BIHAREAN BIOLOGIST 13 (1): 28-31
Article No.: e181306

http://biozoojournals.ro/bihbiol/index.html

Histochemical examinations on integument of four anurans: *Bufo bufo*, *Bufotes variabilis* (Bufonidae), *Pelophylax bedriagae* (Ranidae), *Hyla savignyi* (Hylidae) from Turkey

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Received: 15. January 2018 / Accepted: 22. October 2018 / Available online: 28. October 2018 / Printed: June 2019

Abstract. Here, we aimed to enlighten the intra-specific differences of dermis in terms of thickness of Eberth-Katschenko (E-K) layer in *Bufo bufo*, *Bufotes variabilis*, *Pelophylax bedriagae* and *Hyla savignyi*. Main function of E-K layer is known to be a barrier for water loss. Therefore, we studied *Bufo bufo* and *Bufotes variabilis* for terrestrial, *Hyla savignyi* for semi-aquatic and *Pelophylax bedriagae* for aquatic species based upon variable biotopes for a better understanding of the role of E-K layer. The thickest E-K layer was detected in *Bufo bufo*, thinner in *Pelophylax bedriagae* and *Hyla savignyi*. In *Bufotes variabilis*, E-K layer was not constant. However large calcium depositions were observed in stratum spongiosum of *Bufotes variabilis*.

Key words: Amphibia, Anura, Eberth-Katschenko layer, Histochemistry, Dermis, Epidermis.

Introduction

Integument is an important organ to provide a barrier between external environment and organisms and prevent invasions of pathogens and maintain homeostasis (Sperman 1973, Proksch et al. 2008). The most important property of terrestrial vertebrate evolution is maintaining effective skin barrier against water loss (Lillywhite 2006). The amphibian integument is a variable and adaptable organ which has many functions–such as mechanical protection, chemical defense, sensory perception, ion transport, water absorption, respiration and osmoregulation (Sullivan et al. 2000, Koyama et al. 2001, Azevedo et al. 2005, Azevedo et al. 2006, Azevedo et al. 2007).

Anuran skin, like that of any other vertebrate, comprises of epidermis and dermis. The epidermis is stratified squamous epithelium that is organized in four cell layers; stratum basale or germinativum, stratum spinosum, stratum granulosum and the outermost stratum corneum. Dermis is subdivided into the spongious dermis and the compact dermis formed by mainly collagenous fiber bundles (Duelmann & Trueb 1994, De Brito-Gitirana & Azevedo 2005, Azevedo et al. 2006, Azevedo et al. 2007, Felsemburgh et al. 2009). Various explanations that have been suggested on function of calcification in dermis such as creating barrier against desiccation (Toledo & Jared 1993a, b), serving as calcium deposit, (Bentley 1984), providing protection against predators through hardening of the skin (Baldwin & Bentley 1980), and being dermal skeleton structure remains inherited from ancestor amphibians (Katchburian et al. 2001). Elkan (1968) described a layer in dermis of anuran skin, which was first observed by Eberth (1869) and subsequently by Katschenko (1882). This layer was later named as Eberth-Katschenko (E-K) layer. E-K layer is an acellular structure and consists of glycosaminoglycans and calcium salts (Elkan 1976, Azevedo et al. 2005, Mangione et al. 2011). Possible function of this acellular layer is mineral homeostasis and protection against desiccation (Toledo & Jared 1993, Schwinger et al. 2001, Mangione et al. 2011).

E-K layer's presence, location and structure vary among anurans. (Toledo & Jared 1993, Schwinger et al. 2001, Garcia & Cardozo 2005, Mangione et al. 2011). These variations depend on environment and physical factors (Toledo & Jared 1993, Mangione et al. 2011).

In this study we examined four anurans from three families; terrestrial *Bufo bufo* and *Bufotes variabilis* from Bufonidae, aquatic *Pelophylax bedriagae* from Ranidae and arboreal and semi-aquatic *Hyla savignyi* from Hylidae. Even though they were syntopic species, they inhabit different types of environments and their water dependence varies. We aimed to reveal histomorphological, histochemical characteristics of integument of these species, especially dermal layers for a better understanding of the relationship between water dependence and E-K layer.

Materials and Methods

The present study was carried out according to the animal ethical committee of Ege University, Faculty of Medicine (2012/029). The animals were collected from Eastern Mediterranean Region of Anatolia, Adana, Turkey (Lat. 37.214840; Long. 35.340086). In this study, we histochemically examined integumentary structure of 2 adult *Bufotes variabilis* (Pallas 1769), 1 adult *Bufo bufo* (Linnaeus 1758), 1 adult *Pelophylax bedriagae* (Camerano 1883), and 1 adult *Hyla savignyi* (Audoin 1827). After animals were brought to laboratory with breeding boxes, they were exposed to barbiturate overdose. Skin samples were taken from both dorsal and ventral body parts and fixed in 10% neutral buffered formalin. Thereafter, the samples were processed according to the standard histological protocols for paraffin embedding. Five micrometer thick sections were stained with Hematoxylin-Eosin for general morphology. The Von Kossa technique (Stevens 1972, Prophet et al. 1994) was used to detect calcium. Sections for each specimen were pretreated with 5% nitric acid before sections were stained with Von Kossa (Azevedo et al. 2005).

Results

According to light microscopic observations, dorsal and ventral integuments of experimental animals were composed of epidermis and dermis. The epidermis was composed of a stratified squamous epithelium. Epidermis included in four layers which were the stratum germinativum constituted by

prismatic or cubic cells seated on basal membrane, stratum spinosum composed of polyhedral cells, stratum granulosum with squamous cells, and stratum corneum, a keratinized layer. The dermis was subdivided into a vascularized stratum spongiosum of loose connective tissue and the compact dermis formed by mainly collagenous fiber bundles (Fig 1A, 1B, 1C, 1D). Two different types of dermal glands, mucous and granular glands, were observed in the dorsal and ventral regions of all anurans. Mucous glands were more abundant than granular glands, and granular glands were rarely observed in both the dorsal and ventral sides.

Mucous glands possessed an obvious lumen and were lined by mucocytes. These alveolar glands were filled with flocculent, amorphous secretory material. Granular glands contained homogeneously distributed granules. Beneath the dermis, there is a subcutaneous tissue which is named as tela subcutanea and which is rich for lymphatics and blood vessels (Fig 1D, Fig 2).

After Von kossa staining method, dense calcium deposition was observed on E-K layer of *Bufo bufo* and *Pelophylax bedriagae* (Fig 1A, 1D). In *Bufotes variabilis* large calcium depositions were observed in stratum spongiosum (Fig 1B, 1C). In *Hyla savignyi,* calcium deposition was also observed in the E-K layer (Fig 2).

Discussion

The structure and function of the integument depend on environment and it is first organ to come into contact with environment. Amphibian skin have not been clarified exactly yet (Duellman & Trueb 1994). Anuran skin consists of ectodermal epidermis and mesodermal origin dermis. Epidermis is composed of stratified squamous epithelium, and dermis is composed of stratum spongiosum and stratum compactum (Sampson et al. 1987, Lillywhite et al. 1997, De Brito-Gitirana & Azevedo 2005, Azevedo et al. 2005, Azevedo et al. 2006, Lillywhite 2006, Felsemburgh et al. 2007).

In H-E stained sections, basophilic and acellular E-K layer was observed between the stratum spongiosum and stratum compactum layers of both dorsal and ventral skins in *Bufo bufo* (Fig 1A) and *Pelophylax bedriagae*. E-K layer was shown in many anuran species (Elkan 1968 Azevedo et al. 2005, Mangione et al. 2011). Mangione and Lavilla (2004) stated that E-K layer was contained in stratum spongiosum of *Pleurodema diplolistris*, *P. nebulosum*, *P. tucumanum*, *P. borellii*. However, Elkan (1968) stated that E-K layer was not present in the samples Pipidae family including completely aquatic species such as *Telmatobius*, *Batrachophrynus* (Leptodactylidae). In *Bufotes variabilis*, E-K layer was not constant.

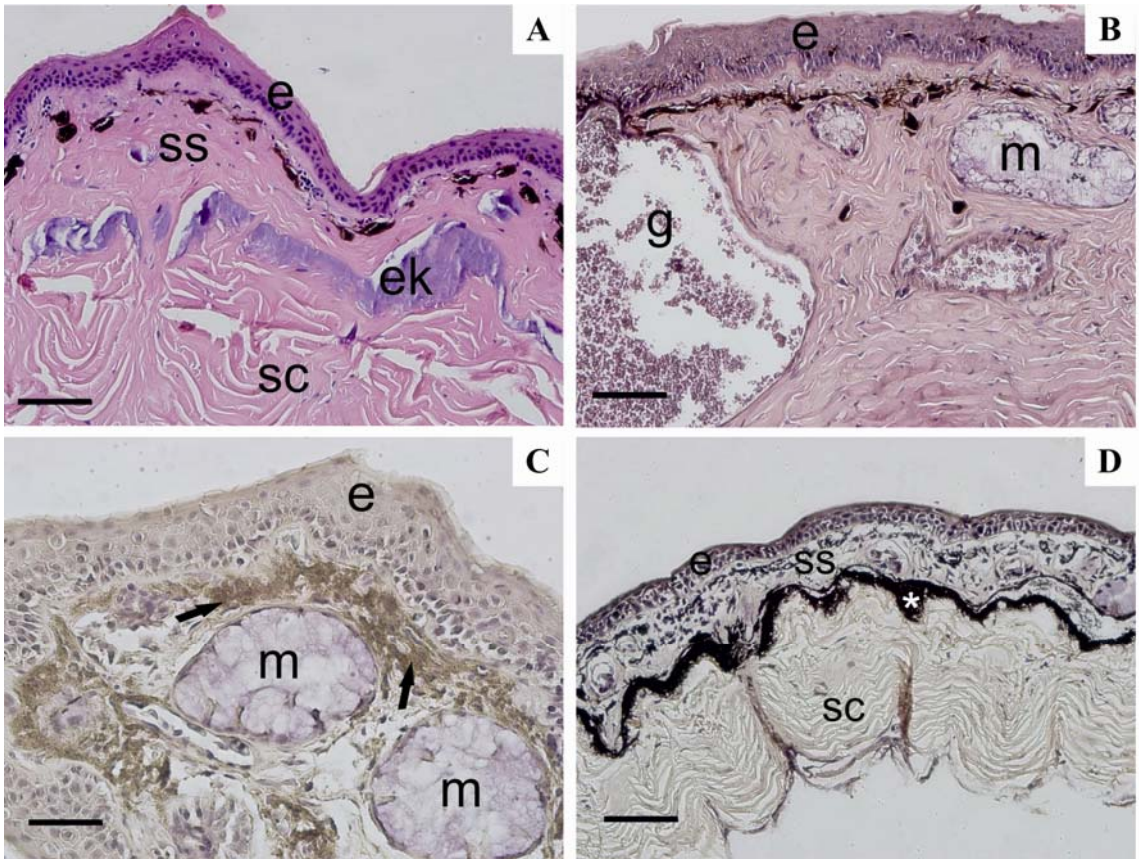

Figure 1. A-B, light micrograph of Hematoxylin-Eosin stained sections of skins. A. *Bufo bufo*, B. *Bufotes variabilis*. C-D, light micrographs of Von Kossa stained sections. Nuclei are counterstained with hematoxylin. C. *Bufotes variabilis*. D. *Pelophylax bedriagae*. e: epidermis, ek: Eberth-Katschenko (E-K) layer, g: granular gland, m: mucous gland, sc: stratum compactum, ss: stratum spogiosum. Black arrow: calcium deposition, Asterisk: Eberth-Katschenko (E-K) layer with calcium. Magnifications; A, B, D 20x, C 40x.

Figure 2. Light micrograph of Hematoxylin-Eosin stained skin sections of *Hyla savigni*. e: epidermis, d: dermis, white arrow: Tela subcutanea; 20x

Elkan (1968) reported that E-K layer might not be seen in the skin of the areas where big glands are present. In *Hyla savignyi*, a slightly basophilic E-K layer was observed between the stratum spongiosum and stratum compactum. To sum up, the thickest E-K layer was detected in *Bufo bufo*, thinner in *Pelophylax bedriagae* (Fig 1D) and *Hyla savignyi*. In *Bufotes variabilis*, E-K layer was not constant.

There are various views on calcium deposition in the dermis (Toledo & Jared 1993a, b, Bentley 1984, Baldwin & Bentley 1980, Katchburian et al. 2001). Azevedo et al. (2005) stated that dermal calcium may have been contributed to hydric balance of the skin by racing with water molecules for biding sites of hyaluronic acid. Our results support the findings that calcified E-K layer has a role at hydric balance by reason of the variable thickness of the layer associated with different biotopes.

We conclude that the presence and thickness of E-K layer in the anuran skin varies among the species and this is closely associated with the biotope of the species. And E-K layer become thicker in terrestrial species, as we showed here between terrestrial *Bufo bufo* and aquatic *Pelophylax bedriagae* and aquatic/arboreal *Hyla savignyi*, respectively. As for *Bufotes variabilis*, E-K layer was not constant. However large calcium depositions were observed in stratum spongiosum of *Bufotes variabilis*. Therefore, thickness of E-K layer and calcium deposition are probably related to defense against desiccation and maintain integument moisture.

Acknowledgements. This study is financially supported by TUBITAK [Project number: 112T913] and EBILTEM [2013BIL013].

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