Borneo Journal of Resource Science and Technology (2024), 14(1): 186-200
DOI: https:doi.org/10.33736/bjrst.6246.2024

The Comparison of the Histological Skin Structures of Common Sunda Toad (*Duttaphrynus melanostictus*) and Grass Frog (*Fejervarya limnocharis*)

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Received: 2 November 2023 Accepted: 17 April 2024 Published: 30 June 2024

ABSTRACT

Anuran skin preserves all functional activities, especially for respiration and water regulation. *Duttaphrynus melanostictus* and *Fejervarya limnocharis* are the common species found in Borneo lowlands and are well-adapted to humans. Hence, they can reproduce quickly and rapidly in great numbers in the urban area. This study aims to select these urban-type anurans and describe the skin structure and glands. Four regions of skin samples were obtained, namely Dorsal Head (DH), Dorsal Centre (DC), Ventral Head (VH) and Ventral Centre (VC). The microscopic slides were prepared accordingly as in the histological techniques including skin grossing, fixing, processing, embedding, sectioning and were stained with Haematoxylin and Eosin staining. The seromucous glands are most prevalent in all four regions for both species. Parotoid glands are clearly visible in the skin structure of *D. melanostictus*, while there is a lack of parotoid glands in *F. limnocharis*. Nonetheless, *F. limnocharis* contains regular rows of glands, whereas the distribution of glands in *D. melanostictus* is scattered. In addition, *D. melanostictus* possess dermal bones, which are absent in *F. limnocharis*. Since anuran skin is a mucosal surface that in constant direct contact with the environment, their adaptations to harsh habitats should be reflected in the skin, particularly in the urban and invasive species in this study.

Keywords: *Duttaphrynus melanostictus*, *Fejervarya limnocharis*, glands, skin histology, urban-types anuran

INTRODUCTION

An anuran's skin consists of epidermis and dermis, which is the largest and heaviest single organ of the body. Their skin can be described as the mucosal surface which will be in constant and direct contact with the aquatic and terrestrial environments that are microbial diverse and laden (Varga *et al.*, 2019). Thus, they must be adapted to the demands of both habitats with their soft, moist integument. All the functional activities of the anuran skin are preserved by the skin of amphibian, which cooperates with the cardiac and respiratory systems (Zainudin *et al.*, 2018). Hence, for efficient cutaneous respiration and reduced evaporative water losses, their skin compensates for them by osmotic reabsorption when in contact with water. In addition, anuran skin is the essential innate organ of immunity, constituting a complex network of physical, chemical, immunological, and microbiological barriers, serving as the first line of deference against pathogens in the environment (Varga *et al.*, 2019). Their skin is composed of a lot of chemical compounds secreted from the glands that may play an important role as a defensive mechanism against potential predators and as a protection against ectoparasites (Moreno-Gómez *et al.*, 2014). Most of the anurans have mucous, granular, and seromucous glands, which granular glands are also called poison glands. In the dermal layer, all these glands play different functions and vary in size and surface area. Interestingly, toads have parotoid glands and warts on their skin. When toads are disturbed, they will secrete a milky and latex-like toxin that makes them smell nasty and harmful to predators (Inger *et al*., 2017). In the same way, toads also possess the granular gland that aids in protecting them from enemies and inflict insects that might harm them.

Duttaphrynus melanostictus, is a stocky and medium-sized to large true toad belongs to the family Bufonidae. The dark crests that border the eyelids and extend downwards on either side of the eye, as well as the round warts of various sizes on the back, make *D. melanostictus* easy to

be recognised (Inger *et al.*, 2017). The body of *D. melanostictus* has a variety of colour of greyish or reddish brown throughout the distribution range, without markings other than the dark edges of the warts (Inger *et al.*, 2017). *Duttaphrynus melanostictus* have black-tipped, hooked toes that lack adhesion pads and webbing on front toes, and only have extremely small webbing on hind toes. In addition, this species lacks the dorsolateral and supratympanic folds (Jaafar *et al.*, 2009).

Fejervarya limnocharis, is a small true frog in the family Dicroglossidae. Long, narrow head, a slender and oval body is the feature of this species. Based on Inger *et al.* (2017), the skin is finely pebbled, with a series of low and interrupted ridges on the back that form a line of bumps down the rump and sides. In addition, *F. limnocharis* lacks a loose skin fold on the outside of the fifth hind toes but does have a small metatarsal tubercle (Jaafar *et al.*, 2009). There are the distinctive brown and white streaks on its lips. According to Inger *et al.* (2017), *F. limnocharis* has darker blotches on its rusty brown to brownish grey in colour back, often with U- or W-shaped marking between the shoulders. Majority of them also have a light stripe down the middle of the back from the nose to the anus.

As anurans exhibit high levels of genetic structure, strong habitat association conferred by ecological and physiological constraints, with low dispersal ability, they can be described as excellent models for evolutionary studies (Avise, 2009). Their skin is sensitive to the climatic factors including temperature and precipitation, which helps in adapting to changes in the environment. However, most histological studies have focused on the family Ranidae, Megophryidae and Rhacophoridae, and there is a lack of histological study on the skin structure of the common Sunda Toad (*Duttaphrynus melanostictus*) and Grass Frog (*Fejervarya limnocharis*). As both *D. melanostictus* and *F. limnocharis* are urban anuran species and well-adapted to human activities and typically inhabit agricultural areas, drains, road verges, lawns, and football pitches, they can be easily found in the lowland of Borneo. This allows them to reproduce and live in large numbers in urban area. Usually, the differences between frog and toad are based on their skin structure. Thus, this study, these urban-type anurans taken from similar habitat and distribution were selected to compare their skin structures. The findings may aid in the understanding of the adaptation of these anuran species to urbanisation.

MATERIALS AND METHODS

Study Areas

A total of three study sites in Sarawak were chosen for sample collection. The study sites consisted of one protected area, Matang Wildlife Centre (1˚36.917’ N, 110˚10.470’ E), and two unprotected areas: UNIMAS (1˚28.013’ N, 110˚25.694’ E) and Mayor Song Swee Guan Park (1˚31.664’ N, 110˚22.409’ E).

Field Technique

The field surveys were carried out at night between 1830 to 2200 hours, during the period of the anurans are most active. Visual Encounter Survey (VES) and sound detection were used to systematically search for anurans. The shine of anurans’ eyes can be detected with the use of headlamps, and their calls can assist to locate them. Important details including the time and date of capture, microhabitat, and the distance from the nearest water source were labelled on the plastic. Besides that, the captured anurans were measured and identified. The body mass, snout-vent length (SVL) and tibia-fibula length (TFL) were taken and recorded (Table 1).

Table 1. Field data of samples analysed in this study for skin structures

No.	Field ID	Species	Date	Hour	Measurements			Age	Gender
					SVL (mm)	TFL (mm)	Weight (g)		
1	UW193	*Fejervarya limnocharis*	2/4/2023	2005	57	29	18	Adult	Female
2	MSSG01	*Duttaphrynus melanostictus*	7/4/2023	1936	70	29	40	Adult	Male
3	MSSG02	*Duttaphrynus melanostictus*	7/4/2023	1943	79	27	42	Adult	Female
4	MSSG04	*Fejervarya limnocharis*	10/4/2023	2025	52	27	12	Adult	Male

Histological Preparations

After completing all measurement and identification procedures, two individuals of *Duttaphrynus melanostictus* and two individuals of *Fejervarya limnocharis* anurans were euthanised with absolute ethanol. The tissues were then taken and preserved in absolute ethanol for the preservation of DNA tissue. The dorsal and ventral skin of the anurans were taken, mounted on filter paper, and fixed with 10% formalin. Six steps were conducted which include skin grossing, fixing, processing, embedding, sectioning, and staining.

The initial step in preparing the slide is skin grossing, in which the skin is cut out and placed in a casket. The essential step in skin grossing is fixation with 10% formalin for 24 hours. The skins were then placed into the processor machine following the standard protocol. During skin embedding, the samples were transferred to the Tissue Embedding Center 'Tissue-Tek˚TEC°' for skin block preparation. The tissues were embedded with paraffin for rigidity. The tissues were more resistant to sectioning because the paraffin penetrates all intercellular spaces and even into the cells (Junqueira & Carneiro, 2005). The block of tissues was trimmed and sectioned when it had been solidified and hardened. The thickness of the ribbons is 4.0 µm. The ribbon with the skin tissue was put in 50 ˚C hot water and mounted on labelled microscope slides.

As the majority of tissues are colourless, it will be challenging to observe the unstained tissues under a microscope (Junqueira & Carneiro, 2005). Thus, the modified Haematoxylin and Eosin (H & E) protocol was used (Sungif, 2017). The transparent appearance of the basic structure became pinkish-red with eosin staining, while haematoxylin stained the acidic structures purplish-blue. The tissue was then mounted using Distryene Plasticizer Xylene (DPX) and covered with a cover slip.

Skin Structure Analysis

All slides were visualised and examined using a Leica ICC50 HD microscope with 40x magnification and Leica LAZ EZ software. Four regions of the skin were analysed (Figure 1 & Figure 2), including the Dorsal Head (DH), Dorsal Centre (DC), Ventral Head (VH) and Ventral Centre (VC). Ten random skin slides in each of the four regions observed represent the dorsal and ventral parts of each skin area of frogs and toads. Thus, there were 40 skin slides (N) for each individual to be analysed, with 10 slides each for DH, DC, VH, and VC. Analysis and measurements were performed using ImageJ 1.15k software, with the glands numeration of 10 slides per region counted in units and the gland area measured in millimetres (mm). The Mann-Whitney Rank Sum Test was used to distinguish between *D. melanostictus* and *F. limnocharis* based on the number of glands present and the area of the glands. The data from the Mann-Whitney Rank Sum Test were analysed by using Statistical Package for the Social Science (SPSS) software.

Figure 1. Dorsal and ventral skin regions of *D. melanostictus* for histological study. The skin regions were denoted as: DH-dorsal head, DC-dorsal centre, VH-ventral head, and VC-ventral centre

Figure 2. Dorsal and ventral skin regions of *F. limnocharis* for histological study. The skin regions were denoted as: DH-dorsal head, DC-dorsal centre, VH-ventral head, and VC-ventral centre

RESULTS

Histology of Anuran Skin

Both *Duttaphrynus melanostictus* and *Fejervarya limnocharis* have two skin layers comprised of the epidermis and dermis (Figure 3). The epidermis layer of the skin in both species consists of stratum corneum, stratum spinosum and stratum germinativum, which serve to distinguish the internal cellular environment from the external physical environment. The stratum corneum is the outermost thin layer of the epidermis, while the innermost thick layer of the epidermis is called the stratum germinativum. In addition, the intermediate layer between the stratum corneum and the regenerative stratum germinativum layer is called the stratum spinosum (Varga *et al.*, 2019). The dermis layer which located below the epidermis layers, is composed of stratum spongiosum and stratum compactum. The stratum spongiosum is the outer layer of the dermis and is composed of loosely packed connective tissue. The loose connective tissue helps in maintaining the presence of glands including seromucous glands, mucous glands, and granular glands. The stratum spongiosum of *D. melanostictus* is thicker than *F. limnocharis*, as the size of glands in *D. melanostictus* is larger than in *F. limnocharis*. Furthermore, there is the presence of ground substance in both species, which is visible at the basal of the stratum spongiosum. Besides, the stratum compactum is made of dense organised connective tissue that is rich in collagen fibres and fibroblasts (Ponssa *et al.*, 2017). Stratum compactum also functions to separate between the skin layer and muscle layer.

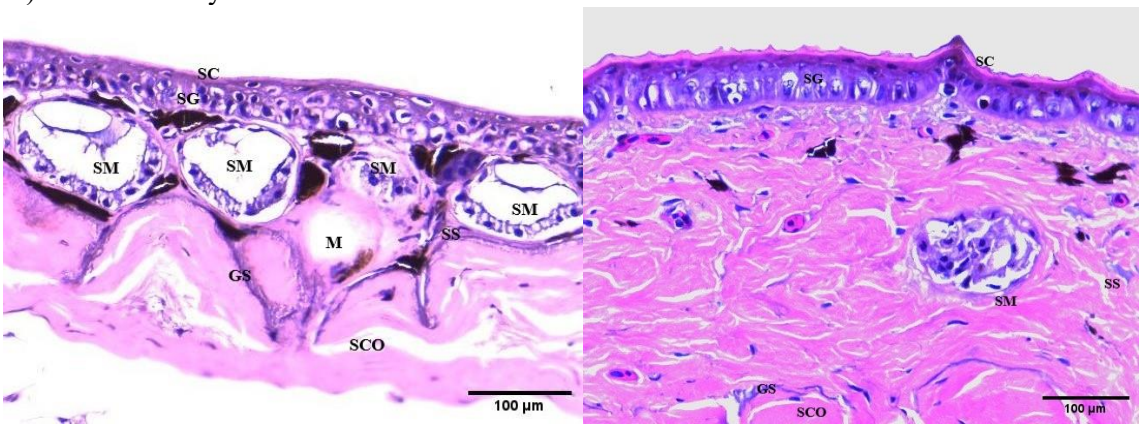

Figure 3. Histological section of the dorsal skin of *Fejervarya limnocharis* (left) and *Duttaphrynus melanostictus* (right), stained using H&E at 40x magnification. M, Mucous Glands; SM, Seromucous Glands; SC, Stratum corneum; SG, Stratum germinativum; SS, Stratum spongiosum; SCO, Stratum compactum; GS, Ground Substance

Histology of Anuran Glands

The anuran skin has several essential glands. In *D. melanostictus*, there are four types of glands present, which are mucous, seromucous, granular, and parotoid glands. *Fejervarya limnocharis*, in contrast, has only three types of glands: mucous, seromucous, and granular glands. The glands of *D. melanostictus* are found to be larger but fewer than the glands of *F. limnocharis*. In addition, the glands in *F. limnocharis* are arranged in orderly rows, while the glands arrangement of *D. melanostictus* is scattered. In contrast to the mucous and granular glands, the seromucous gland is the most prevalent in both species. Seromucous glands are mixed glands that consist of mucous and granular glands. They also include observable nuclei at the two layers of the cells. Seromucous glands also have the acinus that is bordered by simple and squamous-shaped cells, with abundant mitochondria. Besides that, mucous glands lack of cytoplasm in the acinus, giving the character an empty appearance. Despite having the same acinus lined by squamous-shaped cells similar to seromucous glands, this characteristic can distinguish mucous glands from seromucous glands. There is a low quantity of mucous glands found in *D. melanostictus* and *F. limnocharis*. Furthermore, granular glands, also known as poison glands, have a lumen filled with acinus that is made up of vacuolated, heterogeneous, and visible granular material and nuclei (Mills & Prum, 1984). In both species, the granular glands have a larger size than other glands (Figure 4).

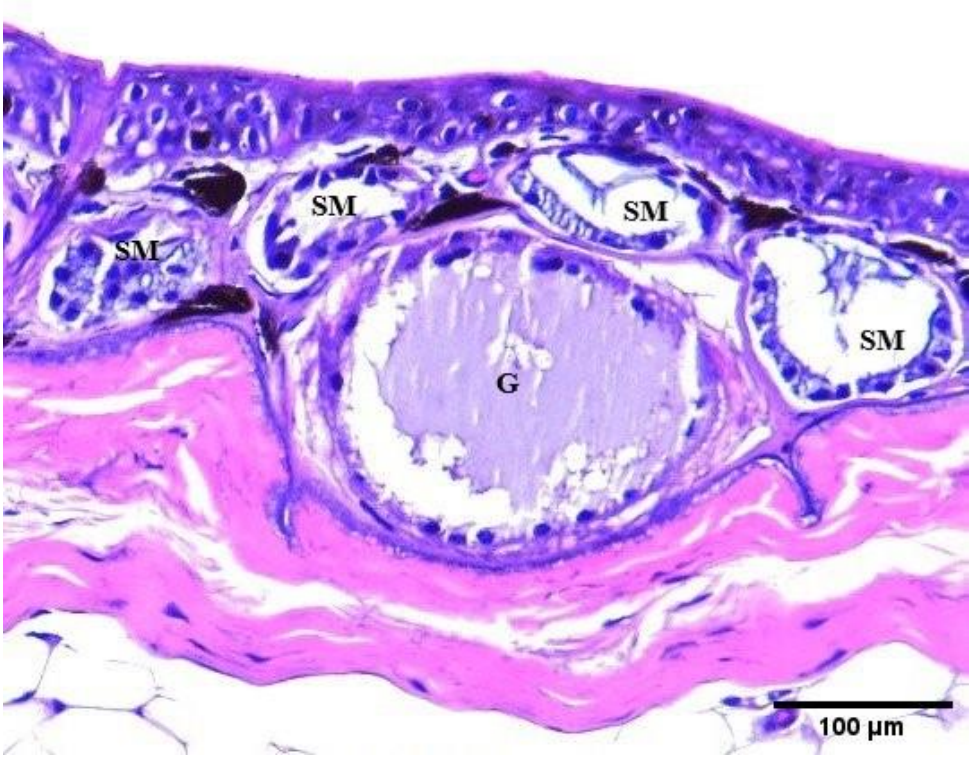

Figure 4. Glands present in the dorsal skin of *Fejervarya limnocharis*, stained using H&E at 40x magnification. SM, Seromucous Glands; G, Granular Gland

The existence of parotoid glands distinguishes the skin structures of *D. melanostictus* and *F. limnocharis*. This gland is found only in toad species, including *D. melanostictus*. Parotoid glands are made up of an accumulation of poison-producing granular alveoli. In this research, the dorsal head of *D. melanostictus* contains the structure of parotoid glands (Figure 5 & Figure 6). Compared to the granular glands in the other dorsal skin of *D. melanostictus*, the granular glands in parotoid glands (dorsal head) are particularly enormous. The dermal bones (Figure 7) found in *D. melanostictus* are also able to differentiate the skin structure between *D. melanostictus* and *F. limnocharis*. The dermal bones are clearly visible in almost all the observed slides of the dorsal parts (dorsal head and dorsal centre) of *D. melanostictus* skin.

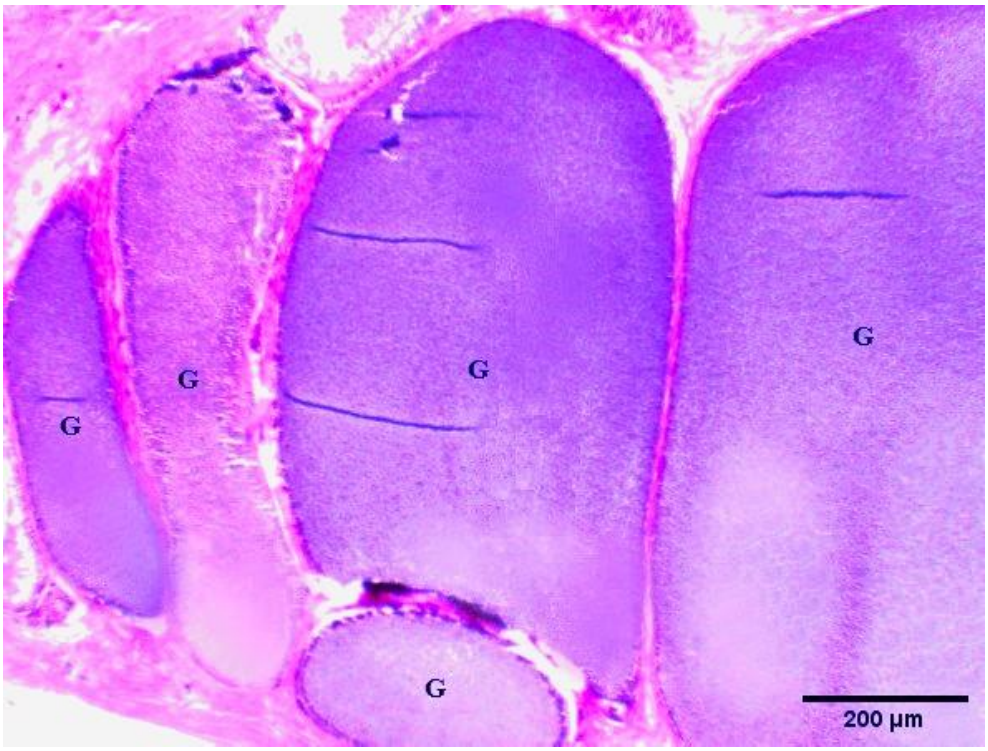

Figure 5. The parotoid gland (dorsal head) of *Duttaphrynus melanostictus* showing the distribution of granular glands, stained using H&E at 4x magnification. G, Granular Gland

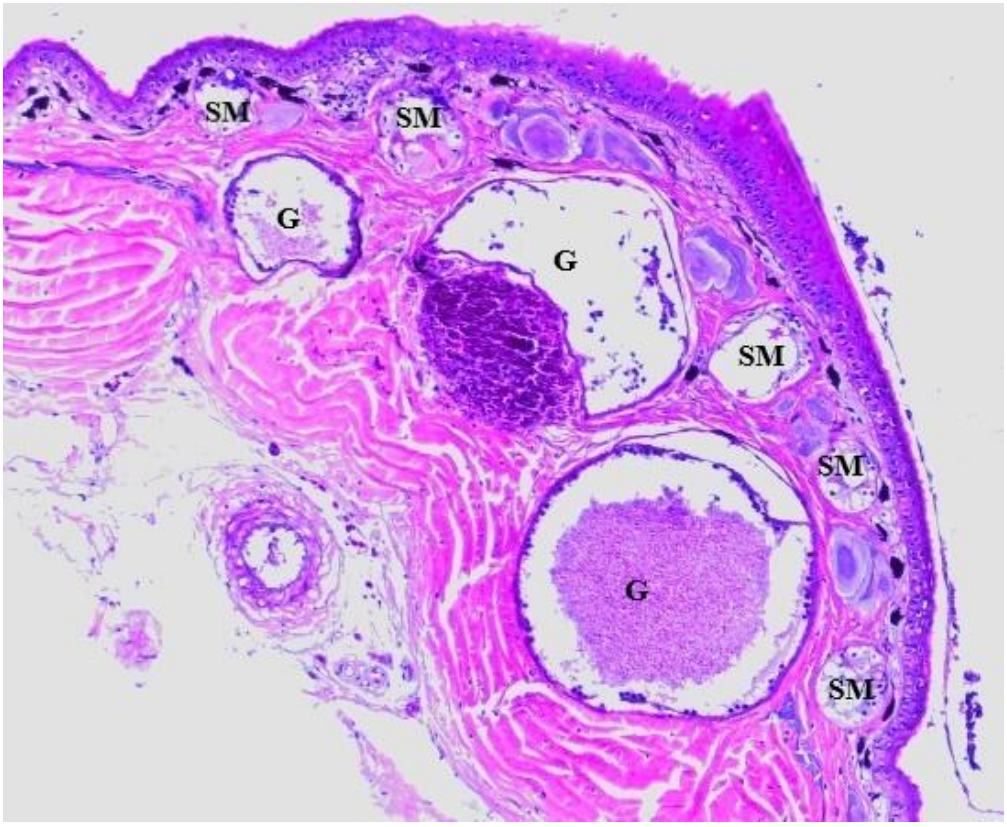

Figure 6. The parotoid gland (dorsal head) of *Duttaphrynus melanostictus* showing the distribution of seromucous and granular glands, stained using H&E at 10x magnification. SM, Seromucous Glands; G, Granular Gland

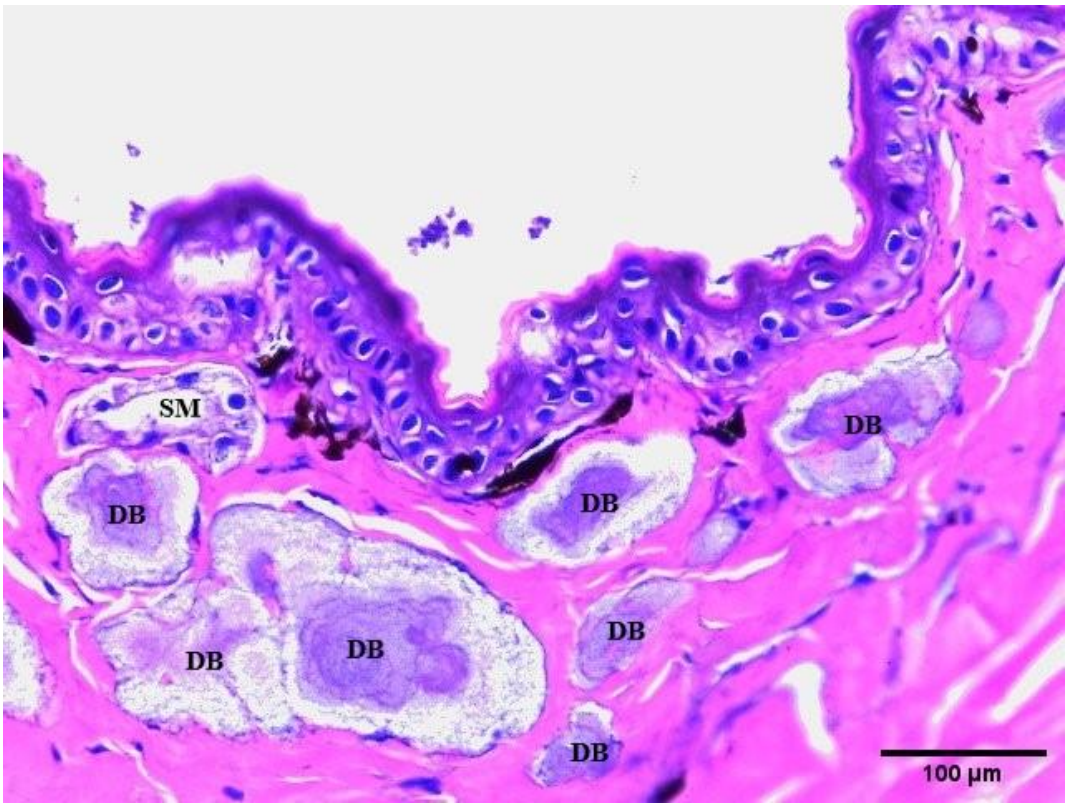

Figure 7. Dermal bone present in *Duttaphrynus melanostictus*, stained using H&E at 40x magnification. SM, Seromucous Glands; DB, Dermal Bone

Glands Numeration Between *Duttaphrynus melanostictus* and *Fejervarya limnocharis*

As the data in this research are not normally distributed and the sample sizes are small, the Mann-Whitney Rank Sum Test is an alternative to the t-test. The Mann-Whitney Rank Sum Test is a type of non-parametric test, in which the ranks of sample data from two independent populations are taken into account. In the dorsal head of *D. melanostictus* and *F. limnocharis* (Table 2 & Table 3), there is sufficient evidence to conclude that there is a significant difference in the seromucous glands present ($U = 8$, $n_1 = n_2 = 20$, $p=0.001< 0.05$ two-tailed) between the two species at 5% significance level. However, there are no statistically significant differences in the number of mucous glands ($U = 180$, $n_1 = n_2 = 20$, $p=0.298>0.05$ two-tailed) and granular glands ($U = 190$, $n_1 = n_2 = 20$, $p= 0.681 >0.05$ two-tailed) between the skin structures of *D. melanostictus* and *F. limnocharis*.

The analysis of gland numeration reveals no significant difference in the granular glands ($U = 191.5$, $n_1 = n_2 =20$, $p= 0.689 >0.05$ two-tailed) at the dorsal centre. Conversely, the significant differences in the mucous and seromucous glands present in the skin structure of *D. melanostictus* and *F. limnocharis* were observed, with values of $U = 160$, $n_1 = n_2 = 20$, $p = 0.037 < 0.05$ two-tailed and $U = 24$, $n_1 = n_2 = 20$, $p= 0.001 <0.05$ two-tailed, respectively. Additionally, it was discovered that the significant difference in the seromucous glands between these two species was found greater than the significant difference in the mucous glands between the skin structures of *D. melanostictus* and *F. limnocharis*.

In the ventral head, there is a significant difference only in the seromucous glands ($U = 15$, $n_1 = n_2 = 20$, $p= 0.001 < 0.05$ two-tailed) between the skin structures of *D. melanostictus* and *F. limnocharis* at 5% significance level. The mucous ($U = 180$, $n_1 = n_2 = 20$, $p=0.152 >0.05$ two-tailed) and granular glands ($U=190$, $n_1 = n_2 = 20$, $p=0.317 >0.05$ two-tailed) do not show statistically significant differences.

Table 2. Summarised data of numeration of glands on the dorsal head, dorsal centre, ventral head, and ventral centre between *Duttaphrynus melanostictus* and *Fejervarya limnocharis*

Skin Area	Types of Glands	Number of Glands		Mean	N	Standard Deviation	Median
		F. limnocharis	*D. melanostictus*				
Dorsal Head	Mucous	3	1	0.1	40	0.30382	0
	Seromucous	88	26	2.85	40	1.86121	2
	Granular	4	3	0.175	40	0.38481	0
Dorsal Centre	Mucous	4	0	0.1	40	0.30382	0
	Seromucous	77	31	2.7	40	1.57219	2
	Granular	3	3	0.15	40	0.42667	0
Ventral Head	Mucous	0	2	0.05	40	0.22072	0
	Seromucous	75	38	2.825	40	1.21713	3
	Granular	0	1	0.025	40	0.15811	0
Ventral Centre	Mucous	6	0	0.15	40	0.42667	0
	Seromucous	77	39	2.9	40	1.35495	3
	Granular	0	2	0.05	40	0.22072	0

Both mucous ($U = 150$, $n_1 = n_2 = 20$, $p= 0.018 < 0.05$ two-tailed) and seromucous glands ($U = 35$, $n_1 = n_2 = 20$, $p=0.001<0.05$ two-tailed) present in the ventral centre of *D. melanostictus* and *F. limnocharis* skin structure show a significant difference at 5% significance level. There is no significant difference in the number of granular glands present in the skin structure between *D. melanostictus* and *F. limnocharis* with the value of $U = 180$, $n_1 = n_2 = 20$, $p=0.152 > 0.05$ two-tailed.

It can be concluded that the number of seromucous glands in four regions (dorsal head, dorsal centre, ventral head, and ventral centre) of *D. melanostictus* and *F. limnocharis* skin structure showed a significant difference and able to distinguish between *D. melanostictus* and *F. limnocharis*.

Table 3. Summarised Mann-Whitney Rank Sum Test statistics of numeration of glands on the dorsal head, dorsal centre, ventral head, and ventral centre between *Duttaphrynus melanostictus* and *Fejervarya limnocharis*

Skin Area	Dorsal Head			Dorsal Centre			Ventral Head			Ventral Centre		
	M	SM	G	M	SM	G	M	SM	G	M	SM	G
Mann-Whitney U	180	8	190	160	24	191.5	180	15	190	150	35	180
Sig.	0.298	0.001	0.681	0.037	0.001	0.689	0.152	0.001	0.317	0.018	0.001	0.152

Notes: M= Mucous Glands, SM= Seromucous Glands, G= Granular Glands

Area of Glands Between *Duttaphrynus melanostictus* and *Fejervarya limnocharis*

The area of glands between *D. melanostictus* and *F. limnocharis* was tested by using the Mann-Whitney Rank Sum Test (Table 4 & Table 5). There is only a statistically significant difference can be observed in the dorsal head, which is the area of seromucous glands ($U = 51$, $n_1 = n_2 = 20$, $p=0.001<0.05$ two-tailed) between *D. melanostictus* and *F. limnocharis* skin structure at 5% significance level. However, the areas of mucous and granular glands showed no significant differences between these two

species, with the value of U = 180.5, $n_1 = n_2 = 20$, p=0.311>0.05 two-tailed and U = 194, $n_1 = n_2 = 20$, p=0.806>0.05 two-tailed, respectively.

In the dorsal centre, *D. melanostictus* and *F. limnocharis* have significant differences in the two types of area of glands, which are the area of mucous (U = 160, $n_1 = n_2 = 20$, p=0.038<0.05 two-tailed) and seromucous glands (U = 47, $n_1 = n_2 = 20$, p=0.001<0.05 two-tailed) at 5% significance level. However, the area of seromucous glands shows an extremely substantial difference between *D. melanostictus* and *F. limnocharis* skin structures compared to the area of mucous gland. Besides, there is also consists of the area of granular glands (U = 187, $n_1 = n_2 = 20$, p=0.541> 0.05 two-tailed), although it shows no significant difference between *D. melanostictus* and *F. limnocharis* skin structures.

Table 4. Summarised data of area of glands (mm^2) on the dorsal head, dorsal centre, ventral head and ventral centre between *Duttaphrynus melanostictus* and *Fejervarya limnocharis*

Skin Area	Types of Glands	Area of Glands (mm^2)		Mean	N	Standard Deviation	Median
		F. limnocharis	*D. melanostictus*				
Dorsal Head	Mucous	0.01128	0.00567	0.0004	40	0.00141	0
	Seromucous	0.15661	0.57413	0.0183	40	0.01717	0.009
	Granular	0.10668	0.27488	0.0095	40	0.02797	0
Dorsal Centre	Mucous	0.02618	0	0.0007	40	0.00222	0
	Seromucous	0.17372	0.5533	0.0182	40	0.01844	0.0119
	Granular	0.0189	0.19588	0.0054	40	0.01976	0
Ventral Head	Mucous	0	0.11316	0.0028	40	0.01479	0
	Seromucous	0.18575	0.78894	0.0244	40	0.03072	0.0126
	Granular	0	0.08813	0.0022	40	0.01393	0
Ventral Centre	Mucous	0.01701	0	0.0004	40	0.00122	0
	Seromucous	0.16496	0.57194	0.0184	40	0.01546	0.0132
	Granular	0	0.18539	0.0046	40	0.02323	0

In the ventral head, there is sufficient evidence to conclude that there is a significant difference between the areas of seromucous glands in *D. melanostictus* and *F. limnocharis* skin structures at 5% significance level. The null hypothesis is rejected as the area of seromucous glands (U = 9, $n_1 = n_2 = 20$, p=0.001<0.05 two-tailed) can prove the difference between these two species. In contrast, the analysis of the areas of mucous and granular glands shows values trending towards similarities between *D. melanostictus* and *F. limnocharis* skin structures, with U = 180, $n_1 = n_2 = 20$, p = 0.152>0.05 two-tailed and U = 190, $n_1 = n_2 = 20$, p=0.317>0.05 two-tailed, respectively.

The areas of mucous (U = 150, $n_1 = n_2 = 20$, p=0.019<0.05 two-tailed) and seromucous glands (U = 20, $n_1 = n_2 = 20$, p=0.001<0.05 two-tailed) in the ventral centre have an effect on the significant difference between *D. melanostictus* and *F. limnocharis* skin structures. There is no significant difference in the area of granular glands in the skin structures between *D. melanostictus* and *F. limnocharis* with the value of U = 180, $n_1 = n_2 = 20$, p=0.152>0.05 two-tailed.

The area of seromucous glands in the dorsal head, dorsal centre, ventral head, and ventral centre in *D. melanostictus* and *F. limnocharis* had been proved to have a highly significant difference in the area of glands present in *D. melanostictus* and *F. limnocharis* skin structure with a significant level of $\propto = 0.05$. It can be said that the area of seromucous glands in *D. melanostictus* has a large difference compared to the area of seromucous glands in *F. limnocharis* skin structure.

Table 5. Summarised Mann-Whitney Rank Sum Test Statistics of area of glands (mm^2) on the dorsal head, dorsal centre, ventral head and ventral centre between *Duttaphrynus melanostictus* and *Fejervarya limnocharis*

Skin Area	Dorsal Head			Dorsal Centre			Ventral Head			Ventral Centre		
	M	SM	G	M	SM	G	M	SM	G	M	SM	G
Mann-Whitney U	180.5	51	194	160	47	187	180	9	190	150	20	180
Sig.	0.311	0.001	0.806	0.038	0.001	0.541	0.152	0.001	0.317	0.019	0.001	0.152

Notes: M= Mucous Glands, SM= Seromucous Glands, G= Granular Glands

DISCUSSION

Urbanisation is accelerating worldwide, which means that the increasing population growth and the demand for basic life have resulted in the cities' inhabitation with the modification of landscapes dominated by architectural structural for human benefit. However, urbanisation has fundamentally altered the composition of wildlife communities, leading to biodiversity loss and the development of more species to urban areas (Bradley & Altizer, 2007). For instance, *Duttaphrynus melanostictus* and *Fejervarya limnocharis* are both urban anuran species, which can be well adapted to an environment disturbed by human activities. Fortunately, the existence of urban green lands aids some species in reducing habitat loss and preserving water runoff from impervious urban surfaces as well as serving as the breeding habitat for amphibians, particularly for anurans (Zhang *et al.*, 2015). As they adapt to urban environments, their skin structure must have some changes and becomes sensitive to the environment. This is due to the fact that the essential role of skin as the anuran's mechanical barrier, medium for ion transports and water regulation, sensor apparatus, part of chemical defence mechanism, respiratory organ, and sodium reservoir, that aid in anuran survival (Barlian *et al.*, 2011).

There are obvious distinguishes in skin morphology, with frogs having a thin layer of moist, soft textured skin and toads having a thick layer of dry, rough skin. Nevertheless, both possess epidermis and dermis layers. In the epidermis, the stratum corneum is formed of a thin layer of keratinized cells (Varga *et al.*, 2019), owing to the anuran skin usually "naked" with the absence of the covering with scales, feathers, or hair characters (Sungif, 2017). The keratinized cells that contain the substance alfakeratin are the alternative cells to assist prevent the loss of humidity and respond to environmental contamination (Barlian *et al.*, 2011). The thickness of the stratum corneum of *F. limnocharis*, nevertheless, is thinner than the stratum corneum of *D. melanostictus*. This is because the skin of *D. melanostictus* is water-tight and consists of warts, cones and spines, which induce the stratum corneum to thicken and become more densely packed with keratins (Elias & Shapiro, 1957). Thus, *F. limnocharis* is more susceptible to environmental contaminants and less tolerable to the resistance of water movement between internal and external environments (Campbell *et al.*, 2012), compared with *D. melanostictus*. *Fejervarya limnocharis* shows antibacterial activity against to environmental contaminants such as *Streptococcus pneumoniae* multidrug-resistant, in contrast to the forest frog, *Limnonectes macrodon* (Suhyana *et al.*, 2015). As a result, the stratum corneum of *F. limnocharis* still has the function of reducing water loss via evaporative dehydration and barrier to environmental contaminants without the existence of warts, cones, and spines in the skin. Hence, this becomes a proof that both *D. melanostictus* and *F. limnocharis* adapt to the urban areas, but *D. melanostictus* can be more tolerant to the warmer ambient temperature. In addition, stratum germinativum is also present as the innermost layer of the epidermis, which is the thick layer of the epidermis in *D. melanostictus* and *F. limnocharis* skin structure. This stratum provides strong adherence to the dermis underneath and conducts cell division (Maderson, 2010), which produces the outermost layer of the epidermis and develops into the uppermost layer of keratin (Sungif, 2017). Furthermore, the stratum spinosum, which serves as the intermediate layer between the stratum corneum and the stratum germinativum, is composed of oval to round terminally differentiating cells in the layer of the epidermis (Varga *et al.*, 2019). Different types of

cells such as epithelial cells, immune cells, as well as chromatophores which function as the producers of pigmentation patterns, are present in this layer (Cömden *et al.*, 2023).

There is also the observation of the dermis layer compasses of stratum spongiosum and stratum compactum. The outermost layer of the dermis, the stratum spongiosum, consists of loosely packed connective tissue. In both *D. melanostictus* and *F. limnocharis*, the loosely packed connected tissue helps to maintain the seromucous glands, mucous glands, and granular glands. Thus, the stratum spongiosum can be described as the gland's storage place. Despite this, the stratum spongiosum of *D. melanostictus* is thicker than *F. limnocharis*. This is due to the skin of *D. melanostictus* containing glands that are larger than those found in *F. limnocharis*, and the area-to-skin ratio of glands in *D. melanostictus* is higher than in the skin of *F. limnocharis*. Furthermore, the existence of stratum compactum in both species' skin structure aids in separating the skin layer and muscle layer. Therefore, it is composed of a substantial density of organized connective tissue that is abundant in collagen fibres and fibroblasts (Ponssa *et al.*, 2017). It can be inferred that the thickness of stratum spongiosum and stratum compactum in *D. melanostictus* is thicker than in *F. limnocharis*, as *D. melanostictus* has the enormous size of glands that require more physical support to maintain in the dermis of the skin.

According to Zainudin *et al.* (2018), the ground substance, which can be observed at the basal of the stratum spongiosum of both species, is a type of spongy moisture agent that serves to provide fluid to the interior of the stratum spongiosum. This is essential for urban-type anurans, as they are mostly in dry and terrestrial environments. This is because some urban-type anurans might not be able to alter their skin properties in response to changing environmental conditions, and the presence of ground substance can aid the skin in maintaining moisture (Zainudin *et al.*, 2018). In the comparison of *D. melanostictus* and *F. limnocharis* skin structure, *F. limnocharis* has a thicker ground substance than those in *D. melanostictus* skin structure. In addition, the less or lack of mucous glands in *D. melanostictus* skin structure can be described as the skin of *D. melanostictus* can gain moisture directly from the substrate (Zainudin *et al.*, 2018). This can be explained that *D. melanostictus* can be more adaptable and survive in terrestrial habitats, compared with *F. limnocharis*. It also can be concluded that *D. melanostictus* has the capacity to adjust and change its skin properties to fit the different types of environments.

The dermal bones were discovered on the dorsal parts of the skin of *D. melanostictus*, consistent with *Pelobatrachus nasutus* (Zainudin *et al.*, 2018), which is a condition corresponds to dermal ossifications. These dermal bones on the head and back of the pumpkin toadlets are visible through particularly thin skin due to fluorescence patterns (Goutte *et al.*, 2019), which may serve as intra-specific communication signals or as reinforcement for their aposematic colouration (Bowler, 2019). Such characteristics aid in alerting prospective predators to their toxicity. This suggests that the dermal bone of *D. melanostictus* may have the same function as the dermal bone in pumpkin toadlets.

All the exocrine glands appear in the stratum spongiosum, including mucous, seromucous, and granular glands, but only the toad species (*D. melanostictus* in this research) has the parotoid glands on the dorsal head skin section. Parotoid glands are the developed glandular accumulation in various body regions, and these glands are located in the dorsum of the head in *D. melanostictus* (Mariano *et al.*, 2019). and consists mainly of large alveoli with a milky secretion (Toledo *et al.,* 1992). Thus, this becomes a reason that the skin of *D. melanostictus* is thicker than *F. limnocharis*, as the parotoid glands near the eyes require physical support (Rais, 2012). The arrangement of alveoli in the parotoid glands of *D. melanostictus* shared a similar structure with *Phryoidis juxtaspera* in a honeycomb-like arrangement (Sungif, 2017). A large variety of alkaloids and steroids are present in the milky and latex-like toxins secreted by parotoid glands (Toledo *et al.,* 1992; Mariano *et al.*, 2019). This toxin acts as the chemical barrier against predators of microbial infection. Consequently, the toad venom produced by the dried toxin secretions of parotoid glands has high medicinal value to traditional Chinese medicine (Yang *et al.*, 2023). In addition, the granular glands in parotoid glands, on the dorsal head of *D. melanostictus* in this research, are enormous.

This explained the numeration of granular glands that had been measured in *D. melanostictus* lower than in *F. limnocharis*. It is because the measurements and calculations were taken when the magnification of the microscope was 40x, while the majority of the granular glands could only be observed in full detail in the microscope at a magnification of 4x or 10x. Apart from the parotoid glands, the granular glands in both species secrete the toxins containing peptides, amines and alkaloids that can protect them from microorganisms that exist on their skin surface and are fatal to prospective predators (Zainudin *et al.*, 2018). According to Rasit *et al.* (2018), this gland also has the potential for medical application, which produces secretion-containing peptides. Thus, the granular glands are most abundant at the dorsal part of the skin since these areas are typically exposed to the environment during both species are foraging, resting, or mating (Rasit *et al.*, 2018). Interestingly, the rate of epithelialisation of anuran skin wound is influenced by the concentration of granular glands (Rasit *et al.*, 2018; Rasit *et al.*, 2023).

In comparison to granular and mucous glands, the seromucous glands are significantly more prevalent in the dorsal and ventral parts of these two species. As seromucous glands are the mixed glands of mucous and granular, they also secrete toxins that serve the same functions as those secreted by the granular glands. Even though *D. melanostictus* has parotoid glands in the dorsal head of the body, the granular and seromucous glands also can aid in immunity to environmental variables. The granular and seromucous glands are the sole protective structures in the skin of *F. limnocharis* due to the absence of parotoid glands. Besides, the seromucous glands are exhibiting over the entire body in the skin structure of *D. melanostictus* and *F. limnocharis*. As the seromucous glands consist of mucous, they help preserve skin moisture and provide wet conditions for all regions of the body (Sungif, 2017). It can be assumed that the more seromucous glands emerge inside the skin of the anuran, the longer duration the anuran can stay and thrive in drier conditions. Additionally, it is possible to say that having seromucous glands is equivalent to compacting both types of glands in one way. This may be the strategy of *D. melanostictus* and *F. limnocharis* to be able to adapt, colonise and inhabit dry environments terrestrial that may lack of water sources or humidity (Razali, 2017).

The mucous glands, nonetheless, have a low quantity found in the skin structure *D. melanostictus* and *F. limnocharis*. The enumeration of mucous glands on the dorsal centre and ventral centre showed only significant differences between *D. melanostictus* and *F. limnocharis*. This is due to the dorsal centre and ventral centre of *D. melanostictus* lacking mucous glands. As the mucous glands are the ones that primarily focus on gas exchange and water balance (Mailho-Fontana *et al.*, 2017), the mucous glands produce a clear secretion that acts as the lubricant in water and is composed of glycoproteins such as mucin, mucinogen, sialic acid and carbohydrate residue including galactose and fructose (Garg *et al.*, 2008). The reason the skin of *F. limnocharis* contains a smaller number of mucous glands may be the adaptation to dry habitat, as their preference for hiding in the marshes or ponds to minimise water loss via evaporation and maintain a stable body temperature (Lillywhite & Licht, 1975). At the same time, it has been hypothesized that *D. melanostictus*, which has fewer or no mucous glands, may adapt to the drier environment by having a large size of the body since it has a greater volume to hold onto water and comparatively less surface area to lose it (Sungif, 2017). However, it also can be argued that the use of skin regions to identify glands may not be appropriate as the ventral head of these two species may not be in direct contact with the substrate.

The area of granular glands presents in *D. melanostictus* and *F. limnocharis* shows insufficient evidence to conclude that the significant differences between these two species on the dorsal head, dorsal centre, ventral head, and ventral centre. This is because of the large size of the majority of the granular glands in the skin structure of *D. melanostictus*, which necessitates the use of the microscope with 4x magnification to observe the whole of the glands. In addition, there is a lack of granular glands in the ventral regions of *F. limnocharis* skin structure. This is due to most granular glands are on the dorsal head, where they serve to protect *F. limnocharis* from potential predators and ectoparasites (Moreno-Gómez *et al.*, 2014). In contrast, there is a statistically significant difference in the area of seromucous glands present in both species' dorsal head, dorsal centre, ventral head, and ventral centre. As the

area of seromucous glands in *D. melanostictus* is about 3.5 times greater than those in *F. limnocharis* skin structure, the difference between these two species can be detected obviously. Compared to the seromucous glands in *F. limnocharis* skin structure, the seromucous glands in *D. melanostictus* revealed less in amount but a larger size. This results in the stratum spongiosum in *D. melanostictus* being thicker than in *F. limnocharis*. This may be due to the large total body size of *D. melanostictus*. Besides, the area of mucous glands on the dorsal centre, ventral head and ventral centre showed a significant difference between *D. melanostictus* and *F. limnocharis* skin structure. This is because the small number of mucous glands in the skin structure of *D.* melanostictus, as it may adapt to dry environments by its large body size.

The seromucous glands are most abundant in all parts of the skin areas (dorsal head, dorsal centre, ventral head, and ventral centre) with significant differences in the number of glands present and the area of the glands present in *D. melanostictus* and *F. limnocharis*. The seromucous glands are arguably the most essential glands required for both species. The seromucous and parotoid glands can demonstrate that the skin structure of *D. melanostictus* is different from *F. limnocharis* skin structure. As these anurans are urban species, the number and area of glands aid in determining their adaptation to the surrounding environments. Thus, this study supports the opinion that the distribution of mucous and seromucous glands in different skin regions can reflect the habits of *Pelobatrachus nasutus* in their natural habitat (Zainudin *et al.*, 2018). According to Rasit *et al.* (2023), the mucous glands are critical to represent the water quality of the habitats, as a low quantity of mucous glands in both species can prove that low water quality in urban areas. This also suggests that the large number of seromucous glands serves to defend against high levels of microbial activity in the water resources, facilitate the diffusion of oxygen through the skin (Rasit *et al.*, 2023), and maintain skin moisture.

CONCLUSION

In conclusion, the presence of parotoid glands can demonstrate that the species is a toad species and seromucous glands are the most abundant glands present in all four analysed skin regions of *Duttaphrynus melanostictus* and *Fejervarya limnocharis*. The dermal bones found in *D. melanostictus* are also able to differentiate them. It is possible to distinguish *D. melanostictus* and *F. limnocharis* based on the variation in the thickness of the epidermis and dermis, as well as gland distribution. In addition, the seromucous glands are the most abundant glands present in all four analysed skin regions of *D. melanostictus* and *F. limnocharis*. The enumeration of seromucous glands allows the seromucous glands to distinguish between these two species. The number and area of glands also can determine their adaptation to the environments. Further study and research might explore more on the different types of urban anuran species. In addition, future studies also can focus on the dermal bone within the skin structure, as there is only little research about this has been done.

ACKNOWLEDGEMENTS

This project was funded by the Malaysian Ministry of Higher Education's Fundamental Research Grant Scheme (FRGS/1/2020/SKK0/UNIMAS/01/1) with a research permit from the Sarawak Biodiversity Council (SBC-2021-RDP-34-AHR) and the Sarawak Forestry Department (WL08/2022). The animal ethics approval (UNIMAS/AEC/R/F07/043) was issued by the Animal Ethics Committee of Universiti Malaysia Sarawak. Special thanks to the Faculty of Resource Science and Technology (FRST) and Department of Pathology, Faculty of Medicine and Health Sciences (FMHS) for technical support.

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