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Morphology and Histochemistry of the Skin of the Mud Loach, *Misgurnus mizolepis*, in Relation to Cutaeneous Respiration

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The skin structure of *Misgurnus mizolepis* was studied based on the micro-anatomical investigation of skin fragments taken from four regions. The epidermis was distinguished by two types of skin glands, a small mucous cell and a large club cell. The mucous cell was acid sulfomucins (some sialo-mucins), but the club cell did not give any histochemical tests for muco-substances. The presence of a well defined lymphatic system with small lymphocytes was established in the stratum germinativum layer of the epidermis. A large number of blood capillaries run very close to each other just below the basement membrane, and a definite area giving AB and PAS positive was present between the basement membrane and scale. These structural features of skin in *M. mizolepis* seem to be closely related with cutaneous respiration.

The mud loach *Misgurnus mizolepis* inhabits muddy swamps, ponds, and ricefields which are subjected to periodic drying. The fishes which live in rivers or streams are able to constantly meet their oxygen demands, and can perform aquatic respiration with their gills. However, some fishes live in warm and stagnant reservoirs or environments undergoing periodic drought which causes reduction of dissolved oxygen in the water. To adapt to these environments, those fishes have developed an additional respiratory apparatus to receive or hold air in the following organs; the intestine (Cobitidae), the skin (Cobitidae, Anguillidae, Nototeridae, Gobiidae), the branchial chambers (Anabatidae, Osphromenidae, Channidae), the swim bladder (Dipnoi), the labyrinthine organ (*Anabas*), and others (Liem, 1967; Johansen, 1970; Niva et al., 1981; Munshi and Hughes, 1986; Moitra et al., 1989; Itazawa and Hanyu, 1991; Ishimatsu et al., 1998; Park and Kim, 2000, 2001). These air breathing fishes are bimodal breathers- they are both air breathing and water breathing. It has been known that the ratio between the aerial and aquatic respiration depends on the oxygen and carbon dioxide contents of the water (Liem, 1967; Ishimatsu et al., 1979, 1998).

The six *Misgurnus* species are small and slender fishes in the Euro-east Asia. Some are additional air-breathing fishes who take up air to supplement their oxygen supply through the skin or intestine. *Misgurnus fossilis* (Jakubowski, 1958) and *M. anguillicaudatus* (Park and Kim 1999) have cutaneous respiration, and *M. fossilis* (Johansen, 1970), *M. anguillicaudatus* (Koyama, 1958; Itazawa and Hanyu, 1991) and *M. mizolepis* (Park and Kim, 2001) have intestinal respiration. In addition to *Misgurnus*, the air respiration by the skin has been known in several taxa, *Monopterus*, *Periophthalmus*, *Heteropneustes*, *Mastacembelus* and *Amphipnous* (Liem, 1967; Johansen, 1970; Mittal and Munshi, 1971; Mittal and Banerjee 1974; Whitear, 1986; Park and Kim, 2000).

Little is known about the structure and histochemistry of the skin as well as the respiration in relation to the skin in *M. mizolepis*. Therefore, the purpose of this study was to examine the structure of the skin, as well as the histochemical nature of its gland cells, and to describe any modifications for cutaneous respiration in *Misgurnus mizolepis*.

Material and Methods

The 10 specimens collected from Dolsan Island in the southwestern coast of Korea ranged from 78.5 mm to 119.2 mm in standard length. The specimens were fixed in 10% neutral buffered formaldehyde. Skin fragments were taken from four regions; top of the

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head, the dorsal, lateral and abdominal regions.

These fragments were dehydrated through a standard ethanol series to 100%, cleared in xylene and then embedded in wax (Paraplast, Oxford). Five µm sections were deparaffinized and stained with Harris' hematoxylin, iron alum hematoxylin, counter-stained with eosin, and Masson trichrome-stained (Gurr, 1956) for general histology. For blood cells the giemsa method was used. Mucins of the gland were demonstrated by alcian blue solution (AB) at pH 1.0 and 2.5 (Steedman, 1950; Lev and Spicer, 1964), and the periodic acid-Schiff (PAS) method with or without prior digestion with diastase/saliva (Lilllie and Greco, 1947). In addition, the PAS technique was employed in combination with AB and vice-versa for neutral and acid mucins. Acid mucin was shown by metachromatic reactions with toluidine blue (Tock and Pearse, 1965). Aldehyde fucshin with AB (Spicer and Meyer, 1960) and high iron diamine (HID) with AB (Spicer, 1965) were used to determine the nature of the acid mucins. Acetylation and deacetylation was performed following Lillie (1954) for the confirmation of hydroxyl group. Methylation and demethylation (Spicer, 1960) were done to confirm the acidic nature of the mucins. Evaluation of the skin was made by light microscopy on the whole mount PAS preparations, and hematoxylin and eosin preparations.

Results

The integument of *M. mizolepis* could be divided into three principal layers, the epidermis, the dermis, and the subcutis (Fig. 1A). The epidermis was composed of three layers (outermost layer, middle layer and stratum germinativum), and the dermis was composed of two layers (stratum laxum and compactum).

Epidermis

The epidermis could be divided into three layers- the outermost layer, the middle layer, and the stratum germinativum, (Figs. 1A and 1E to 1G). The epidermis was thickest at the abdominal region (approximately 195.5 µm), and the thinnest in the lateral region (approximately 136.3 µm). The dorsal region was 180.8 µm and the top of the head region was 162.5 µm.

Outermost layer; This layer was composed of polygonal cells and a few flattened cells, which were arranged in one to six rows of cells (Fig. 1A). Between these cells, small spherical or flask-shaped unicellular mucous glands were discernible (Figs. 1A to 1F). The mucous cells have a large spherical body and a short narrow neck that opens on the surface by a wide pore. They have a basal, spherical or oval nucleus with a thin rim of slightly basophilic cytoplasm, pushed at the periphery of the cell due to the heavy accumulation of its basophilic secretions (Figs. 1A, 1E and 1F). Its secretory matter was highly vacuolated and basophilic. A few lymphocytes were present between the epidermal cells and mucous cells.

Middle layer; This layer was composed mainly of voluminous club cells and a few flask or spherical mucous cells (Figs. 1A to 1E). In between these skin glands the elongated spindle-shaped epidermal cells were found to be vertical. The boundary of these cells was usually not clear as it greatly expanded or stretched out due to the collateral pressure of these glands. The club cells arranged in three to four layers were oval or spherical in shape (Figs. 1A and 1E). They were more numerous. They were usually uninucleate with an oval nucleus, and sometimes binucleate with the two nuclei very close to each other (Fig. 1E). Their cytoplasms were finely granular or more or less homogeneous in nature and invariably showed some shrinkage due to the fixation. Some cells had a few vacuoles in their cytoplasm. The main thickness of the epidermis was due to the middle layer, particularly the skin glands.

Stratum germinativum; This layer was composed of a single layer of cuboidal cells on a thin basement membrane which was PAS- and AB-positive, took green color in Masson trichrome and gave γ-metachromasia with toluidine blue. Each basal cell had a prominent, lightly stained, centrally placed spherical or oval nucleus, and their cytoplasm was homogeneous. The average height and width of the basal cells were approximately 7.8 µm and 3.8 µm, respectively. There were small oval or round lymphatic spaces in between the cuboidal cells (Fig. 1G). Inside these spaces one or two small lymphocytes were found and they had deeply stained nuclei, surrounded by small amounts of faintly stained cytoplasm. They were stained purple by AB, blue by PAS and giemsa staining. The lymphocytes penetrated the middle layer, often reaching up to the outer cell layers of the epidermis.

The pit organs, single or in groups of two to three were distributed on the surface of the skin (Fig. 1F). Each pit organ was a pear-shaped structure, sunk below the epithelial cells lying directly on the basement membrane. In the intraepithelial layer, there were blood vessels serving the sense organs of the epidermis.

Dermis

The dermis consisted of a relatively thin upper layer of loose vascular connective tissue, stratum laxum, and a thick lower compact layer, stratum compactum (Figs. 1A, 1D and 1H).

Stratum laxum; This layer was well differentiated by the presence of loose connective tissue in which blood capillaries abound and by the presence of a thin scale, which was lodged in pockets in the connective tissues

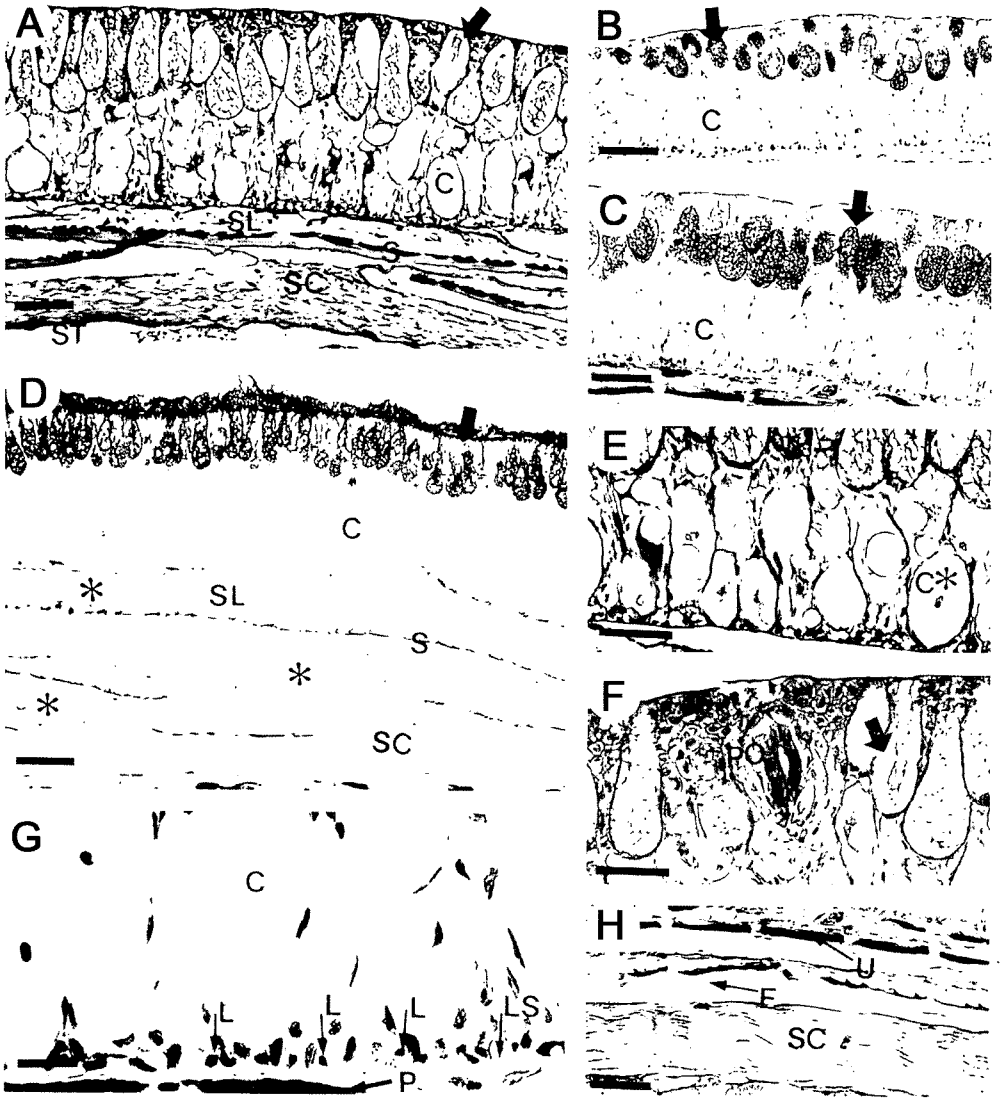

Fig. 1. Structure of the skin of *Misgurnus mizolepis*. A, The dorsal skin with haematoxylin and eosin. The skin is composed of epidermis, dermis and subcutis (ST). The epidermis has epidermal glands of mucous cell (arrow) and club cell (C) and the dermis has stratum laxum (SL) containing scale (S) and stratum compactum (SC). B, The ventral skin stained with alcian blue pH 2.5. The mucous cells (arrow) are positive and the club cells (C) are negative. C, The dorsal skin with periodic-acid-Schiffs reaction. The mucous cells (arrow) are strongly positive and the club cells (C) are negative. D, The dorsal skin with AB (pH 2.5)-PAS reaction. The mucous cells (arrow) are strongly positive and the club cells (C) are negative. Definite areas (*) in the stratum laxum (SL) are positive: S, scale; SC, stratum compactum. E, The dorsal skin with haematoxylin and eosin. Club cells are generally uninucleate, sometimes binucleate (C*). F, The dorsal skin with haematoxylin and eosin. The epidermis has pit organ (PO): Arrow, mucous cell. G, The dorsal skin with Masson trichrome stain. The stratum germinativum has several lymphocytes (L) in lymphatic space (LS) and there are pigment cells (P) under the basement membrane. H, The dorsal skin with haematoxylin and eosin. The scales consist of a upper bony layer (U) and inner fibrillary plate (F): SC, stratum compactum. Scale bars = 25 μm (A-D), 20 μm (E, F), and 12 μm (G, H).

(Figs. 1A, 1D and 1H). A layer of loose connective tissue below the basement membrane was richly supplied with blood capillaries, which were connected to blood vessels of the stratum compactum and subcutis by capillaries running between the scale pockets. The loose connective tissue of the stratum laxum was mainly composed of thin collagen bundles, stained green in Masson trichrome stained preparations. A layer of sparsely distributed pigment cells was found below the basement membranes and also in the connective tissue pockets on the underside of the scales (Fig. 1G).

A definite area was present between the basement membrane and the scales (Fig. 1D). The area was PAS positive and diastase resistant, AB positive, giemsa positive, and it gave γ-metachromasia with toluidine blue. With Masson trichrome stain it was negative. These materials were present in some free space in which the scales were lodged. The scales consisted of two distinct layers-the upper bony layer and the inner fibrillary plate (Figs. 1A and 1H). However, the top of the head had no scale. The bony

layer carried concentrically arranged ridges of striae on its free surface, while the lower fibrillary plate was mainly composed of parallel collagen fibers. The upper bony layers of the scale was basophilic in nature, and gave red colour with PAS technique, γ-metachromasia with toluidine blue, appeared blue in alcian blue and a deep green color in Masson trichrome stained preparations. The lower fibrillary plate was eosinophilic, weakly PAS positive, alcian blue negative and gave strong orthochromasia with toluidine blue. With the Masson trichrome stain preparations, it showed a green color.

Stratum compactum; This layer was characterized by the presence of bundles of coarse collagenous fiber arranged compactly in several layers (Fig. 1A and 1H). A few collagen fiber bundles ran vertically at intervals. This layer was weakly PAS positive and took a deep green color in Masson trichrome stained preparations for collagen. Between them were a few pigment cells and blood capillaries. The pigment cells were distributed sparsely on the inner part of this layer

Subcutis

This was the innermost and thinest layer of the skin and was situated in between the stratum compactum and the muscle (Fig. 1A). A large number of nerves and blood vessels were found in this layer. In hematoxylin and eosin preparations this layer invariably showed numerous empty spaces which were occupied by fat cells (Fig. 1A).

Histochemistry of the skin gland

Mucous cell; The flask-shaped or spherical unicellular glands were predominantly distributed in the outermost layer of the skin but were sparse in the middle layer. The mucous cells were various, reaching 15-100 μm in height as described in the cells of the outermost layer. These cells gave a deep red color reaction with PAS technique, which was diastase resistant, γ-metachromasia with toluidine blue, and blue with AB at pH 1.0 and 2.5 (Table 1). The mucous cells, giving red color with aldehyde fucshin and black color with high iron diamine, were likely to be sulfomucins. In mild methylation/AB and acetylation/PAS techniques they were negative, and in methylation/saponification-AB they stained blue. Their nuclei were purple or red with AB-PAS reaction and Masson trichrome stain.

Club cell; The club cells with oval or spherical shape were present only in the middle layer of the epidermis. Each club cell was approximately 27.5-75.0 μm in height and was arranged in three to four rows of cells. With hamatoxylin/eosin stained preparations, the nuclei of club cells showed blue and the cytoplasm was stained light pink. However, they did not give any histochemical tests for mucosubstances (Table 1). The club cell took a greenish color with Masson trichrome staining and orthochromasia with toluidine blue.

Table 1. A summary of the histochemical tests performed to show the nature of the gland cell of the skin in *Misgurnus mizolepis*

Staining employed	Gland cell	
	Mucous cell	Club cell
Hematoxylin & iron	±B	+++PN
Weigerts rion hematoxylin	+++B	-
Masson trichrome	-	++G
PAS	++R	-
PAS after digestion in malt diastase/PAS	++R	-
AB (1.0)	+B	-
AB (2.5)	++B	-
AB/PAS	++B, BR, R	-
PAS/AB	++B, BR, R	Ortho
Toluidine blue	γ-meta	-
Acetylation/PAS	-	-
Methylation/AB	-	
Methylation/Saponication/AB	+B	
Aldehyde fucshin	+R	
Aldehyde fucshin/AB	++BR	
High iron diamine	+N	
High iron diamine/AB	+++BN	

B, blue; BN, bluish black; BR, bluish red; G, green; N, black; PN, pink; R, red; +, increasing intensity of reaction; ±, fairly present; -, absent.

Discussion

Cutaneous respiration has been known in several fishes as an air-breathing organ of dual breathing fishes (Jakubowski, 1958; Liem, 1967; Johansen, 1970; Mittal and Munshi, 1971; Mittal and Banerjee 1974; Mittal et al., 1980; Whitear, 1986; Park and Kim, 1999). In particular, the capacity of cutaneous respiration depends on the structure of the skin and the degree of its vascularization in relation to their habitats. The skin of air breathing fish has certain features, such as a thicker epidermis due to large glandular cells *(Misgurnus, Monopterus)*, intraepithelial capillaries (*Periophthalmus*), and well-developed vascularization (*Heteropneustes*). These structures may be related to the amphibious habit of the fishes (Jakubowski, 1958; Liem, 1967; Johansen, 1970; Mittal and Munshi, 1971; Mittal and Banerjee 1974; Whitear, 1986; Park and Kim, 1999, 2000). The skin structure of *M. mizolepis* was characterized by a thick epithelial layer having two types of glands, a small mucous cell and a large club cell. Also there was a thin superficial layer consisting of 1 to 6 rows of cells, the small scales located inside the dermis, a well-defined lymphatic system, a definite area showing AB and PAS positive staining, and a large number of blood capillaries just under the basement membrane. These results suggest that *M. mizolepis* is closely related to cutaneous respiration. The epidermis of *M. mizolepis* was thick, approximately 97.5 to 113.5 μm. In the thickness, the epidermis of *M. fossilis* was 182 μm (Jakubowski, 1958), *M. anguillicaudatus* 162.8 μm, *Heteropneustes fossilis* 98 μm, *Mastacembelus pancalus* 44 μm, *Amphipnous cuchia* 119 μm (Mittal & Munshi, 1971), and *Monopterus albus* 75 μm (Liem, 1967) (Table 2).

Table 2. A summary of thickness of the dorsal epidermis in cutaneous respiration fishes

Species	Epidermis			Literature
	Average	Minimum	Maximum	
Monopterus albus	75	-	-	Liem (1967)
Notopterus notopterus	167	130	138.7	Mittal and Banerjee (1974)
Heteropneustes fossilis	98	64	130	Mittal and Munshi (1971)
Mastacembelus pancalus	44	7	39	Mittal and Munshi (1971)
Amphipnous cuchia	119	108	127	Mittal and Munshi (1971)
Misgurnus fossilis	182	-	-	Jakubowski(1958)
Misgurnus anguillicaudatus	162.8	-	-	Park and Kim (1999)
Misgurnus mizolepis	113	97.5	113.5	Present study

The size and abundance of mucous cells play an important role in supporting and maintaining the normal relationship of the cutaneous respiration. The stratum laxum layer of the dermis in *M. mizolepis* had a definite area with acid mucopolysaccharides. This area was found just under the basement membrane or present in some of the free space in which scales were lodged. The presence of mucopolysaccharides in the stratum laxum correlated with the semiterrestrial ecological habits of fish (Mittal & Munshi, 1971). Rogers (1961) reported that 1 g of mucopolysaccharides could bind or release 200 g or 500 g of water. Thus, it was considered that the presence of mucopolysaccharides in the stratum laxum was an adaptive modification for cutaneous respiration of *M. mizolepis*. They had a vascularization system consisting of a pit organ and lymphatic space in the epidermis. A pit organ of a pear-shaped structure was present below the epithelial cells and lay directly on the basement membrane. Jakubowski (1958) described the pit organ as the looplike vessels serving the sense organs of the epidermis in *M. fossilis* and considered that the vascularization of the looplike vessels may be proof of air breathing. A well-defined lymphatic system with a series of lymph spaces containing small lymphocytes was present in the stratum germinativum layer of the epidermis. These lymphocytes penetrated the intraepithelial layer. Such structures were not reported in *M. fossilis* (Jakubowski, 1958) but were confirmed to be present in *M. anguillicaudatus* (Park and Kim, 1999). The lymphatic system functions to supply nutrition to the stratum germinativum for cell proliferation and to protect the epidermis from microorganisms or foreign proteins (Mittal and Munshi, 1971).

M. mizolepis also had small scales embedded in the superficial layer of the dermis. The small scales, rudimentary scales or absence of scales were found in a burrowing and a mud-dwelling fish (*Amphipnous*, *Monopterus*) and were considered as an adaptation to its peculiar mode of life (Liem, 1967; Mittal and Munshi, 1971; Whitear, 1986). The blood capillaries of the skin are very important to air breathing fishes. The epidermis of amphibious fishes, *Periophthalmus cantonensis* (Tamura et al., 1976) and *Periophthalmus modestus* (Park and Kim, 2000) has intraepithelial capillaries, whereas the skin of *M. fossilis*, *M. anguillicaudatus, Anguilla*, *Amphinous*, and *Monopterus* has the blood vessels in the dermis (Jakubowski, 1958; Liem, 1967; Lethbridge and Potter, 1982; Park and Kim, 1999). In this case, the diffusion of oxygen took place readily across the mucous coat of the epithelium, though the blood vessels were situated in the deep dermis (Mittal and Munshi, 1971; Perry and McDonald, 1993).

The epidermis of *M. mizolepis* has two epidermal glands, the mucous cell and the club cell. As have been reported in other air breathing fishes, these skin glands contained a lot of water, and oxygen may easily penetrate them towards the deeper layers of the skin (Jakubowski, 1958; Mittal and Munshi, 1971). Letterer (1959) and Rogers (1961) have reported that mucus substances had great ability to bind a large amount of water. Hora (1934) and Mittal et al. (1980) have shown that the mucus has a remarkable power for precipitating mud held in suspension in water, and that the mucus secreted by the skin in air breathing fishes may also be used to keep the skin clear for respiration. As in Table 3, the club cell was proteinaceous in nature as reported in other fishes (Whitear 1986, Agrawal and Mittal 1992, Zaccone et al. 2001). As listed by Agrawal and Mittal (1992), their functions are mainly protective and have been associated with the secretion of pheromones. Otherwise, the nature of the mucous cell was acidic sulphated mucopolysaccharides (Mittal and Munshi, 1971; Mittal and Banerjee 1974; Mittal et al., 1980). The mucous cell of *M.*

Table 3. A summary of histochemical nature of mucous cell in cutaneous respiration of dual breathing fishes.

Species	Histochemical nature	Literatures
Monopterus cuchia	Strongly acidic sulphated mucopolysaccharides	Mittal et al. (1980)
Notopterus notopterus	Acidic sulphated mucopolysaccharides	Mittal and Banerjee (1974)
Heteropneustes fossilis	Weakly acidic sulphated mucopolysaccharides	Mittal and Munshi (1971)
Mastacembelus pancalus	Strongly acidic sulphated mucopolysaccharides	Mittal and Munshi (1971)
Amphipnous cuchia	Strongly acidic sulphated mucopolysaccharides	Mittal and Munshi (1971)
Misgurnus anguillicaudatus	Acidic mucopolysaccharides	Park and Kim (1999)
Misgurnus mizolepis	Acid sulfomucins (some sialomucins)	Present study

anguillicaudatus of family Cobitidae was acid mucopolysaccharides in nature (Park and Kim, 1999). In our histochemical observation the mucous cell of *M. mizolepis* was also acidic sulfomucins in nature and the acidic mucosubstances seem to be characteristic of cutaneous respiration in dual breathing fishes.

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