrews, *T. verrucosus* was being attacked but never seriously injured, as shown in a study of Brodie et al. (1984). The shrews however, chewing or eating bits of the skin of *T. verrucosus* started to regurgitate within seconds and when macerated skin of *T. verrucosus* was injected intraperitoneally into mice, they exhibited toxic symptoms and died within minutes (Brodie et al., 1984). These experiments showed that the secretions of the skin glands of *T. verrucosus* have the potential to truly harm or even kill would-be predators. Although the newts may suffer wounds and smaller injuries during the encounter behavior, the toxic skin secretions seem to be an effective protective factor that prevents being eaten by most potential predators.

The present study comprises the findings of two age groups, juveniles and adults. All examined individuals were completely metamorphosed and the major morphological and physiological changes of the larval skin were fulfilled.
The general constitution of the skin in both age groups was alike. In general the skin of *T. verrucosus* showed several adaptations associated with the amphibious life and the close connection to an aquatic environment. The secretion of cutaneous glands ensures the various tasks performed by the skin. The skin of *T. verrucosus* is a poorly keratinized organ, composed of a few cell layers, whereof only the outermost cell layer is keratinized. As the amphibian skin is water permeable and important in respiration and osmoregulation (Fox, 1994; Stebbins and Cohen, 1997; Clarke, 1997; Fontana et al., 2006), protective thickening or other modifications of the skin which would provide a mechanical protective barrier, are limited. The secretions of cutaneous glands are an advantage as they make the skin of amphibians quite slippery and therefore decrease lesions by abrasion and promote escape from aggressors (Stebbins and Cohen, 1997). *T. verrucosus* inhabits both terrestrial and aquatic habitats, but is more closely confined to water than allied species (Seglie et al, 2003). This is also reflected in the organization of the skin, as neuromast organs of the lateral-line system are present in all stages of life of *T. verrucosus*, even in adult individuals. Neuromasts

function as mechanoreceptors, sensitive to water currents, and are therefore present only in aquatic animals. They are known among amphibians in aquatic larvae, aquatic adult urodels, adult pipid frogs and adult salamandrids that are aquatic after a terrestrial stage (Duellman and Trueb, 1994). Additionally the skin of *T. verrucosus* is well supplied with capillaries forming an extensive network of blood vessels. This underlines the role of the skin as a respiratory organ in *T. verrucosus.*

The entire skin of juvenile and adult *T. verrucosus* was interspersed with the main cutaneous glands: mucous and granular. Whereas granular glands are thought to be the site of skin toxin production and associated with defence (Noble, 1931; Stuhr, 1936; Quay, 1972; Delfino, 1976; Nowak and Brodie, 1978; Neuwirth et al., 1979; Daly, 1995; Toledo and Jared, 1995; Alvarez et al., 2005; Daly et al., 2005; Arifulova et al., 2007) mucous glands are generally participated in several crucial functions.

The cutaneous glands of amphibians have received considerable attention from morphologists. All adult extant amphibians are believed to posses two main types of dermal glands: mucous and serous/granular glands (Duellman and Trueb; 1994, Fontana et al, 2006; Fox, 1994; Houck and Sever, 1994). Helf and Stark (1941) raised the question whether the two types of glands develop from separate “nests” or if granular glands represent simply a further differentiation of the mucous type. In the past there has been controversy concerning this question but the results of Helf and Stark (1941) as well as of Delfino et al. (1982) are very clear. The mucous and granular glands arise from distinctly separate glandular nests and no transition stages or other indications of glandular transformation from one type to the other have been observed. Mixed glands, with mucous and granular units, are reported in the literature as a constant feature for some Urodela (Delfino et al., 1982; Brizzi et al, 2002). This poses the question whether the mixed glands represent transformations of mucous and/or granular glands or are separate gland lines that have evolved independently in several species and exhibit special functions. The study of Delfino et al. (1982) evidence that the mixed units represent a further independent gland line. Giovanni Delfino is one of the leading experts in amphibian exocrine glands of and a lot of his work concerns the development and organization of the glandular tissue in amphibians. His analyses of development of cutaneous glands in *Salamandrina terdigitata* show that previous Anlagen are formed by blastocytes in the epidermis and evolve into complex functional units. The anlagen can be identified by the presence of the secretory elements, even before secretion occurs.

However, *T. verrucosus* shows at least three different acinar, multicellular cutaneous gland types: mucous glands (MG) and two different types of granular glands (GG1 and GG2). The

identified granular glands differ in several morphological and histostaining properties from MGs.

Mucous glands in amphibians are involved in fundamental physiological activities. During the transition from water to land in tetrapod evolution, multicellular mucous glands presumably arose to keep the skin moist to avoid dehydration in the new environment (Toledo and Jared, 1995). Mucous glands in *T. verrucosus* are widely distributed on the body and mainly responsible for producing mucopolysaccharides and mucoproteoglycans, the main components of the slippery amphibian mucus. Mucous glands in adult *T. verrucosus* reacted mainly positively to PAS and slightly to alciane blue, indicating the presence of primarily neutral carbohydrates but acidic carbohydrates as well. In the mucous glands of amphibians, a mixture of acidic and neutral mucopolysaccharides is common. For instance, some species of *Aneides* and *Plethodon* (Staub and Paladin, 1997; Hecker et al, 2003) as well as *Eurycea* (Sever, 1989) secrete both, neutral and acidic mucus. However, in *T. verrucosus* as well as in the closely related species *P. waltl* most of the mucous gland cells show a strong reaction to the PAS-test, pointing to a majority of neutral mucosubstances (according to Quay, 1972; Bueno et al., 1981; Fox, 1994; Heiss et al., 2009).

Interestingly, the mucous glands in juvenile *T. verrucosus*, though resembling those in adults, exhibit no acidic carbohydrates. Nevertheless, goblet cells und multicellular glands that open into the oral cavity of the investigated juvenile *T. verrucosus* do contain acidic carbohydrates. Presumably the necessity of acidic mucus is only given in adult *T. verrucosus* and therefore not developed in juveniles. The functional differences between acidic and neutral mucus are uncertain and not properly studied yet. Getchell et al. (1984) proposed in a work of salamander oral mucosa, that neutral mucus forms a thin watery surface layer, whereas acidic mucus comprises a thicker surface layer. It may be beneficial to produce a diluted, watery surface film during the growth process of *T. verrucosus*, whereas tough mucus may have more advantages in adult stages. As discussed above the composition of the mucus can definitely be modified within the mucous glands during life. The potential of changing the mucous composition as an adaptation to certain conditions may be advantageous in respect of changing environmental conditions.

Mucous glands of *T. verrucosus* are typically small glands and feature a central lumen, which is continuous with the excretory duct that opens through a pore to the exterior. Light microscopic findings of mucous glands of *T. verrucosus* are very similar to the mucous glands of other amphibians (e.g., Bueno et al., 1981; Delfino et al., 1982; Brizzi et al, 2002; Hecker et al., 2003; Fontana et al., 2006; Heiss et al., 2009). All functions of mucous glands, usually

involved in fundamental physiological activities, may account for their uniform appearance and homogeneity throughout the class (Brizzi et al., 2002). Nonetheless, present findings of mucous glands of *T. verrucosus* indicate, that besides age-related variations there are regional ones as well. It could be assumed that on different parts of the body, mucous glands can provide different secretions that may result various functions. This paints a wider morphofunctional scenario and the adaptive potential of mucous glands may have more biological importance as previously thought.

Secretory cells encircling a central lumen build up the mucous gland. As shown by LM, the cells in the apical gland pole are smallest and increase in size towards the basal pole. Ultrastructural investigations revealed that the smallest cells, located in the apical pole, are especially organelle-rich. They exhibit great amounts of endoplasmatic reticula, Golgi apparati and mitochondria, which are necessary for the great amount of secretory granules they produce. Towards the basal gland pole, the number and size of vesicles within the secretory cells increase. The basalmost located cells are stocked with big sized vesicles, still display organelles in the few vesicle-free areas. Such observations lead to the hypothesis that apical cells reflect an immature status of development whereas advanced stages of maturation are represented in cells of the basal gland pole. Therefore it seems likely that secretory cells within mucous glands proliferate from apical to basal (in accordance to Heiss et al., 2009).
Glandular secretion filling in the mucous gland lumen was seen in nearly all sections observed. While the secretory product within the glandular cells is stored in vesicles, such vesicles were mostly lacking in the MG-lumen. It seems likely, that mucous secretion is largely merocrine. As the lumen of the gland is continuous with the excretory duct, we can assume that mucus reaches the body surface permanently, resulting from luminal pressures generated by continuous exocytosis activity of the secretory cells. Hoffman and Dent (1978) argued that mucous glands secrete their contents in a merocrine manner continuously onto the skin and that phasic gland stimulation seems likely.
In the past, there has been uncertainty about the presence of a distinct myoepithelial sheath in mucous glands. Some authors reported that a myoepithelial sheath is absent or, if present, poorly developed (Dawson, 1920; Quay, 1972; Reyer et al., 1992; Brizzi et al., 2002). Brizzi et al. (2002) suggested that a myoepithelium directly innervated is a feature, generally not found in mucous units.
In *T. verrucosus,* a distinct myoepithelium which surrounds the mucous gland is present. This pattern is also observed in the closely related species *Pleurodeles waltl* (Heiss et al., 2009).

The myoepithelial sheath of mucous glands is thin and hardly visible using light microscopy but clearly recognizable with transmission electron microscopy. Studies which included electron microscopic findings in general revealed the presence of a mucous gland myoepithelium (e.g., Voute, 1963; Ernst, 1973). Several recent findings indicate that a myoepithelial sheath is a consistent feature of amphibian mucous glands. Hoffman and Dent (1978) postulated that a MG-myoepithelium of the red-spotted newt is rather vestigial and without functional significance, because of its thin appearance. The MG-myoepithelium in *T. verrucosus* is clearly thinner than that of GGs but is has to be mentioned that MGs are much smaller as well than GGs. In any case, it seems likely that muscular contraction on mucous glands can lead to glandular discharge and it is supposed here that the MG-myoepithelium features an important biological role.

Secretory cells of mature mucous glands are probably regularly exchanged by a continuous proliferation from apical to distal and exhausted basally located secretory cells are replaced. Once developed, mucous glands are characterized by continuous exocytosis activity of their secretory cells, a glandular discharge mechanism that fits the main function of primarily keeping the skin moist. Based on the present findings, it is suggested that a constant merocrine secretion of mucus into the glandular lumen and consequently onto the body surface is true in *T. verrucosus*. Additionally a luminal discharge of a greater amount of mucus all at once seems possible, due to the presence of a well-developed myoepithelial sheath.

Granular glands are the second main cutaneous gland type in *T. verrucosus.* As in other amphibians (Quay, 1972; Toledo and Jared, 1995), they are much larger than mucous glands and in contrast to the latter, they produce mainly proteinaceous components (in accordance to Le Quang Trong, 1973; Reyer et al., 1992; Fontana et al., 2006; Heiss et al., 2009). Amphibians are known for the wide range of bioactive substances they produce in their granular glands, including amines, peptides and a great number of alkaloids (Daly, 1995; Daly et al., 2005). Probably most granular glands were originally used for regulative functions and gained later a multiplicity of aims, producing diverse bioactive substances, including toxins (Duellman and Trueb, 1994). Granular glands of *T. verrucosus* share some common features with mucous glands as they are multicellular, acinar glands located in the dermis, that open to the skin surface by an excretory duct. They are build up by a neck region containing regenerative stem cells, a secretory unit containing the secretory cells, and the surrounding myoepithelium as the contractile sheath.

While MGs in juveniles have the same size and general organization as in adults, the appearance of granular glands differs from juveniles to adults. Most conspicuous is the difference in size and the maturation state. Granular glands in juveniles are much smaller than in adults, compared in equivalent body areas and many show an immature state, whereas adults feature predominantly mature granular glands. This is applicable for all granular glands observed, most conspicuous for the GG2s of the dorsal edge of the tail and less discernable for the GG1s of the parotoid area. Maturing glands become apparent, as the single secretory cells within one gland differ from one another pertaining to their granule appearance and partially staining properties. Fully developed GGs are big and assigned by organelle-poor secretory cells, still separated by cell membranes. They comprise a great amount of granules, featuring all approximately the same size and shape in every secretory cell. The small quantity of organelles present in mature granular glands leads to a state of suspended activity and advanced maturation.

In granular gland history the gland is presumably emptied by contraction of the myoepithelium and the broken secretory cells are then renewed and mature to become functional again. In contrast to mucous glands, secretory cells of granular glands are supposably not regularly exchanged by continuous proliferation from apical to distal. Granular glands are rather characterized by long biosynthetic and maturational processes where a great amount of secretory cells develop and mature. This process seems to progress very slowly indicating that the production of secretions, repulsive to predators is extensive and complex. Like loaded weapons, ready for expulsion, the specific bioactive secretions are stored within the granular glands. The rapid contraction of the myoepithelial sheath leads to the expulsion of the granular contents in a holocrine way (Navas et al., 1982; Delfino et al., 1995). A distinct, directly innervated myoepithelial sheath that regulates secretions of granular products is present in *T. verrucosus* and has been consistently observed in the granular glands of both Anurans and Urodeles (Hoffman and Dent, 1977; Hoffman and Dent, 1978; Reyer et al., 1992; Brizzi et al., 2002).

T. verrucosus possess two distinct types of granular glands: GG1s and GG2s. As described in detail in the results, GG2s in *T. verrucosus* differ in several histostaining and morphological characters from GG1s. Summarized, GG2s are only present in restricted areas of the skin: the dorsal and ventral edges of the tail. Different substances are present in the secretory cells of GG2s as they stain differently with Coomassie brilliant blue compared to GG1s. This is evident both during gland development as well as in mature GG2s. In contrast to ordinary

GG1s the secretory granules of mature GG2s are smaller and distributed rather patchy throughout the gland and do not fill up in the entire gland. This distinct patchy distribution of granules in GG2s is a conspicuous characteristic for this type of gland. Further differences to ordinary GG1s, distributed elsewhere concern their large size, their oval appearance and their horizontal elongation, in relation to the skin surface. Only ontogenetic analysis of the skin glands would ascertain if GG2s represent a different gland line but all findings so far support the theory that serous gland polymorphism is true for *T. verrucosus.*

Delfino et al. (1998) suggests that the presence of different serous gland types within one species can be regarded as a characteristic feature for at least some species of hylids and bufonids. Likewise Heiss et al. (2009) reported serous gland polymorphism in the Salamndrid *P. waltl*, where the granular glands in the tail resemble a distinct type of granular glands, similar to *T. verrucosus*.

It seems likely that the presence of different types of granular glands is a common feature within the amphibians. The types of biologically active substances found in amphibians appear to have phylogenetic significance (Daly, 1995; Delfino et al., 1994). Delfino et al. (2002) emphasize the phylogenetic importance of ultrastructural similarities between serous products in anurans. He suggests that closely related species manufacture serous granules with similar or identical morphological traits, providing the derivation from comparable secretory pathways. The GG2s in the closely related Salamandrids *T. verrucosus* and *P. waltl* share several structural and histostaining similarities and hence the occurrence of GG2s is maybe an ancestral characteristic representative for the monophylum *Tylototriton*, *Echinotriton* and *Pleurodeles*. The literature contains no histological findings about the glandular tissue of Echinotriton to verify or falsify this hypothesis. However, the GG2s in the tail in both species represent a distinct granular gland type.

The presence of enlarged granular glands in the tail is a feature commonly found in some urodels. Some authors state the occurrence of these glands as an important antipredator adaptation (Brodie et al., 1984; Duellman and Trueb, 1994). Others speculate that they fulfill a dual function: nutrient storage and predator deterrence. Williams and Larson (1986) provide evidence that the caudal glands of *Ambystoma macrodactylum columbianum* stores both, toxins and nutritive proteins. The proteins are stored to be used as an energy source avoiding starvation. Hecker et al. (2003) describe modified serous glands in the caudal integument of *Plethodon cinereus* which are used for scent marking. Moreover caudal sexually dimorphic glands (hedonic glands) are mentioned for some members of the Plethodontidae. These enlarged glands are supposed to produce pheromones as a source of female attraction and

hence are involved in courtship behavior (Brizzi et al., 1996; Sever, 1989; Staub and Paladin, 1997).

GG2s in the tail of *T. verrucosus* are similar in occurrence in both males and females. As there is no sexual dimorphism in *T. verrucosus*, the role of GG2s in courtship behavior seems unlikely. Most probably, the presence of these glands is associated with defensive behavior. *T. verrucosus* uses the tail actively during defensive postures and several morphological features, especially the presence of a well developed myoepithelial sheath adapted for fast expulsion of glandular contents, underline the function in repelling would-be predators. Similar to the GG1s, which are definitely assigned as poison glands (Quay, 1972; Delfino, 1976; Neuwirth et al., 1979; Duellman and Trueb, 1994; Daly, 1995; Stebbins and Cohen, 1997; Toledo and Jared, 1995; Alvarez et al., 2005; Arifulova et al., 2007), it's suggested that GG2s have similar functions.

Like mentioned, in addition to granular glands of ordinary size distributed homogenously throughout the skin, some regions exhibited concentrations of enlarged granular glands. *Tylototriton verrucosus* bears large and distinct parotoids, a glandular mid-dorsal ridge on the trunk, rows of lateral warts and dorsal and ventral tail edges with concentrations of such enlarged granular glands (in accordance to Brodie et al., 1984). In this study, the highest density of granular glands and the largest glands in adults were found in the dorsal edge of the tail. Almost the entire tail was stocked with enormous GG2s. They could reach maximum diameters of up to 1550 µm and therefore can be added to the largest skin glands ever found in terrestrial salamanders. They differ in size only little from *Salamandra salamandra*, who is supposed to have the largest skin glands of any terrestrial salamander, with maximum diameters ranging between 1500-1800 µm (Phisalix, 1922).

Like *T. verrucosus*, many terrestrial salamanders have concentrations of enlarged granular glands along the tail dorsum, in the parotoid region or in lateral warts (Brodie, 1983). Several members of Ambystomatidae like *Ambystoma gracile* and *A. maculatum* as well as members of the genus *Salamandra*, such as *S. salamandra* and *S. atra* exhibit glandular parotoids. A lot of species of the Ambystomatidae have concentrations of granular glands along the tail dorsum and members of *Salamandra* exhibit concentrations of granular glands in rows along the body (Brodie, 1977). Such clusters of granular glands are known to be important in defense against predators (Duellman and Trueb, 1994). Most species with concentrations of granular glands use defensive postures to display these glandular regions to the aggressor. This behavior increases the likelihood that first predator contact would occur with the most

unpalatable parts of the body and therefore the avoidance would be most rapid and intense. Probably, this causes the predator to learn to avoid similar prey after initial contact (Nowak and Brodie, 1978; Brodie, 1983). Furthermore, such anti-predator posturing also serves to increase the apparent size of the salamander to intimidate or distract the potential predator (Brodie, 1977).

When tapped or touched in experiments, *T. verrucosus* exhibits various antipredator postures and secretions on the body surface can appear within seconds. The first response to a threatening stimulus is often immobility. According to Brodie (1977), immobility could be a precursor to several antipredator displays. In addition, immobility might reduce the intensity of further predator attacks and the potential of injuries.

Further anti-predator postures in *T. verrucosus* include body arching in connection with extension of the limbs and erection of the ribs (Brodie et al., 1984 and this study). This is beneficial as it increases the likelihood of a predator contacting the unpalatable glandular concentrations of the mid-dorsal ridge and of the lateral warts.

The tail, which is studded with huge GG2s, is intensively deployed during defensive behavior in adult *T. verrucosus.* During these defensive postures, the body often keeps still while the tail is held in an elevated position, wagged and undulated or even swung forcibly into the attacking stimulus. A similar behavior is also found in other salamandrids with presumably noxious or toxic skin secretions of the tail (Brodie, 1977). Moving the tail may confuse the predator by changing the characteristic shape of the salamandrid and the predator's attention is attracted to this most noxious, moving part of the salamander (according to Brodie, 1977).

In sum the body of *T. verrucosus* was always orientated in the way to display each of the glandular regions with concentrations of enlarged granular glands towards the attacking stimulus.

Morphological features are closely associated with defensive behavior to enhance the survival rate, as demonstrated above. The coloration of many amphibians is often closely integrated in protective mechanisms either by avoiding detection by camouflage or by signaling to predators to be unpleasant or dangerous through aposematic coloration (Stebbins and Cohen, 1995; Duellman and Trueb, 1994). Usually, amphibians that have noxious or toxic skin secretions tend to have aposematic coloration, often displayed during anti-predator-posturing as visual warning. A predator will associate the aposematic coloration with the distasteful properties of the prey and will refrain from such colored prey in the future.

The light brown to orange coloration of the individuals of *T. verrucosus* examined in the present study is associated with concentrations of enlarged granular glands. Since there is often a relationship between the presence of bright colors and skin toxins, it is generally assumed that such colors function as a warning signal to potential predators (Stebbins and Cohen, 1995). This seems to be applicable for *T. verrucosus*. Especially during defensive postures the tail is elevated to display bright ventral colors: a warning signal. Besides the warning function of aposematic coloration, bright colors on the other hand might draw the attention of the aggressor, without experience with similar prey, to the most noxious parts of the salamander. However, T. *verrucosus* exhibits predominantly dark brown coloration offering good camouflage on the moist forest floors and pools, this species naturally inhabits. The pigmentation of *T. verrucosus* makes it cryptic on the dorsal surface and the aposematic colors only become visible when the animal assumes one of the defensive postures. Colors that tend to match those of the substrates on which the animals live are important to their avoidance of visual recognition by predators.

The existence of two distinct color morphs assigned to *T. verrucosus* has not been generally recognized and overlooked in the past. In 1995, a taxonomic review of *T. verrucosus* was published by Nussbaum and co-workers. Primarily, based on differences in coloration, *T. shanjing* was separated from *T. verrucosus,* as a discrete species. Until this time, most, if not all authors considered *T. verrucosus* and *T. shanjing* to be the same species and publications until that time did not distinguish between these two species. Nowadays, *T. verrucosus* is restricted to the brown form with bright orange coloration mainly on the ventral edge of the tail. *T. shanjing* is considered as the orange-patterned form.

The distinct color patterns in *T. shanjing* and *T. verrucosus* are specially interesting if set in relation with granular gland concentrations. While *T. verrucosus* is mainly uniformly brown, exactly all regions of the enlarged granular gland concentrations shown in this study display a bright orange aposematic coloration in *T. shanjing*. The genetic and taxonomic relationships are not fully resolved but these two highly distinctive forms obviously have evolved along independent trajectories (Nussbaum et al., 1995). Zhang et al (2007) supposed that the genetic difference of these two species is low and it therefore seems likely to me, that granular gland occurrence, distribution and toxicity are similar in both species. I suppose that the closely related species *T. shanjing* and *T. verrucosus* mainly rely on two different antipredator strategies. *T. verrucosus*, a highly aquatic species, relies mainly on cryptic coloration to avoid visual recognition. By contrast, *T. shanjing* is a predominantly terrestrial species and uses

pronounced aposematic coloration to warn about its noxious properties. A potential interpretation is that living on land involves being faced with a greater amount of predators. Clearly pointing to the noxious properties by aposematic warning coloration may avoid predator attack and enhance the survival rate on land.

Beside the presence of clusters of enlarged skin glands, associated defensive postures and related coloration the closely allied salamandrid genera *Pleurodeles* and *Echinotriton* feature another unique anti-predator adaptation. Being attacked, these newts protrude their sharply pointed and elongated ribs through their skin to use them as ”stinging tools” to injure predators. This behavior clearly decreases palatability and increases survival rate (Novak and Brodie, 1978; Brodie, 1983; Brodie et al., 1984; Heiss et al., 2008, 2010). The use of the ribs as ‘concealed’ weapons’ however is only known from these two phylogenetically closely related salamander genera. It is generally accepted that the two sister taxa *Tylototriton* and *Echinotriton*, together with *Pleurodeles*, represent a monophyletic branch within the salamandrids (Weisrock et al., 2006). Interestingly, while *Pleurodeles* and *Echinotriton* protrude their ribs, *Tylototriton* does not. It has elongated ribs, associated with the lateral warts. Erection of the ribs leads to expansion of the warts, but the ribs are blunt and not able to penetrate the skin. However, some Tylototriton fossils are known to have had long sharp ribs with elongated epipleural processes (Nussbaum and Brodie, 1982). So possibly, extinct species of *Tylototriton* were able to protrude their ribs as well. It seems, therefore, that the use of ribs as ‘concealed’ weapons within this monophyletic clade is rather ancestral than derived and only *Tylototriton* has lost this ability through time (Heiss et al., 2010). If comparing the glandular tissue in the skin of *P. waltl* and in *T. verrucosus*, it becomes apparent that the glandular tissue in *T. verrucosus* is much more developed as in *P. waltl*. It is likely that this feature in *T. verrucosus* resembles the compensation to the loss of skin protruding ribs.

Salamanders of the Salamandridae have evolved a remarkable variety of antipredator adaptations that help them avoid being eaten by predators. Most adaptations are dependent upon the presence of noxious skin secretions which is probably the most important means of repelling predators within terrestrial salamanders.
In fact little is known on the exact mode of regeneration and discharge properties of poison glands as well as on the reorganization of the myoepithelial sheath after expulsion. Future studies will focus on this topic, combined with ontogenetic analysis of the skin glands.

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LITERATURE CITED

Alvarez BB, Delfino G, Nosi D, Terreni A. 2005. Ultrastructure of poison glands of South American frogs: A comparison between *Physalaemus albonotatus* and *Leptodactylus chaquensis* (Anura: Leptodactylidae). J Morphol 263(2):247-258.

Arifulova I, Delfino G, Dujsebayeva T, Fedotovskikh G, Nosi D, Terreni A. 2007. Serous cutaneous glands in the South American horned frog *Ceratophrys ornata* (Leptodactylformes, Chthonobatrachia, Ceratophrydae): ultrastructural expression of poison biosynthesis and maturation. J Morphol 268:690-700.

Brandon RA, Labanick GM, Huheey JE. 1979. Relative palatability, defensive behavior, and mimetic relationships of red salamanders (*Pseudotriton ruber*), mud Salamanders (*Pseudotriton montanus*), and red efts (*Notophthalmus viridescens*). Herpetologica 35(4):289-303.

Brizzi R, Calloni C, Delfino G, Lotti S. 1996. The male cloaca of *Salamandra lanzai* with notes on the dorsal glands in the genera *Salamandra* and *Mertensiella* in relation to their courtship habits. Herpetologica 52(4):505-515.

Brizzi R, Delfino G, Pellegrini R. 2002. Specialized mucous glands and their possible adaptive role in the males of some species of *Rana* (Amphibia, Anura). J Morphol 254(3):328-341.

Brodie ED Jr, Hensel JL, Johnson JA. 1974. Toxicity of urodele amphibians *Taricha*, *Notophthalmus*, *Cynops* and *Paramesotriton* (Salamandridae). Copeia(2):506-511.

Brodie ED Jr. 1977. Salamander antipredator postures. Copeia(3):523-535.

Brodie ED Jr. 1983. Antipredator adaptations of salamanders: Evolution and convergence among terrestrial species. In: Margaris NS, Arianoutsou-Faraggitaki M, Reiter RJ, editors. Plant, Animal, and Microbial Adaptations to Terrestrial Environment. New York: Plenum Publishing Corporation.

Brodie ED Jr, Nussbaum RA, Digiovanni M. 1984. Antipredator adaptations of Asian salamanders (Salamandridae). Herpetologica 40(1):56-68.

Brodie ED Jr, Smatresk NJ. 1990. The antipredator arsenal of fire salamanders: Spraying of secretions from highly pressurized dorsal skin glands. Herpetologica 46(1):1-7.

Bueno Ch, Navas P, Aguirre JA, Aijon J, Lopezcampos JL. 1981. Skin mucous glands of *Pleurodeles waltlii* Mich. Histochemical and ultrastructural study. Arch Biol 92(1):67-72.

Clarke BT. 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol Rev 72(3):365-379.

Daly JW. 1995. The chemistry of poisons in amphibian skin. P Natl Acad Sci USA 92(1):9-13.

Daly JW, Spade TF, Garaffo HM. 2005. Alkaloids from amphibian skin: A tabulation of over eight-hundred compounds. J Nat Prod 68:1556-1575

Dawson AB. 1920. The integument of *Necturus maculosus*. J Morphol 34(3):487-589.

Delfino G. 1976. Structural and ultrastructural aspects of cutaneous granular glands in *Bombina variegata* (L.) (Amphibia Anura Discoglossidae). Monitore Zool Ital 10(4):421-448.

Delfino G, Brizzi R, Calloni C. 1982. Development of ctaneous glands in *Salamandrina terdigitata* (Lacepede, 1788) (Amphibia Urodela); findings by light and electron microscopy. Z Mikrosk Anat Forsch 96(6):948-971.

Delfino G, Brizzi R, Calloni C. 1994. Serous cutaneous glands in the tree frog *Hyla arborea arborea* (L.): origin, ontogenic evolution, and possible functional implications of the secretory granule substructure. Acta Zool-Stockholm 75(1):27-36.

Delfino G, Brizzi R, Feri L. 1995. Chemical skin defence in *Bufo bufo*: an ultrastructural study during ontogenesis. Zool Anz 234(2):101-111.

Delfino G, Brizzi R, Jantra S, Feri L. 1995. Post Golgian maturative processes during the biosynthesis of poison secretion in cutaneous glands of the European common toad *Bufo bufo*. J Nat Toxins 4(2):97-113.

Delfino G, Brizzi R, Kracke-Berndorff R, Alvarez B. 1998. Serous gland dimorphism in the skin of *Melanophryniscus stelzneri* (Anura Bufonidae). J Morphol 237(1):19-32.

Delfino G, Brizzi R, Nosi D, Terreni A. 2002. Serous cutaneous glands in New World hylid frogs: An ultrastructural study on skin poisons confirms phylogenetic relationships between *Osteopilus septentrionalis* and *Phrynohyas venulosa*. J Morphol 253(2):176-186.

Duellman WE, Trueb L. 1994. Biology of Amphibians. Baltimore, Maryland: The Johns Hopkins University Press.

Ernst VV. 1973. Digital pads of tree frog, *Hyla cinerea*. II. The mucous glands. Tissue Cell 5(1):97-104.

Erspamer V. 1994. Bioactive secretions of the amphibian integument. In: Heatwole H, Barthalmus GT, editors. Amphibian Biology, Vol. 1. The integument: Chipping Norton, NSW: Surrey Beatty & Sons. p 178-350.

Fox H. 1994. The structure of the integument. In: Heatwole H, Barthalmus GT, editors. Amphibian Biology, Vol. 1. The integument: Chipping Norton, NSW: Surrey Beatty & Sons. p 1-92.

Fontana MF, Ask KA, Macdonald RJ, Carnes AM, Staub NL. 2006. Loss of traditional mucous glands and presence of a novel mucus-producing granular gland in the plethodontid salamander *Ensatina eschscholtzii*. Biol J Linn Soc 87(3):469-477.

Getchell ML, Rafols JA, Getchell TV. 1984. Histological and histochemical studies of the secretory components of the salamander olfactory mucosa: effects of isoproterenol and olfactory nerve section. Anat Rec 208(4):553-565.

Hecker L, Madison DM, Dapson RW, Holzherr V. 2003. Presence of modified serous glands in the caudal integument of the red-backed salamander (*Plethodon cinereus*). J Herpetol 37(4):732-736.

Heiss E, Wolfram MT, Redl E, Schwaha T, Weisgram J. 2008. Defense mechanism of *Pleurodeles waltl* (Amphibia, Urodela) against predators. Annual of Konstantin Preslavsky University, Shumen Vol XVIII B 6 Faculty of Natural Science:99-108.

Heiss E, Natchev N, Rabanser A, Weisgram J, Hilgers H. 2009. Three types of cutaneous glands in the skin of the salamandrid *Pleurodeles waltl*. A histological and ultrastructural study. J Morphol 270(7):892-902.

Heiss E, Natchev N, Salaberger D, Gumpenberger M, Rabanser A, Weisgram J. 2010. Hurt yourself to hurt your enemy: new insights on the function of the bizarre antipredator mechanism in the salamandrid *Pleurodeles waltl*. J Zool 280:156-162.

Helf OM, Stark W. 1941. Studies on amphibian metamorphosis. XVIII. The development of structures in the dermal plicae of *Rana sylvatica*. J Morphol 68(2):303-327.

Hoffman CW, Dent JN. 1977. Effects of neurotransmitters upon the discharge of secretory product from cutaneous glands of the red-spotted newt. J Exp Zool 202(2):155-161

Hoffman CW, Dent JN. 1978. Morphology of mucous gland and its responses to prolactin in the skin of the red spotted newt. J Morphol 157(1):79-97.

Houck LD, Sever DM. 1994. Role of the skin in reproduction and behaviour. In: Heatwole H, Barthalmus GT, editors. Amphibian biology, Vol. 1. The integument. Chipping Norton NSW: Surrey Beatty & Sons. pp 351-381.

Karnovsky, MJ. 1965. *A* formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol. 27:137A-138A

Kiernan JA. 2003. Histological And Histochemical Methods: Theory and Practice, 3rd ed. New York: Oxford University Press.

Le Quang Trong Y. 1967. Structure et développement de la peau et des glandes cutanées de *Nectophrynoides occidentalis* Angel. Arch Zool Exp Gen 108:589-610.

Le Quang Trong Y. 1973. Structure et dévelopment de la peau et des glandes cutanées de *Bufo regularis* Reuss. B Soc Zool Fr 98:449-485.

Navas P, Bueno C, Hidalgo J, Aijon J, Lopez-Campos JL. 1982. Secretion and secretory cycle of tegumentary serous glands in *Pleurodeles waltlii* Mich. Basic Appl Histochem 26(1):7-15.

Neuwirth M, Daly JW, Myers CW, Tice LW. 1979. Morphology of the granular secretory glands in skin of poison dart frogs (Dendrobatidae). Tissue Cell 11:755-771.

Noble GK. 1931. The biology of the amphibia. New York and London: McGraw-Hill.

Noble GK, Noble ER. 1944. On the histology of frog skin glands. Trans Am Micro Soc 63:254-263.

Nowak RT, Brodie ED Jr. 1978. Rib penetration and associated antipredator adaptations in the salamander *Pleurodeles waltl* (Salamandridae). Copeia(3):424-429.

Nussbaum RA, Brodie ED Jr. 1982. Partitioning of the salamandrid genus *Tylototriton* Anderson (Amphibia, Caudata) with a description of a new genus. Herpetologica 38(2):320-332.

Nussbaum RA, Brodie ED Jr, Datong Y. 1995. A taxonomic review of *Tylototriton verrucosus* Anderson (Amphibia, Caudata, Salamandridae). Herpetologica 51(3):257-268.

Phisalix M. 1922. Animaux Venimeux et Venins. Vol. 2. Masson, Paris.

Porter KR. 1972. Herpetology. Philadelphia: W. B. Sounders Company.

Quay WB. 1972. Integument and environment: glandular composition, function, and evolution. Am Zool 12(1):95-108.

Reyer RW, Liou W, Pinkstaff CA. 1992. Morphology and glycoconjugate histochemistry of the palpebral glands of the adult newt, *Notophthalmus viridescens*. J Morphol 211(2):165-178.

Romeis B. 1989. Mikroskopische Technik, 17th ed. Böck P, editor. München, Wien, Baltimore: Urban u. Schwarzenberg

Seglie D, Roy D, Giacoma C, Mushahiddunnabi M. 2003. Distribution and conservation of the himalalayan newt (*Tylototriton verrucosus*, Urodela, Salamandridae) in the Darjeeling District, West Bengal (India). Russian Journal of Herpetology 10(2):157-162.

Sever DM. 1989. Caudal hedonic glands in salamanders of the *Eurycea bislineata* complex (Amphibia, Plethodontidae). Herpetologica 45(3):322-329.

Staub NL, Paladin J. 1997. The presence of modified granular glands in male and female *Aneides lugubris* (Amphibia: Plethodontidae). Herpetologica 53(3):339-344.

Stebbins RC, Cohen NW. 1997. A Natural History of Amphibians. New Jersey, West Sussex: Princeton University Press.

Steinfartz S, Vicario S, Arntzen JW, Caccone A. 2006. A Bayesian approach on molecules and behavior: Reconsidering phylogenetic and evolutionary patterns of the Salamandridae with emphasis on *Triturus* newts. J Exp Zool, Part B-Molecular and Developmental Evolution 308B(2):139-162.

Stuhr ET. 1936. A toxicological study of the cutaneous secretions of the salamander, *Triturus torosus* (Rathke).J Am Pharm Assoc 24:117-119.

Toledo RC, Jared C. 1995. Cutaneous granular glands and amphibian venoms. Comp Biochem Phys A 111(1):1-29.

Tsuruda K, Arakawa O, Kawatsu K, Hamano Y, Takatani T, Noguchi T. 2002. Secretory glands of tetrodotoxin in the skin of the Japanese newt *Cynops pyrrhogaster*. Toxicon 40(2):131-136.

Voute CL. 1963. An electron microscopic study of the skin of the frog (*Rana pipiens*). J Ultrastruct Res 52:497-510.

Weisrock DW, Papenfuss TJ, Macey JR, Litvinchuk SN, Polymeni R, Ugurtas IH, Zhao E, Jowkar H, Larson A. 2006. A molecular assessment of phylogenetic relationships and lineage accumulation rates within the family Salamandridae (Amphibia, Caudata). Mol Phyl Evol 41(2):368-383.

Westheide W, Rieger R. 2004. Spezielle Zoologie Teil 2: Wirbel- oder Schädeltiere. Heidelberg, Berlin. Spektrum Akademischer Verlag.?

Williams TA, Larsen JH. 1986. New function for the granular skin glands of the eastern long-toed salamander, *Ambystoma macrodactylum columbianum*. J Exp Zool 239:329-333.

Zhang M, Rao D, Yu G, Yang J. 2007. The validity of red knobby newt (*Tylototriton shanjing*) species status based on mitochondrial Cyt b gene. Zoological Research 28(4):430-436.

APPENDIX

Tab. 1. Embedding in Paraffin.

Operating procedure/solution		Duration
Fixation: Bouin's solution		1month
Rinsing: 70% alcohol		1day
Dehydration:	70% alcohol	2h
	80% alcohol	2h
	90% alcohol	2h
	96% alcohol	2h
	100% alcohol	1h
Infiltration:	isopropyl alcohol	3 days
Resin infiltration: soft paraffin (48°)		9h
Resin infiltration: paraffin (60°)		18h

Tab. 2. Embedding in LR white resin.

Operating procedure/solution		Duration
Fixation: Bouin's solution		1month
Rinsing: 70% alcohol		1day
Dehydration:	70% alcohol	2h
	80% alcohol	2h
	90% alcohol	2h
	96% alcohol	2h
	100% alcohol	1h
Resin infiltration:	100% alcohol:100%LR white	2h
	100% LR white	12h
Polymerization:	100% LR white (60°)	20h

Tab. 3. Embedding in Agar Low Viscosity Resin (LV).

Operating procedure/solution	**Duration**
Prefixation : Karnovsky-solution	12h
Rinsing with Wash-solution	30min
Postfixation: Osmium-solution	2h
Rinsing with Aq. Bidest.	30min
Dehydration: 70% alcohol	2h
80% alcohol	2h
90% alcohol	2h
96% alcohol	2h
100% alcohol	1h
Rinsing with acetone	30min
Rinsing with acetonitrile	30min
Infiltration: acetonitrile:LV = 1:1	2h
acetonitrile:LV = 1:3	3h
100% LV	16h
Prepolymerization: 100% LV (40°)	3h
Polymerization: 100% LV (60°)	16h

Tab. 4. Treatment for scanning electron microscopic investigations.

Operating procedure/solution	**Duration**
Fixation: Bouin's solution	1month
Rinsing: 70% alcohol	1day
Dehydration: 70% alcohol	1h
80% alcohol	1h
90% alcohol	1h
96% alcohol	1h
100% alcohol	1h
Infiltration: 100% alcohol:100% acetone = 1:1	1h
Infiltration: 100% acetone	1h
Critical point drying	6h
Gold-plating	250sec

Tab.5: Staining procedure for Alciane blue-PAS staining; modified after Romeis (1989)

Dye/ Chemical	**Duration**
Xylene	5min
Xylene	5min
Isopropyl alcohol	5min
Isopropyl alcohol	5min
Alcohol 90%	3min
Alcohol 80%	3min
Alcohol 70%	3min
Aqua dest.	3min
Azocarmine	4min
Aniline alcohol	3sek
Acetic acid alcohol	30sek
Tungsten acid	20min
Tungsten acid	20min
Tungsten acid	20min
Aqua dest.	3min
Aniline blue	8min
Aqua dest.	3min
Alcohol 96%	3min
Alcohol 96%	3min
Alcohol 96%	3min
Alcohol 100%	3min
Alcohol 100%	5min
Isopropyl alcohol	5min
Xylene	5min
Xylene	5min

Tab.6: Staining procedure for Azan staining after Heidenhain; modified after Romeis (1989)

Dye/ Chemical	**Duration**
Xylene	5min
Xylene	5min
Isopropyl alcohol	5min
Isopropyl alcohol	5min
Alcohol 90%	3min
Alcohol 80%	3min
Alcohol 70%	3min
Aqua dest.	3min
Acetic acid 3%	3min
Alciane blue 8GX 2,5%	13min
Acetic acid 3%	3min
Aqua dest.	5min
Periodic acid 1%	10min
Aqua font.	5min
Aqua dest.	Rinse
Schiff's reagent	15min
Sulfite solution	2min
Sulfite solution	2min
Sulfite solution	2min
Aqua font.	5min
Aqua dest.	Rinse
Alcohol 70%	3min
Alcohol 80%	3min
Alcohol 90%	3min
Alcohol 100%	3min
Alcohol 100%	5min
Xylene	5min
Xylene	5min

Tab.7: Staining procedure for Haemalaune-Eosine staining after Mayer; modified after Romeis (1989)

Dye/ Chemical	**Duration**
Xylene	5min
Xylene	5min
Isopropyl alcohol	5min
Isopropyl alcohol	5min
Alcohol 90%	3min
Alcohol 80%	3min
Alcohol 70%	3min
Aqua dest.	3min
Haemalaune	13min
Aqua font.	10sek
Aqua dest.	rinse
Eosine	5min
Aqua dest.	rinse
Alcohol 70%	rinse
Alcohol 80%	rinse
Alcohol 90%	3min
Alcohol 100%	3min
Alcohol 100%	5min
Isopropyl alcohol	5min
Xylene	5min
Xylene	5min

Tab.8: Staining procedure for Coomassie brilliant blue staining; modified after Kiernan (2003).

Dye/ Chemical	**Duration**
Xylene	5min
Xylene	5min
Isopropyl alcohol	5min
Isopropyl alcohol	5min
Alcohol 96%	3min
Alcohol 96%	3min
Alcohol 100%	5min
Coomassie brilliant blue	15min
Acetic ethanol	5min
Alcohol 96%	rinse
Alcohol 100%	3min
Alcohol 100%	3min
Isopropyl alcohol	5min
Xylene	5min
Xylene	5min

Tab. 9. Formula for 0,1 M buffered Karnovsky-solution;
modified after Karnovsky (1965).

0,1 M buffered Karnovsky-solution

A. 10 ml 25% glutaraldehyde
B. 20 ml 10% formaldehyde
C. 50 ml buffered solution
D. 20 ml Aq. bidest.
E. add muriatic acid until pH = 7,4

0,2 M buffered solution (Karnovsky)

A. 6,42 g CaCo
B. 6 g sugar
C. 150 ml Aq. bidest.
D. add muriatic acid until pH = 7,4

0,1 M wash solution (Karnovsky)

A. 50 ml buffered solution
B. 50 ml Aq. bidest.

1% Osmium solution in 0,1 M buffered solution (Karnovsky)

A. 0,1g Osmium tetroxide mixed with
B. 5ml Aq. bidest.; rest over night and add
C. 5ml Buffered solution (Karnovsky) the next day

Tab. 10. Formula for Bouin`s-solution (Romeis, 1989).

Bouin`s-solution

A. 15 ml saturated diluted picric acid solution
B. 5 ml 35% formaldehyde
C. 1 ml glacial acid

Tab. 11. Formula for Agar Low Viscosity Resin (LV)-hard.

Agar Low Viscosity Resin (LV)-hard

A. 48g LV Resin
B. 8g VH1 Hadener
C. 44g VH2 Hadener
D. 2,5g LV Accelerator