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ARTICLE

Bactericidal activity of skin mucus and skin extracts of *Catla catla* and *Channa striatus*

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ABSTRACT Fishes counteract certain microbial attacks in water by producing antimicrobial proteins/peptides in their skin surface. The present study focused on screening the bactericidal activity of skin and skin mucus extracts of *Catla catla* and *Channa striatus*. The bactericidal activity was assessed against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Aeromonas hydrophila*, *Staphylococcus aureus* and *Bacillus coagulans* by disc diffusion method. The minimal inhibitory concentration was also determined. Protein profiles in skin and skin mucus extracts were analyzed by SDS-PAGE. Samples from both fishes showed antibacterial activity. Detailed analysis of individual protein and peptide would throw light on their medicinal importance to be used against pathogenic microbes. **Acta Biol Szeged 64(1):11-16 (2020)**

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Introduction

Fishes have great economic value due to their taste and rich protein content. In an aquatic environment, a myriad of pathogenic and non-pathogenic organisms is present. Occasionally, fish cultivation results in enormous loss because of infectious diseases caused by the pathogenic microorganisms. Antibiotics are being utilized to manage these diseases; however, pathogens develop resistance against several antibiotics (Lalumera et al. 2004). At the same time, fishes possess excellent defense system against the pathogens by producing biochemically diverse secretions which mainly act on bacterial membranes and induce cell lysis.

The mucus layer on the surface of the fish is constantly replaced, which possibly prevents stable colonization by parasites, bacteria and fungi. Skin secretions have a broad range of polypeptides with antimicrobial properties (Uthayakumar et al. 2012). The bioactive substances like lysozyme, lectins, proteolytic enzymes, flavoenzymes, immunoglobins, C-reactive proteins, apolipoprotein A-1 and antimicrobial peptides are constitutively expressed in the mucus to provide immediate protection to fish against potential pathogens (Kitani et al. 2008).

Further, the mucus layer of the fish skin is presumed to perform several other functions, viz., acts as a lubricant, serves as a barrier for microbial entry, maintains osmoregulation, plays a role in locomotion and pheromone communication (Hellio et al. 2002). By nature, antimicrobial peptides (AMPs) are secreted by the fish skin and function as a first line defense against the microbial attacks. They protect the fish against a wide variety of bacterial, fungal, viral, and other pathogenic infections by disruptive "lytic" or pore-forming "ionophoric" actions (Smith et al. 2010). Fish epidermal mucus AMPs have demonstrated a broad spectrum of activity that is 10-100 times more potent than that of their amphibian counterparts against various fish and human pathogens (Park et al. 1998).

Proteins or peptides present in the fish skin mucus form pores on the bacterial membrane that cause oozing out of cellular contents. This alters the regular ionic gradients of membrane and eventually leads to the death

of bacteria (Fernandes et al. 2004; Silphaduang et al. 2006). Antimicrobial proteins and peptides could also be a source of potential natural antibiotics for pharmaceutical applications (Ying-xia et al. 2008).

In developing countries, most of the ponds in rural as well as urban localities are contaminated by the discharge of sewage water and dumping of solid wastes. Fishes are enduring these adverse conditions by making antimicrobial substances/ peptides in their skin. Interestingly, the secretion and composition of skin mucus are altered in conformity with the changing environment, especially to microbial exposure and hyperosmolarity (Zuchelkowski et al. 1981; Arulvasu et al. 2012). Attempts are being made to identify bioactive principles in the fish skin mucus to obtain potent new antimicrobial agents; several earlier studies described such antimicrobial activity of skin and skin mucus extracts of freshwater fishes (Kumari et al. 2011; Haniffa et al. 2013; Islam et al. 2014; Patil et al. 2015). The present study was aimed to screen the bactericidal activity of skin mucus and skin mucus extracts of *Catla catla* and *Channa striatus*.

Materials and methods

Fish collection and maintenance

Healthy fishes of *Catla catla* (450 g in weight; 20 cm in length) and *Channa striatus* (250 g in weight; 28 cm in length) were collected from the nearby reservoir (Pilavakal dam, Srivilliputtur, India) and brought to the laboratory for the collection of mucus and skin. Only healthy fishes were sampled; dead fish or fish with skin lesions were not chosen for the experiment.

Collection of skin and skin mucus samples

Fish skin mucus was collected carefully from dorsal side of the fish by a sterile scalpel. Then, the skin was removed with sterile scissor and forceps. The samples were stored immediately at -20 °C until further use.

Preparation of mucus and skin extracts

The collected samples were homogenized separately in 100 mM extraction buffer (ammonium bicarbonate, pH 7.8) at 1 mg/mL on ice-cold condition in a glass homogenizer. Insoluble substances were removed by centrifugation at 10 000 rpm for 10 min at 4 °C and the supernatant was collected for further analysis (Anbuchezhian et al. 2011).

Antimicrobial sensitivity

Preliminary screening of skin mucus and skin extracts for their antimicrobial efficacy was carried out against five Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25619, *Aeromonas hydrophila* ATCC 7966, *Proteus vulgaris* ATCC 6380 and *Klebsiella pneumoniae* ATCC 29665) and two Gram-positive (*Bacillus coagulans* ATCC 7050 and *Staphylococcus aureus* ATCC 9144) bacteria. All the microbial strains, except *Aeromonas hydrophila*, were maintained in Luria-Bertani (LB) broth 37 °C. *Aeromonas hydrophila* was grown in nutrient broth at 37 °C.

The antimicrobial activity of skin and skin mucus extracts was determined by agar disc diffusion method (Bauer et al. 1966). Briefly, 100 µl of overnight grown bacterial culture was uniformly seeded on agar plates. Wells of 5 mm diameter were made on the agar using a sterile cork borer. 100 µl each of skin mucus and skin extracts were added to individual wells. Same quantity of extraction buffer, Ammonium bicarbonate, was added to another well which served as control. The agar plates were kept at 37 °C for 24 h and antimicrobial activity was determined by measuring the diameter of zone of inhibition.

Minimum inhibitory concentration (MIC)

MIC was carried out by broth microdilution method as described by Subramanian (2008) with slight modification. 50 µl of skin mucus and skin extracts were added individually to sterile 96 well microtitre plate in different concentrations. To this, 50 µl of overnight grown microbial culture was added. Finally, 50 µl of sterilized Muller-Hinton broth containing 2% NaCl was added to all the wells and incubated at 37 °C for 16-18 hours. After incubation, the wells were observed for microbial growth. The minimal inhibitory concentration was determined by the formation of turbidity and/or button-like structure at the bottom of the well.

SDS-PAGE

The overall protein content of skin mucus and skin extract was calculated by following the method of Bradford (1976). Electrophoresis was performed as described by Laemmli (1970) using 12% separating and 5% stacking gel. 50 µg of protein samples were loaded on to the well. The protein bands were visualized by staining with Coomassie brilliant blue (R250). Molecular mass of bands in the gel was determined by protein standard markers (Protein Molecular Weight Marker-Medium range, Genei, Bangalore).

Results

In the present study, bactericidal activity of skin and skin mucus of *C. catla* and *Ch. striatus* was assessed. The antibacterial activity of mucus and skin extracts of *C. catla* against the tested microorganisms was observed in the following order: *B. coagulans* > *K. pneumoniae* > *A.*

Table 1. Antibacterial activity of skin mucus and skin extracts of *C. catla*. Control: ammonium bicarbonate buffer.

Bacterial pathogens		Diameter of inhibition zone (mm)	
		mucus	skin
Gram negative bacteria			
1.	*E. coli*	10	12
2.	*P. aeruginosa*	0	0
3.	*K. pneumoniae*	13	18
4.	*P. vulgaris*	0	0
5.	*A. hydrophila*	12	15
Gram positive bacteria			
1.	*S. aureus*	0	0
2.	*B. coagulans*	14	20

hydrophila > *E. coli*. Three pathogens such as *P. aeruginosa*, *P. vulgaris* and *S. aureus* exhibited resistance against the mucus and skin extracts (Table 1). For *Ch. striatus,* the outcome was different: *E. coli* > *S. aureus* > *B. coagulans* > *P. aeruginosa* > *A. hydrophila*. *K. pneumoniae* and *P. vulgaris* were not affected by both the samples taken from *Ch. striatus* (Table 2).

The mucus and skin extracts of *C. catla* and *Ch. striatus* were further assayed for minimum inhibitory concentration (Table 3 and Table 4). The extracts showed a broad range of activity against the tested microorganisms. The respective MIC values of mucus and skin extract of *C. catla* against pathogens are as follows: *E. coli* (49.65 μg ml^{-1} and 35.07 μg ml^{-1}), *K. pneumoniae* (66.20 μg ml^{-1} and 17.54 μg ml^{-1}), *A. hydrophila* (49.65 μg ml^{-1} and 35.07 μg ml^{-1}) and *B. coagulans* (16.54 μg ml^{-1} and 17.54 μg ml^{-1}). For *Ch. striatus,* the MIC values are: *E. coli* (6.14 μg ml^{-1} and 4.88 μg ml^{-1}), *P. aeruginosa* (18.42 μg ml^{-1} and 4.88 μg ml^{-1}), *A. hydrophila* (24.56 μg ml^{-1} and 14.65 μg ml^{-1}), *S. aureus* (6.14 μg ml^{-1} and 4.88 μg ml^{-1}) and *B. coagulans* (6.14 μg ml^{-1} and 14.65 μg ml^{-1}).

Table 2. Antibacterial activity of epidermal mucus and skin extracts of *Channa striatus*. Control: ammonium bicarbonate buffer.

Bacterial pathogens		Diameter of inhibition zone (mm)	
		mucus	skin
Gram negative bacteria			
1.	*E. coli*	12	16
2.	*P. aeruginosa*	9	12
3.	*K. pneumoniae*	0	0
4.	*P. vulgaris*	0	0
5.	*A. hydrophila*	8	10
Gram positive bacteria			
1.	*S. aureus*	11	13
2.	*B. coagulans*	10	12

Table 3. MIC of skin mucus and skin samples of *C. catla*.

Bacterial pathogens		MIC of mucus (μg)	MIC of skin (μg)
1	*E. coli*	49.65	35.07
2	*P. aeruginosa*	NI*	NI
3	*K. pneumoniae*	66.20	17.54
4	*P. vulgaris*	NI	NI
5	*A. hydrophila*	49.65	35.07
6	*S. aureus*	NI	NI
7	*B. coagulans*	16.54	17.54

*NI: No inhibition.

The total protein content in the mucus and skin sample was 0.033 ± 0.1 and 0.035 ± 0.0 mg/ml, respectively, for *C. catla*, and 0.031 ± 0.0 mg/ml and 0.090 ± 0.1 mg/ml, respectively, for *Ch. striatus.* The protein profile of *C. catla* revealed the presence of intense protein bands with different molecular masses such as 90.0, 65.0, 40.0, 32.0, 28.0 and 15.0 kDa (Fig. 1). The electrophoretic analysis of *Ch. striatus* samples showed the presence of proteins with molecular masses of 98.0, 49.0 and 38.0 kDa (Fig. 2).

Discussion

Fishes are living in microbe-rich habitat for which they are bestowed with good defense mechanism in their skin mucus. Skin mucus contains antimicrobial peptides as weapons for killing the pathogens. It has been documented that naturally occurring proteins or glycoproteins of non-immunoglobulin nature are present in fish skin and mucus that react with a diverse array of environmental antigens and may confer an undefined extent of natural immunity to fish (Balasubramanian et al. 2012). Pieces of evidences suggest that occurrence of infections in

Table 4. MIC of mucus and skin sample of *Ch. striatus*.

Bacterial pathogens		MIC of mucus (μg)	MIC of skin (μg)
1	*E. coli*	6.14	4.88
2	*P. aeruginosa*	18.42	4.88
3	*K. pneumoniae*	NI*	NI
4	*P. vulgaris*	NI	NI
5	*A. hydrophila*	24.56	14.65
6	*S. aureus*	6.14	4.88
7	*B. coagulans*	6.14	14.65

*NI: No inhibition.

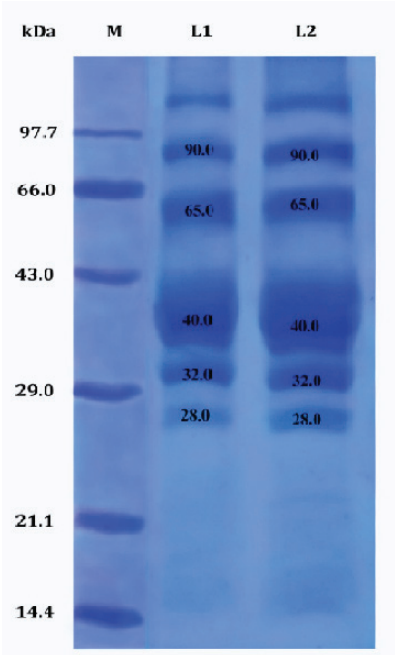

Figure 1. Protein profiles of mucus and skin extracts of *C. catla*. M: marker; L1: mucus; L2: skin

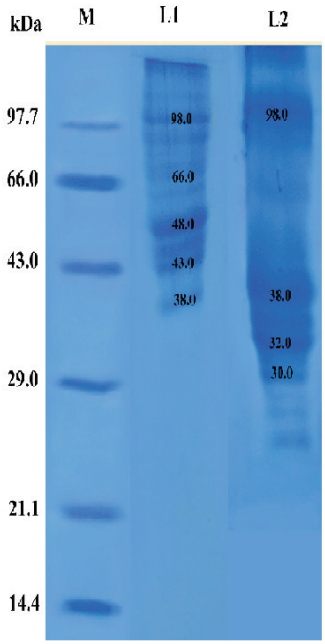

Figure 2. Protein profiles of mucus and skin extracts of *Ch. striatus*. M: marker; L1: mucus; L2: skin

fishes are very rare (Mayer and Hamann 2004; Fuochi et al. 2017). Thus, it makes many scientists to figure out the defense mechanism in fish skin mucus. In the present study, antibacterial effects of proteins from the skin and mucus extracts of *C. catla* and *Ch. striatus* were investigated. The bactericidal assays indicated that the crude mucus and skin extracts of both fishes showed a strong inhibitory effect on both Gram-positive and Gram-negative bacteria. This corroborates the works of Hellio et al. (2002) in which the potential antimicrobial activity of epidermal mucus and epidermal extracts of thirteen fish species were studied. It was reported that both skin and skin mucus play a major role in bactericidal activity. Similar kind of results were obtained by Elavarasi et al. (2013) in the skin mucus and skin extract of freshwater fishes, *Clarias batrachus* and *Tilapia mossambicus*.

The bactericidal activity of fish skin and skin mucus could be accounted for the counteraction of the negatively charged membrane and positively charged antimicrobial peptides. This causes the aggregation of antibacterial proteins to develop pores on the membrane. The mechanism of action of each peptide is distinct even though they belong to same structural class (Merrifield et al. 1994). It is also suggested that the antibacterial activity of fish mucus may be attributed to the antibacterial glycoproteins and their ability to kill bacteria by forming large pores in the target membrane (Ebran et al. 1999; Wei et al. 2010).

In the present study, the effect of skin mucus and skin extracts on bacterial growth was monitored by a liquid growth inhibition assay. The samples of *C. catla* showed a broad range of activity against pathogens. It inhibited the growth of *B. coagulans* at very low concentrations viz.,16.54 µg/ml and 17.54 µg/ml, respectively. The minimal inhibitory concentrations against *A. hydrophila* were found to be 49.65 µg/ml and 35.07 µg/ml, respectively. Among seven pathogens tested, five microorganisms were inhibited by the mucus and skin extracts of *C. catla* and *Ch. striatus*.

As another phase of the present study, electrophoretic analysis of skin and skin mucus of *C. catla* and *Ch. striatus* was carried out. The results showed the presence of proteins with molecular masses of 90.0, 65.0, 40.0 and 32.0 kDa in both skin and skin mucus of *C. catla*. In *Ch. striatus*, 98.0, 49.0 and 38.0 kDa mass proteins were identified. The following antibacterial proteins have so far been found in skin secretions or mucus (although most of them have been poorly characterized): hydrophobic proteins with 27 and 31 kDa from carp (*C. carpio*), 45 kDa protein from eel (*Anguilla anguilla*). 65 kDa protein from rainbow trout (*O. mykiss*) and a 49 kDa protein from doctor fish (*Tinca tinca*). Presumably, these antibacterial proteins form ion channels in the bacterial membrane and kill both Gram-positive and Gram-negative bacteria (Ebran et al. 1999, 2000). Anbuchezian et al. (2011) discovered two peptides, 13.6 kDa and 13.9 kDa, from the catfish mucus. Rao et al. (2015) stated that the low molecular mass proteins in the fish mucus play a major role in bactericidal activity.

Similarly, the mucus secretion of the fresh water spiny eel (*Mastacembelus armatusis*) was proteinaceous and showed potential hemolytic activity (Uthayakumar et al. 2012). Bijalwan et al. (2017) also found an antimicrobial protein (46 kDa) in the skin and muscle homogenate of *Labeo rohita*. These results support the present findings that different AMPs may be responsible for the antibacterial activity of skin mucus and skin extracts of *C. catla* and *Ch. striatus*. Further investigation is needed to purify the specific proteins from mucus and skin extract of these fishes and to characterize them in detail including their exact mode of action.

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References

Anbuchezhian R, Gobinath C, Ravichandran S (2011) Antimicrobial peptide from the epidermal mucus of some estuarine cat fishes. World Appl Sci J 12(3):256-260.

Arulvasu CS, Babu G, Dhanasekaran G (2012) Effect of crude and partially purified epidermal mucus proteins of marine catfish *Tachysurus dussumieri* on human cancer cell line. J Acad Ind Res 1(4):164-169.

Balasubramanian S, Prakash M, Senthilraja P, Gunasekaran G (2012) Antimicrobial properties of skin mucus from four freshwater cultivable fishes (*Catla catla*, *Hypophthalmichthys molitrix*, *Labeo rohita* and *Ctenopharyngodon idella*). Afr J Microbiol Res 6(24):5110-5120.

Bauer SW, Kirby WM, Sherris JC, Thurck M (1966) Antibiotic susceptibility testing by standardized single disc method. Am J Clin Pathol 45(4):493-496.

Bijalwan K, Verma SK, Mathur A (2017) Isolation and purification of protein from skin and muscles homogenate of *Labeo rohita* (Rohu). Int J Curr Microbiol App Sci 6(8):1493-1500.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1-2):248–254.

Ebran N, Julie S, Orange N, Auperin B, Molle G (2000) Isolation and characterization of novel glycoproteins from epidermal mucus: correlation between their pore-forming properties and their antibacterial activities. Biochim Biophys Acta 1467(2):271-280.

Ebran N, Julie S, Orange N, Saglio P, Lemaitre C, Molle G (1999) Pore-forming properties and antibacterial activity of proteins extracted from epidermal mucus of fish. Comp Biochem Physiol A Mol Integr Physiol 122(2):181-189.

Elavarasi K, Ranjini S, Rajagopal T, Rameshkumar G, Ponmanickam P (2013) Bactericidal proteins of skin mucus and skin extracts from fresh water fishes, *Clarias batrachus* and *Tilapia mossambicus*. Thai J Pharm Sci 37:194-200.

Fernandes JM, Molle G, Kemp GD, Smith VJ (2004) Isolation and characterization of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Dev Comp Immunol 28(2):127-138.

Fuochi V, Li Volti G, Camiolo G, Tiralongo F, Giallongo C, Distefano A, Petronio, G, Barbagallo I, Viola M, Furneri PM, Di Rosa M (2017) Antimicrobial and antiproliferative effects of skin mucus derived from *Dasyatis pastinaca* (Linnaeus, 1758). Mar Drugs 15(11):342.

Haniffa MA, Jeyasheela P, Milton MJ (2013) *In vitro* antibacterial activity of tissue extracts from four channids against enteric pathogens. Int J Agric Technol 9(6):1437-1445.

Hellio C, Pons AM, Beaupoil C, Bourgougnon N, Gal YL (2002) Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus. Int J Antimicrob Agents 20(3):214-219.

Islam M, Hossain M, Islam S, Khondoker S, Khatun A (2014) Competitive antibacterial activity of two Indian major carps and two Chinese carps fish mucus against common pathogenic bacteria at aquaculture pond. Int J Fish Aquat Stud 2:158-162.

Kitani Y, Kikuchi N, Zhang G, Shizaki I, Shimakura K, Shiomi K, Nagashima Y (2008) Antibacterial action of L-amino acid oxidase from the skin mucus of rockfish *Sebastes schlegelii*. Comp Biochem Physiol Part B: Biochem Mol Biol 149(2):394-400.

Kumari U, Nigam AK, Mitial S, Mitial AK (2011) Antibacterial properties of the skin mucus of the freshwater fishes, *Rita rita* and *Channa punctatus*. Eur Rev Med Pharmacol Sci 15(7):781-786.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680-685.

Lalumera GM, Calamari D, Galli P, Castiglioni S, Crosa G, Fanelli R (2004) Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. Chemosphere 54(5):661-668.

Mayer AM, Hamann MT (2004) Marine pharmacology in 2000: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action.

Mar Biotechnol 6(1):37-52.

Merrifield RB, Merrifield EL, Juvvadi P, Andreu D, Boman HG (1994) Design and synthesis of antimicrobial peptides. In Antimicrobial Peptides. Ciba Foundation Symposium 186. Wiley, Chichester.

Park IY, Par CB, Kim MS, Kim SC (1998) Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. FEBS Letters 437(3):258–262.

Patil RN, Kadam JS, Ingole JR, Sathe TV, Jadhav AD (2015) Antibacterial activity of fish mucus from *Clarias batrachus* (Linn.) against selected microbes. Biolife 3(4):788-791.

Rao V, Marimuthu K, Kupusamy T, Rathinam X, Arasu MV, Al-Dhabi NA, Arockiaraj J (2015) Defense properties in the epidermal mucus of different freshwater fish species. Aquacult Aquarium Conserv Legis 8(2):184-194.

Silphaduang U, Colorni A, Noga EJ (2006) Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish. Dis Aquat Organ 72(3):241-252.

Smith VJ, Desbois AP, Dyrynda EA (2010) Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. Mar Drugs 8(4):1213-1262.

Subramanian S, Ross NW, MacKinnon SL (2008) Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comp Biochem Physiol B Biochem Mol Biol 150(1):85-92.

Uthayakumar V, Ramasubramanian V, Senthilkumar D, Priyadarisini VB, Harikrishnan R (2012) Biochemical characterization, antimicrobial and hemolytic studies on skin mucus of fresh water spiny eel *Mastacembelus armatus*. Asian Pac J Trop Biomed 2(2):S863-869.

Wei OY, Xavier R, Marimuthu K (2010) Screening of antibacterial activity of mucus extract of Snakehead fish, *Channa striatus* (Bloch). Eur Rev Med Pharmacol Sci 14(8):675-681.

Ying-xia Z, Aihui Z, Riga M, Yong-can Z, Shifeng W (2008) Purification and antimicrobial activity of antimicrobial protein from brown-spotted grouper, *Epinephelus fario*. Zool Res 29(6):627-632.

Zuchelkowski EM, Lantz RC, Hinton DE (1981) Effects of acid-stress on epidermal mucous cells of the brown bullhead *Ictalurus nebulosus* (LeSeur): A morphometric study. Anat Rec 200(1):33-39.