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HISTOLOGICAL STUDY OF SKIN STRUCTURE VARIATION ACROSS DIFFERENT BODY REGIONS OF FOUR-LINED TREE FROG IN SARAWAK (*Polypedates leucomystax*) AMPHIBIA: ANURA: RHACOPHORIDAE

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Abstract: The frog skin is essential for its respiration, to retain chemical toxins, osmoregulation, and protection. Each of the frogs' skin glands has a specific function. Therefore, variations in skin traits in different body regions of frogs may be in response to their environmental adaptability. Tree frogs encounter the challenges posed by disruption of natural habitats. In order to our understanding of the function of glands and gland variations across distinct body regions, we examine skin structure in the Bornean Four-lined Frog, *Polypedates leucomystax*. Using Haematoxylin and Eosin staining techniques and a light microscope, the skin of selected adult frogs was investigated. The results revealed that the various body regions of *P. leucomystax* exhibited the same trait in terms of the layers of the epidermis and dermis within their skins. In the skin of *P. leucomystax*, three type of glands, namely mucous, serous, and seromucous glands, release their contents onto the epidermal surface via an intra-epithelial duct. The thickness of the epidermal layer and the distribution of glands differ between the dorsal and ventral areas, implying an underlying strategy of adaptation. The presence of seromucous glands is abundant in every part including dorsal and ventral for this species' special adaptability. The observed patterns of serous, mucous, and seromucous gland distribution across distinct skin regions could potentially mirror the behaviours of this species within its natural habitat. These discoveries may aid in understanding how the skin structure fulfil the ecological needs of the species, particularly within the modified settings of fragmented environments.

Keywords: *Polypedates leucomystax*, frog skin histology, dorsal, ventral, glands.

Introduction

Amphibian skins contain numerous chemical compounds, serving a crucial function in safeguarding against potential predators and warding off ectoparasites (Daly *et al*., 2005). These characteristics preserve the coordinated functionality of frog skin and collaborates harmoniously with the cardiac and respiratory systems (Zainudin *et al*., 2018). From a histological perspective, frog skin consists of epidermal and dermal layers. In contrast to the skin of most other vertebrates, anuran skin is typically devoid of covering such as scales, feathers, or hair, rendering it mostly naked. This characteristic also renders them vulnerable to environmental pollutants, particularly those stemming from agriculture, industry, and pharmaceuticals (Sungif, 2017). The susceptibility of frogs to their dynamic environment is influenced by factors such as the thickness and composition of its epidermis or dermal layer, and the distribution and quantity of its glands (Zainudin *et al*., 2018). In frogs, there are three distinct categories of specialised skin glands–mucous, seromucous, and granular glands–each fulfilling unique roles. Mucous glands allow frogs to stay hydrated and prevent excessive water loss through the skin. The seromucous and other skin glands play a role in preserving moisture, safeguarding against environmental stressors, and assisting in temperature regulation. Granular glands within the frog skin serve to establish a chemical defence mechanism by generating and discharging toxic substances, effectively deterring predators and potential dangers.

According to Cushman (2006), amphibian populations face significant threats from habitat loss and fragmentation, which are some of the foremost challenges they encounter. Habitat fragmentation can have a substantial impact on tree frogs, particularly those residing in arboreal environments. When forests are "divided" into isolated fragments, these smaller habitats might not offer sufficient resources or appropriate living conditions for tree frogs. Given its adaptation to life in trees and other vegetation, *Polypedates leucomystax* (Gravenhorst, 1829) is a frog species that is prone to adapt to the adverse impacts of forest fragmentation (Razali, 2017). It possesses specialised adhesive toe pads that enable it to adhere to diverse surfaces, thereby supporting its arboreal habits (Inger *et al.*, 2017). This species demonstrates adaptability to alterations in its environment through the possession of strong limbs which empower it to adeptly traverse and navigate its arboreal habitat. These limbs furnish the essential strength and agility required for successful climbing and leaping between trees. Hence, the species serves as a valuable model for comprehending the potential impact of habitat modifications on the biological attributes of amphibian species.

Despite the documented population trajectory of a frog species in its natural habitat and its diverse traits that facilitate adaptation to changing environments, our understanding of how their biological traits interact with various surroundings is still limited. Toledo and Jared (1995) documented variations in the structure of frog skin across different species. Is there a difference in the skin structure of *P. leucomystax* as a reaction to environmental changes? Therefore, we posited that the arrangement of skin attributes across various body regions of the *P. leucomystax* species alters in accordance with the skin's function within its native habitat. Nevertheless, the morphological characteristics of tree frog skin, especially those from the dorsal (head and central region) and ventral surfaces (head and central region), lack comprehensive documentation. The present study aims to investigate the skin structure of *P. leucomystax* (Figure 1) across its distinct body regions, with the intention of evaluating variations in skin composition. These findings could contribute to our understanding of how skin structures serve the ecological requirements of the species, particularly in the altered conditions of fragmented environments.

Figure 1: Four-lined tree frog, *Polypedates leucomystax*

Materials and Methods

Data Collection

Five tree frogs were collected from Kampung Maludam (1°39'17.0"N, 111°01'34.7"E), Ranchan, Serian (1°08'38.6"N, 110°35'03.0"E), and Kampung Mulu (4°07'55.2"N, 114°55'08.4"E) (an unprotected area) in Sarawak. Sampling was carried out uniformly across all study sites between 18:00 and 22:30 hours. The samples were collected using active method collection samples, namely visual encounter (VES) and acoustic encounter (AES) surveys. Onsite recordings were made for microhabitat data of the captured frogs, including details such as substrate type, horizontal and vertical positions, and vegetation types. The frogs were manually caught before placing them into plastic bags, subsequently transported to the base camp for the purpose of conducting morphological measurements of their snout-vent and tibia lengths, and body weight.

Procedure for Processing Samples

Five *Polypedates leucomystax* were captured, two frogs from Kampung Maludam, one from Ranchan, Serian, and another two from Kampung Mulu. We collected 10 skin sections, namely Dorsal Head (DH), Dorsal Centre (DC), Ventral Head (VH), and Ventral Centre (VC) as shown in Figure 2 (A) and Figure 2 (B).

After euthanising the frogs, their skin was carefully removed and subsequently preserved in a solution of 10% formalin. The specimens were handed to the Universiti Malaysia Sarawak Zoological Museum Department of Zoology. In order to facilitate the creation of slides for histological analysis, a series of six steps were undertaken. These steps encompass skin grossing, skin fixation, skin processing, skin embedding, skin sectioning, and skin fishing. The dorsal and ventral skins of the frogs were carefully separated and spread evenly on filter paper. The first step in slide preparation is skin grossing, where the skin is removed and placed in a casket. A critical aspect of skin grossing is fixation, performed using 10% formalin for 24 hours. Following fixation, the skin samples were processed in a machine adhering to the standard protocol. During the embedding stage, the samples were transferred to the Tissue Embedding Centre, "Tissue-Tek°TEC°" for skin block preparation. The tissues were embedded in paraffin to provide rigidity. Once the tissue blocks solidified and hardened, they were trimmed and sectioned. The ribbons produced were cut to a thickness of 3.5 µm. These ribbons, containing the skin tissue were floated on 60°C hot water and mounted onto labelled microscope slides. The tissues were then stained following

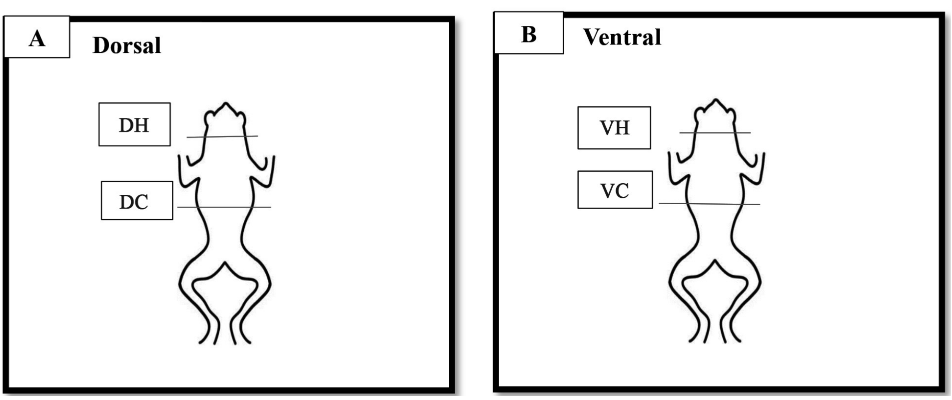

Figure 2: Skin regions of the frog; (A) dorsal and (B) ventral. Dorsal Head (DH), Dorsal Centre (DC). For the ventral regions, Ventral Head (VH), Ventral Centre (VC)

the Haematoxylin and Eosin staining protocol to aid in identifying glands (Sungif, 2017). Photographs of each section were captured at 40x magnification using a light microscope.

Results

Epidermis

Histological study was performed on the extracted skins from five individuals of *Polypedates leucomystax*. The result shows that *P. leucomystax* has an epidermis and a dermis for its skin. The epidermis consists of stratum granulosum, stratum spinosum, and stratum germinativum. The epidermis displayed a stratified structure composed of thin, flattened cells that are not keratinised. Typically, this arrangement has four to six layers of keratinocytes. The outermost layer, known as the stratum granulosum is the initial layer consisting of closely packed stretched cells and it may display a slightly granulated texture due to the presence of keratohyalin granules within the cells. The stratum spinosum encompasses the few middle cell layers primarily characterised by the presence of spherical to orbicular cells. The last layer comprises circular cells that constitute the stratum germinativum within the epidermis.

All of these layers function in synergy to uphold the structural and operational integrity of the skin. Comparable features were noted throughout the epidermis in various body regions, with the exception of the epidermal thickness. Specifically, the dorsal skin, as illustrated in Figures 3 (A) and (B), exhibited thinner thickness with three to five layers of cells as compared to the ventral skin, as depicted in Figures 5 (A) and (B). On the dorsal head, excretory tubes were seen connecting to mucous glands, as shown in Figures 3 (A) and (B).

Dermis

In the dermis, two strata comprising stratum spongiosum and stratum compactum layers as depicted in Figures 3 (A) and (B), Figures 4 (A) and (B), Figures 5 (A) and (B), and Figures 6 (A) and (B) were examined. The dermal layer comprises two distinct layers that align with the overall thickness of the skin. The stratum spongiosum, composed of loose connective tissue is located directly below the basal germinative layer of the epidermis. The continuity of the basement membrane in this strata is disrupted by the presence of glands situated between the epidermal basal layer and the spongy dermis. Within the loose connective tissue, we observed the presence of mucous, seromucous, and serous glands, which will be discussed in detail later. Thus, the thickness of this layer can be affected by the size of the glands. Melanin pigment cells, also known as melanophores and myo-epithelial cells disrupt the anterior portion of this layer [Figures 3 (A) and (B), Figures 4 (A) and (B)]. The stratum compactum is characterised by dense irregular connective tissue abundant in collagen fibres arranged in undulating segments that run parallel to the epidermis. Abundant muscular fibres are distributed throughout this layer and blood vessels can be seen near the layer’s base.

Type of Glands as Frog Skin Traits

Three types of glands, namely mucous, seromucous, and serous glands were notably prominent in the stratum spongiosum just beneath the epithelium. The structure of mucous glands is relatively simple, comprising a single-layered epithelium and some have a duct that directs towards the surface of the skin. Seromucous glands exhibit greater complexity compared with mucous glands, featuring a larger structure with multiple layers. These glands contain both mucous and serous cells. These seromucous glands are found abundant in every part of *P. leucomystax* as depicted in Figures 3 (A) and (B), Figures 4 (A) and (B), Figures 5 (A) and (B), and Figures 6 (A) and (B). Serous glands stand out as the most intricate of the three types and they are characterised by their multi-layered structure and their granular appearance as a result of the presence of secretory vesicles. The serous and mucous secretory cells exhibit a semilunar arrangement opposite the ductal end and they contain granules (Mills & Prum, 1984). For *P. leucomystax*, the mucous glands are more plentiful in the dorsal regions than in the ventral regions, while serous glands are more dominant in the ventral regions. The size of mucous glands was observed to be mostly quite small [Figures 4 (A) and (B)] and some are slightly bigger, especially on the dorsal head [Figures 3 (A) and (B)]. The serous glands were notably larger in the ventral regions, both head and centre [Figures 5 (A) and (B), Figures 6 (A) and (B)]. According to Moreno-Gomez *et al.* (2014), serous glands form a glandular oval compartment, typically larger than mucous glands, and exhibit syncytial walls with distinct features. Both glands release their contents onto the epidermal surface via an intra-epithelial duct that leads to a stoma. Both the mucous and serous glands, as well as the intercalated ducts are surrounded by an intermittent layer of myoepithelial cells. These cells exert pressure on the lumens of the acinus and ducts, facilitating the efficient expulsion and release of

Dorsal Head

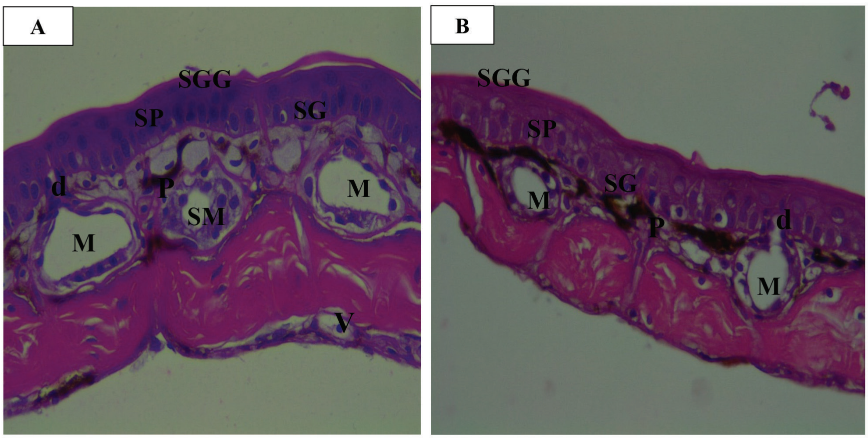

Figure 3: (A) and (B) pictures of *Polypedates leucomystax* skin stained using H&E (100 µm, 40x magnification). Mucous gland (M); seromucous gland (SM); blood vessels (V); duct (d); melanophores (P); stratum granulosum (SGG); stratum spinosum (SP); stratum germinativum (SG)

Dorsal Centre

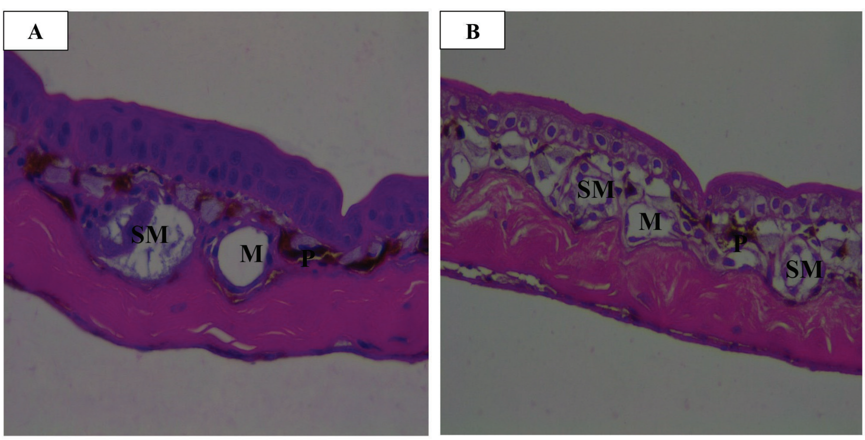

Figure 4: (A) and (B) pictures of *Polypedates leucomystax* skin stained using H&E (100 µm, 40x magnification). Mucous gland (M); seromucous gland (SM); melanophores (P)

Ventral Head

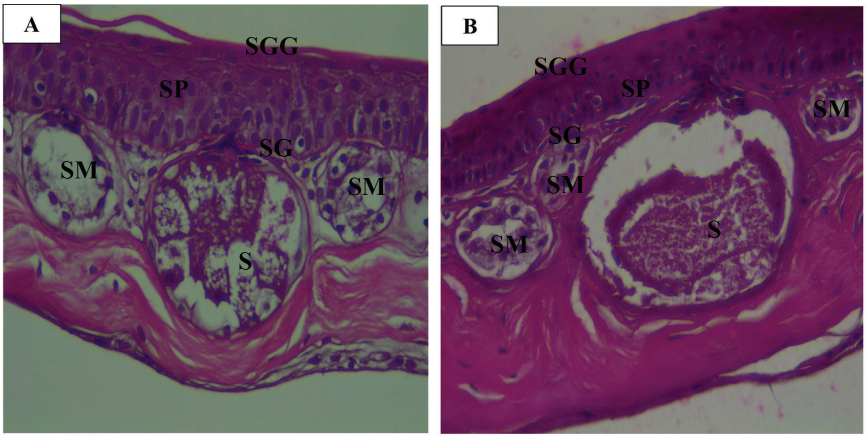

Figure 5: (A) and (B) pictures of *Polypedates leucomystax* skin stained using H&E (100 µm, 40x magnification). Serous gland (S); seromucous gland (SM); stratum granulosum (SGG); stratum spinosum (SP); stratum germinativum (SG)

Ventral Central

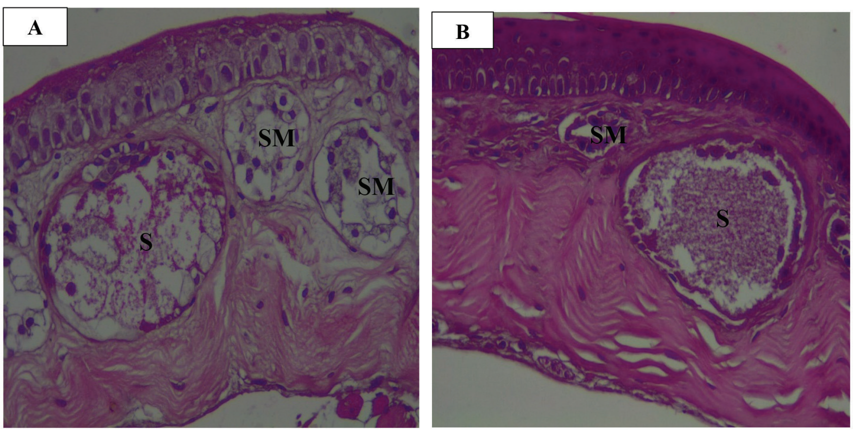

Figure 6: (A) and (B). Pictures of *Polypedates leucomystax* skin stained using H&E (100 µm, 40x magnification). Serous gland (S); seromucous gland (SM)

their contents. The presence of melanophores surrounding the glands on both their apical and lateral surfaces was observed as well. In the absence of the epidermis, melanophores provide protection to the entire spongiosum stratum that accommodates all the glands, safeguarding them from direct contact with the external environment (Zainudin *et al*., 2018). Nevertheless, there is variation in the thickness of melanophores between different regions. The dorsal regions exhibited a thicker layer of melanophores while both ventral regions did not display any presence of this pigment. Blood vessels connected to the exocrine function of the glands could also be observed.

Selection of Microhabitat by *Polypedates leucomystax*

One frog was found in a primary rain forest while the other four were found in the agricultural area. Next, at the horizontal position, two individuals were found at a temporary pond on vegetation, one individual on a rock at the intermittent stream, and two individuals were distant from the water. At the vertical position, one individual was found sat on the rock, two individuals were on seedlings while the other two were in the grass. For the microhabitat substrate, one individual was found on the bank rock while the other four individuals were found on the leaf of the plant. This implies that *P. leucomystax* relies on the presence of plants and water bodies for foraging and breeding, with a preference for laying their eggs on leaves that extend over bodies of water. This species utilises plant leaves and rocks as substrates.

Discussions

Urbanisation brings about significant changes in the makeup of wildlife communities, resulting in a reduction of biodiversity and a rise in the prevalence of species that flourish within urban environments (McKinney, 2002). According to Bradley and Altizer (2007), the global scale of urbanisation is on the rise, bringing ecological repercussions that transcend the confines of urban areas. *Polypedates leucomystax*, for example, is an arboreal species capable of thriving in both urban and non-urban environments, including agricultural areas where it demonstrates adaptation to human-disturbed habitats. As they undergo adaptation to habitat fragmentation, it is likely that their skin structure undergoes modifications to become more responsive to the environment. This is because the skin serves a vital role as an anuran's mechanical barrier, facilitates ion transport and water regulation, acts as a sensory apparatus, contributes to chemical defence mechanisms, functions as a respiratory organ, and serves as a sodium reservoir–all of which play crucial roles in the survival of anuran (Barlian *et al*., 2011). The abundance of seromucous glands and the arrangement of serous and mucous glands on various skin regions reflect the behaviour of this species in its natural environment. The study contributes to our understanding of how habitat loss, particularly due to fragmentation, affects wildlife. The frog's skin must remain consistently moist due to its elevated rates of evaporative water loss. *Polypedates leucomystax* have evolved to excel in arboreal environments, possessing specialised toe pads with adhesive qualities that enable them to grip a variety of surfaces, including leaves, branches, and even vertical surfaces.

This adaptation enhances their ability to manoeuvre effectively within their tree-dwelling habitats and evade potential ground-based predators. In this instance, the serous glands serve a significant role as a defence mechanism, with numerous serous glands on the ventral regions working to safeguard the skin surface from microbial infections resulting from direct bodily contact. The watery secretions emitting from the serous glands form a delicate film on the frog's skin, potentially serving as a protective shield against certain pathogens and parasites. This thin layer of protection has the potential to lower the likelihood of infections and diseases. The serous glands on the dorsal surface also play a crucial role in providing protection against predators (Zainudin *et al*., 2018). The functions of these glands in the *P. leucomystax* species are similar with those observed in other aquatic amphibian species, as reported in the skin glands of salamanders (Pereira *et al*., 2018).

However, in the tree frog, the study observed abundance of mucous glands in their dorsal regions compared with their ventral region. Mucous glands generate a slippery mucous-like secretion that plays a vital role in maintaining the moisture of a frog's skin. This is of paramount importance because tree frogs, including *P. leucomystax*, rely on their skin for respiration. The presence of moisture on the skin's surface enhances the efficiency of gas exchange, enabling them to respire effectively even when they are not in the water. During the day, *P. leucomystax* often seeks shelter in vegetation such as trees, shrubs, or tall grasses. They can conceal themselves among leaves or within tree hollows, a strategy employed to evade predators and preserve their moisture levels. This finding is consistent with that of Seebacher and Alford

(2002), who demonstrated that microhabitat selections can impact the dehydration rate of anurans. Hence, canopy gaps play a crucial role in maintaining a cool environment and elevating humidity levels, as they effectively restrict the penetration of sunlight (Zainudin *et al*., 2018). Thus, the mucous layer also aids in moisture retention, safeguarding the frog from desiccation in arid or dry climates. This is especially significant for tree frogs that dwell in diverse environments, encompassing both damp and arid regions.

The results further indicate that seromucous glands are plentiful throughout the entire region. *P. leucomystax* displays versatility in its choice of habitat and can thrive in various environments, both natural and disrupted habitats. This adaptability enables their survival in regions impacted by human activities such as agriculture and urban development. Tree frogs can also be found residing on tree bark and occasionally perching in elevated positions within the trees, where humidity levels are lower. According to Warburg (1989, 1997), many amphibians have adapted to water scarcity or the absence of water in xeric habitats through a combination of behavioural and physiological adjustments. In terrestrial habitats, the abundant seromucous glands help them retain moisture while in aquatic environments, they prevent the frog from becoming too waterlogged by shedding excess water.

Additionally, the presence of plentiful seromucous glands helps prevent dehydration during periods of dry weather, ensuring the frog stays well-hydrated and prepared to respire efficiently once conditions become favourable. This discovery aligns with Razali (2017) suggestion of a high presence of seromucous glands within the *Polypedates* genus. The discovery also indicated that there are differences in the melanin pigment layer across various body regions of *P. leucomystax*. Melanophores are pigment cells responsible for skin colouration and are typically located in the dorsal region. While the colouration of the dorsal part of *P. leucomystax* can be variable, it is common for them to display a tone of green or brown, often accompanied by four narrow dark stripes that run along their back (Inger *et al*., 2017). This coloration serves as effective camouflage in their forested habitats. Melanin offers shielding against the detrimental effects of ultraviolet (UV) radiation from the sun by absorbing and dispersing UV rays. This diminishes the potential for DNA damage and harm to skin cells. Hence, it would appear that the pigment layer on the dorsal regions is thicker because these areas are more exposed to the external environment.

Conclusions

This study has unveiled variations in skin characteristics between the dorsal and ventral regions, with a notable focus on differences in epidermal thickness and the distribution of glands. The presence of mucous glands is more at the dorsal part while the presence of serous glands is more at the ventral region. Meanwhile, the presence of seromucous glands is abundant in dorsal and ventral regions which indicates the special characteristic of this *Polypedates leucomystax* that can inhabit both natural and disrupted habitats. The discovery suggests that there is potential for further exploration into the function of the skin structure in the dorsal skin region, as it may play a crucial role in adapting to ecological changes in the environment. Hence, it is proposed that this study could be the foundation to establish a comprehensive database to better understand the impacts of habitat fragmentation on anurans.

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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