



Research Paper

Mucous cell responses in branchial rakers of *Astyanax altiparane* (Characidae) after exposure to low concentrations of LAS-based detergents

Accepted 8th April, 2018

ABSTRACT

Most water bodies worldwide are polluted, predominantly by wastewater containing detergents of both domestic and industrial origins. Studies have shown that exposure to detergent can cause histochemical and even physiological changes in an animal's body, especially in gills, skin and liver of fish and, occasionally leading to death. Gill rakers are present in the gill arch and along with it, play an important role in food selection and in the protection of gills, preventing lesions caused by solid particles. Although a lot is known about the effects of pollutants in gills, very little is investigated in regard to gill rakers. By virtue of the role gill rakers play in fish health, this study aimed to identify possible histochemical changes in the first left gill arch of *Astyanax altiparane* (Lambeth) caused by exposure to 01 ppm (01 mg/L) of biodegradable detergents diluted in water for one and five months. The first month of exposure resulted in hyperplasia of mucous cells, but with low mucous secretion on gill rakers. After five months of experiment, the number of mucous cells decreased due to hypersecretion of mucous. The mucous also plays a role in the defense against toxic agents and assists ionic balance mechanisms in fish.

Fernanda Passarini Lopes¹, Bruno Fiorelini Pereira^{2*}, Rebeca Mamede da Silva Alves¹, Dimitrius Leonado Pitol³, Priscilla Hakime Scalize³ and Flavio Henrique Caetano¹

¹Instituto de Biociência, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP - Campus de Rio Claro - SP - Brazil.

²Centro de Ciências Biológicas e da Saúde, Universidade Federal do Oeste da Bahia-UFOB, Campus Barreiras, Barreiras, Brasil.

³Faculdade de Odontologia de Ribeirão Preto - FORP/USP, Brazil.

*Corresponding author. E-mail: brunofp22@hotmail.com.

Key words: Gill raker, hyperplasia of mucous cells, hypersecretion, defense, osmoregulation.

INTRODUCTION

Gill rakers are organs present in the ventral region of gill arches (Rodrigues and Bemvenuti, 2001; Sampaio and Goulart, 2011), whose main function is to capture and select food (Oliveira Ribeiro and Menin, 1996; Sergipense et al., 1999; Sampaio and Goulart, 2011). However, in anastomid fish, gill rakers do not play their common roles, such as protection of arches, gill filaments and primary lamellae against injuries caused by solid elements (Oliveira Ribeiro and Menin, 1996; Machado, 1999; Rodrigues and Menin, 2006). In this case, it is the mucous secreted by specialized cells present in the entire body that perform the different functions rather than gill rakers themselves (Fonseca Neto and Spach, 1999; Machado, 1999; Eiras-Stofella and Charvet-Almeida, 2000; Kumari et al., 2005). The mucous, therefore, acts as a protective cover (Eiras-Stofella and Charvet-Almeida, 1998).

Mucous cells have neutral or slightly acidic glycoproteins, indicative of some acidity control of mucous cells over their secretion (Lima et al., 2009), which occurs according to the viscosity grade (Paulino et al., 2012). The mucous protects the organs in which they reside, being gills or gill rakers, of pathogens, solid particles and toxic substances, and acts in osmoregulation (Misra et al., 1987; Saboia-Moraes et al., 1996; Machado, 1999; Araújo et al., 2001; Lima et al., 2009; Duncan et al., 2010; Saboia-Moraes et al., 2011) in a process of continuous renewal (Hara and Thompson, 1978).

The neutral mucous, which is composed of sugars and sialic acids, serves to protect against possible injury due to the passage of solid particles. Acid mucous, on the other hand, which is composed of sialomucins and sulphomucins, while exerting the same protection function performed by

neutral mucus, is also responsible for the retention of captured food (Duncan et al., 2010; Paulino et al., 2012). However, Saboia-Moraes et al. (2011) point out that neutral mucous usually assists in chemical protection, while acid mucous acts against mechanical abrasion.

Detergents are chemical agents whose toxicity increases according to the chain length (Brown et al., 1968). Few studies address toxicity values of biodegradable detergents. According to Roy (1988a), LD50 for various groups of fish varies between 7 and 22.5 ppm in a vast majority of studies of fish liver and gills.

Given the importance of gill rakers in the capture and selection of food and also in the defense of gills, this study aimed to detect histological changes in response to exposure to low concentrations of biodegradable detergents, keeping in mind that river pollution occurs with low doses.

MATERIALS AND METHODS

This project was approved by the local ethics committee. Sixty individuals of the species *Astyanax altiparanae* in the same stage of development were obtained from the ICMBio / CEPTA - Instituto Chico Mendes (Pirassununga, SP), originally sampled in Mogi Guaçu. The fish were kept in two polyethylene tanks of 500 L capacity inside a greenhouse and fed with the same feed provided in ICMBio / CEPTA - Mendes Institute.

Half of the fish composed the control group (CG), that was kept in water from the artesian well on *campus* (UNESP Rio Claro - São Paulo, Brazil - 22 ° 24 '36 "S, 47 ° 33'36" W). Such water is treated with chlorine before becoming available for use in obedience to the legislation by regional authorities (SABESP, 2012), but it was recirculated to reduce chlorine levels prior to experimentation (Pereira et al., 2014). The remaining fish comprised the detergent group (DG) and were kept in a dilution of a mixture of ten brands of commercial biodegradable detergents in the same artesian well water, in a 1: 1000000 ratio.

Water from both groups was analyzed according to the Standard Methods for the Examination of Water and Waste water (Apha, 2005). The parameters are listed in Table 1.

Exposure time was set to five months with two sampling campaigns, one after thirty days from the beginning of experimentation and one after the fifth month. Fish were anaesthetized with benzocaine solution before each sampling campaign (0.1 g of benzocaine in 1 ml of ethyl alcohol for each 100 mL of deionized water) to ease the suffering during the dissection process.

Light microscopy

For histological analyses, 24 subjects were sacrificed, 6 from each group in every sampling campaign. The first left

Table 1: Chemical parameters of water analysis from the control and detergent groups.

Parameter	Control group	Detergent group
HCO ₃ ⁻ (mg/L)	1.2	4.9
Li ⁺ (mg/L)	<0.01	<0.01
Na ⁺ (mg/L)	17.5	20.1
NH ₄ ⁺ (mg/L)	10.7	2.15
K ⁺ (mg/L)	6.39	6.68
ClO ₂ ⁻ (mg/L)	<0.01	<0.01
F ⁻ (mg/L)	0.25	0.23
CL ⁻ (mg/L)	8.56	5.56
NO ₂ ⁻ (mg/L)	<0.04	1.15
NO ₃ ⁻ (mg/L)	121	106
PO ₄ ³⁻ (mg/L)	18.9	9.33
SO ₄ ²⁻ (mg/L)	11.6	10.4
Acetato (mg/L)	<0.10	<0.10
C ₂ O ₄ ²⁻ (mg/L)	<0.03	<0.03
Al (mg/L)	0.10	0.079
Ba (mg/L)	0.075	0.092
Ca (mg/L)	18.6	17.8
Cd (mg/L)	<0.010	<0.010
Co (mg/L)	<0.010	<0.010
Cr (mg/L)	<0.010	<0.010
Cu (mg/L)	<0.010	0.010
Fe (mg/L)	0.021	0.029
Mg (mg/L)	3.69	4.55
Mn (mg/L)	0.27	0.21
Ni (mg/L)	<0.010	0.066
P (mg/L)	6.79	3.46
Pb (mg/L)	<0.020	<0.020
Si (mg/L)	5.72	6.30
Sr (mg/L)	0.12	0.12
Zn (mg/L)	0.84	0.50
Temperature (°C)	15.6	15.4
Conductibility (µS/cm)	282	237
pH	5.39	6.04
LAS concentration (mg/L)	0	0.375

Note: Analyses are based on the standard methods for the examination of water and wastewater, 21st edition, 2005.

gill arches of all animals were removed and fragments were fixed in aqueous Bouin, paraformaldehyde 0.4% and neutral formalin. The material was stained with PAS, Alcian Blue (AB) pH 1.0 and 2.5 simultaneously and separately, for the detection of mucous cells according to Beçak and Paulete (1976), and Von Kossa, for the visualization of mitochondria-rich cells according to Pereira and Caetano (2009).

Mucous cells that reacted to the PAS technique, AB pH = 2.5, PAS/AB pH = 2.5/AB pH 1.0 and AB pH = 1.0, were

Table 2: Means and standard deviation of the four types of mucous cells for each group at one month of experiment and statistical results (p) of the Mann-Whitney test for CGs and DGs. Note significant differences for cell types 2 and 4 between groups.

Type	Control Group	Detergent Group	Statistical results (p)
	1 month	1 month	
Type 1 Mucous cell [PAS ⁺]	M= 31.1 SD= 14.8	M= 39.5 SD= 19.9	ns
Type 2 Mucous cell [AB ⁺ (pH= 2.5)]	M= 9.4 SD= 7.8	M= 18.6 SD= 3.7	< 0.05
Type 3 Mucous cell [PAS/AB ⁺ (pH= 1.0)/AB ⁺ (pH= 2.5)]	M= 8.6 SD= 9.3	M= 28.5 SD= 17.2	< 0.05
Type 4 Mucous cell [AB ⁺ (pH= 1.0)]	M= 10.7 SD= 11.1	M= 16.3 SD= 8.6	ns

Notes: SD= standard deviation, M= mean, ns= non-significant.

respectively named type 1, 2, 3 and 4, according to Paulino et al. (2012).

Statistical analysis

Mucous cells present in 10 gill rakers of approximately the same size were considered for statistical analyses. Average count scores of the above cells from control and detergent groups were compared with the F test for homogeneity of variance (Berquó, 1981; Zar, 1999). Normality was verified by the Shapiro-Wilk test and, since no group passed the test, the Mann-Whitney test was used to compare the differences between groups (Berquó, 1981; Zar, 1999; Ayres, 2011). Analyzes were run in the BioEstat 5 software (Ayres et al., 2007).

RESULTS

Thirty-day analysis

The Mann-Whitney test found no significant changes in mucous cell number for types 1 and 4 ($p > 0.05$). However, types 2 and 3 proliferated in DGs ($p < 0.05$) (Table 2) (Figure 1) as compared with CGs (Figure 2). Mucous cells present in gill rakers of the DG fish also reacted to the Von Kossa technique (Figures 3 and 4).

Five-month analysis

Mucous cell counts of types 2, 3 and 4 showed no significant difference between groups ($p < 0.05$), unlike type 1, which suffered a reduction in DGs as compared with CGs ($p < 0.05$)

according to the Mann-Whitney test (Table 3). Also, mucous cells present in the rakers of DG fish reacted to Von Kossa (Figures 5 and 6), including cells from the distal region of rakers during the process of content release (Figure 6). Mucous cells in the gill rakers of subjects from the CG did not respond to Von Kossa (Figure 7); however, no mitochondria-rich cells were visualized with this technique either, in both experimental groups. The decrease in mucous cell number (Table 3) in the DG can be directly observed as compared with CGs fish due to mucous hypersecretion (Figures 8 and 9, 10).

DISCUSSION

No significant changes in mucous cell numbers were found with the Mann-Whitney test for types 1 and 4 in the first month of the experiment ($p > 0.05$), meanwhile types 2 and 3 proliferated in the DG ($p < 0.05$) (Table 2). In the fifth month of experiment, no significant differences between groups were found for types 2, 3 and 4 ($p > 0.05$), unlike type 1 mucous cells, which reduced in number in DGs in relation to CGs ($p < 0.05$) (Table 3).

Roy (1988b) found different results in the operculum, arch and gill filament epithelium of *Rita rita* exposed to SDS detergent: stressed fish secreted mainly neutral mucous, while those free of stress secreted acid mucus. However, Saboia-Moraes et al. (2011) warn that when comparing the effects of pollutants on gills and gill rakers, they may react differently to the same stimulus; for example, gill rakers are less sensitive to salinity variations as compared with gills. Thus, gill raker responses to stress caused by detergent exposure could be acid mucous production instead of neutral mucous as reported for gills of *Rita rita* (Roy, 1988b).

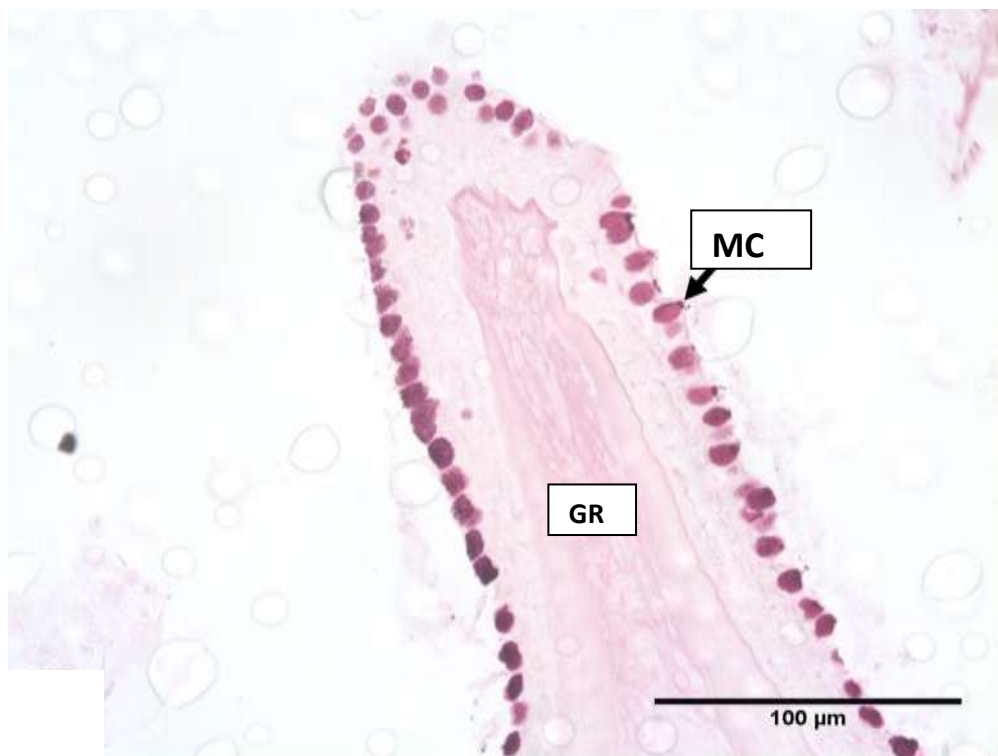


Figure 1: Gill rakers of *A. altiparanae* after 30 days of experimentation. Mucous cells (MC) scattered on the gill raker surface (GR) from the detergent group. TB: taste bud. PAS technique. Scale bar = 100 μm.

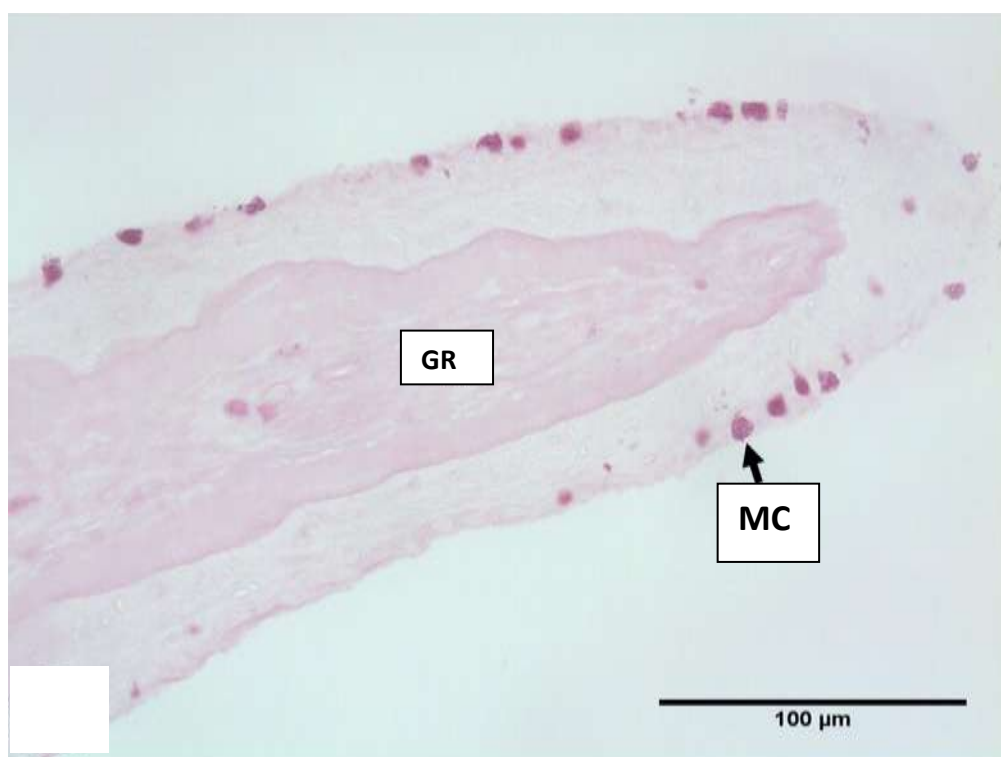


Figure 2: Gill rakers of *A. altiparanae* after 30 days of experimentation. Fewer mucous cells positively reacting to PAS in the gill raker surface (GR) from the control group. Scale bar = 100 μm.

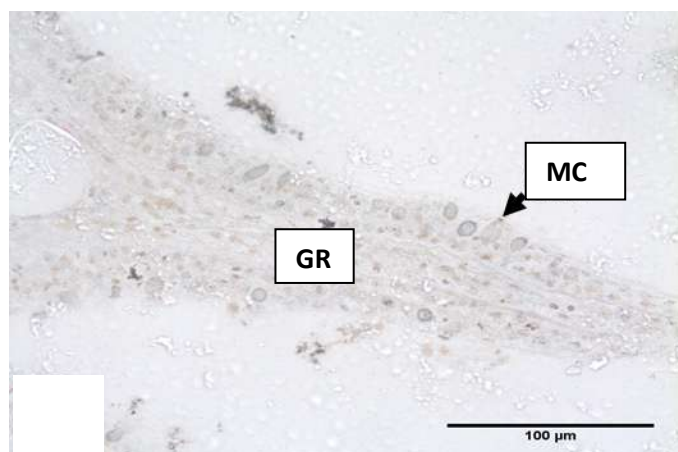


Figure 3: Gill rakers of *A. altiparanae* after 30 days of experimentation. Mucous cells (MC) in gill raker (GR) of the detergent group that reacted to the Von Kossa technique. Scale bar = 100 μ m.

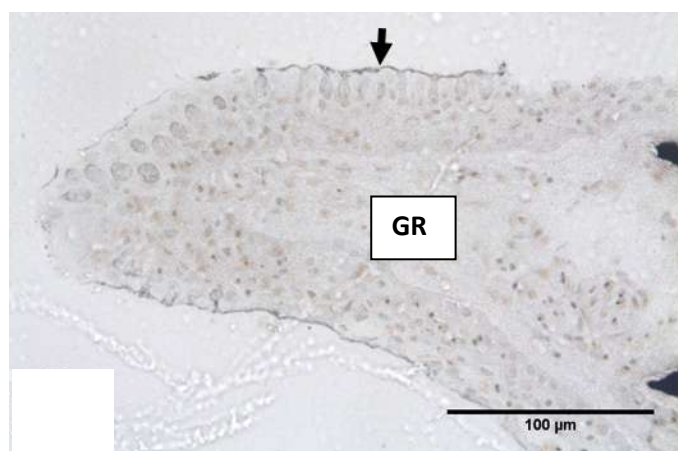


Figure 4: Gill rakers of *A. altiparanae* after 30 days of experimentation. Mucous cells releasing content (arrow). Von Kossa technique. GR: gill raker. Scale bar = 100 μ m.

Table 3: Means and standard deviation of the four types of mucous cells for each group at five months of experiment and statistical results (p) of the Mann-Whitney test for CGs and DGs. Note significant differences for cell type 1 between groups.

Type	Control group	Detergent group	Statistical results (p)
	5 months	5 months	
Type 1	M= 24.8	M= 11.0	< 0.05
Mucous cell [PAS ⁺]	SD= 10.8	SD= 5.6	
Type 2	M= 14.2	M= 6.2	ns
Mucous cell [AB ⁺ (pH= 2.5)]	SD= 12.8	SD= 5.3	
Type 3	M= 20.8	M= 11.8	ns
Mucous cell [PAS/AB ⁺ (pH= 1.0)/AB ⁺ (pH= 2.5)]	SD= 6.9	SD= 12.4	
Type 4	M= 14.1	M= 6.1	ns
Mucous cell [AB ⁺ (pH= 1.0)]	SD= 7.6	SD= 3.0	

Notes: SD= standard deviation, M= mean, ns= non-significant.

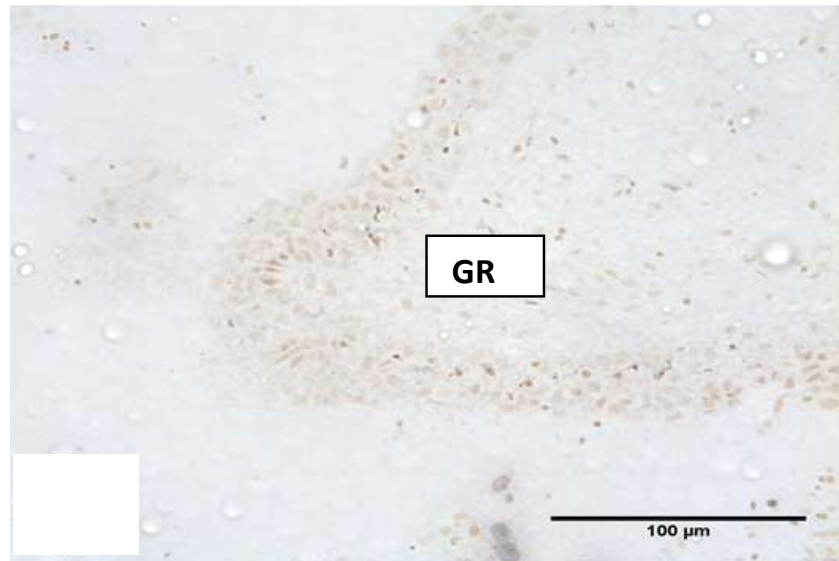


Figure 5: Gill rakers of *A. altiparanae* after 30 days of experimentation. Non-reactive gill raker from the control group. Von Kossa technique. GR: gill raker. Scale bar = 100 μm.

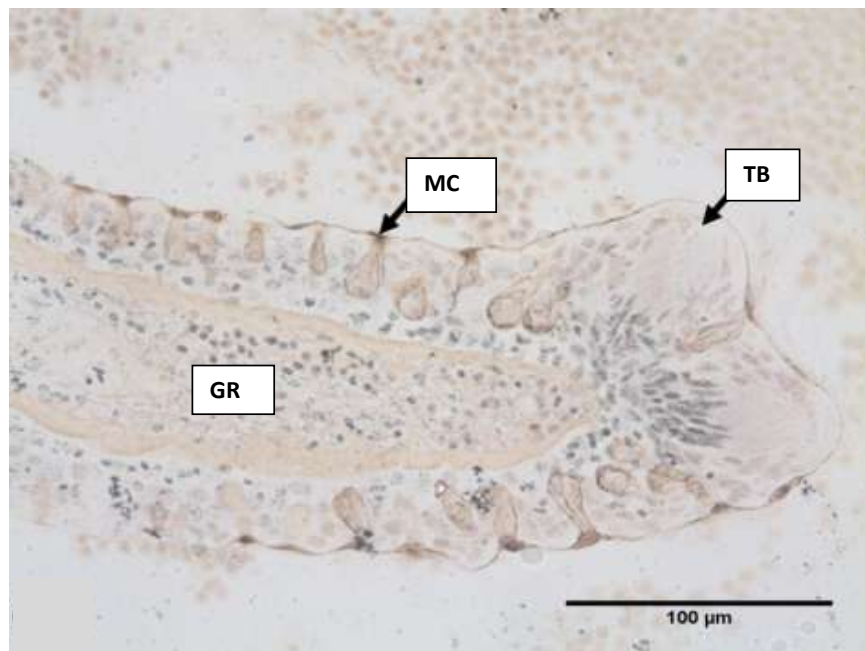


Figure 6: Gill rakers of *A. altiparanae* after 5 months of experimentation. Non-reactive gill raker from the control group. Von Kossa technique. TB: taste bud. Scale bar = 100 μm.

Duncan et al. (2010) point out that the viscosity of the mucous glycoconjugates determines its role in the body when released. In having a lower viscosity, neutral glycoconjugates are important to protect and lubricate the body against injury, whilst acid mucous, presenting a

higher viscosity, has the role of fixing small organic and inorganic material and defending the body against injury and pathogen proliferation (Duncan et al., 2010).

Accordingly, when gill rakers came into contact with detergent in the first 30 days, many of the acid mucous cells

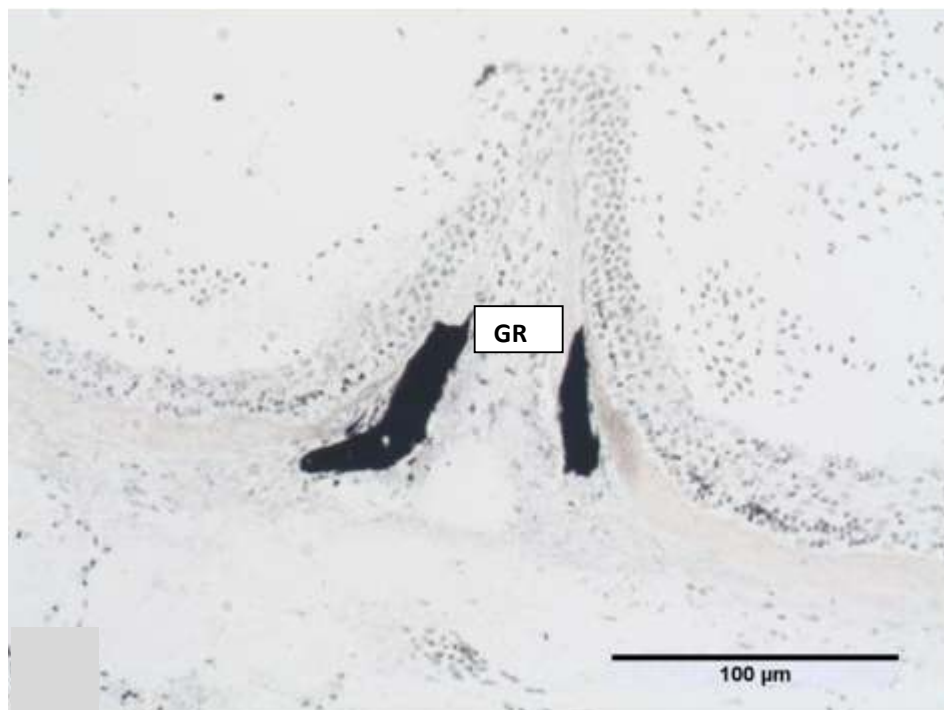


Figure 7: Gill rakers of *A. altiparanae* after 5 months of experimentation. Mucous cells releasing content, as can be observed on the borders (light brown) of the gill raker (GR) apex. Detergent group. Von Kossa technique. Scale bar = 100 μm.

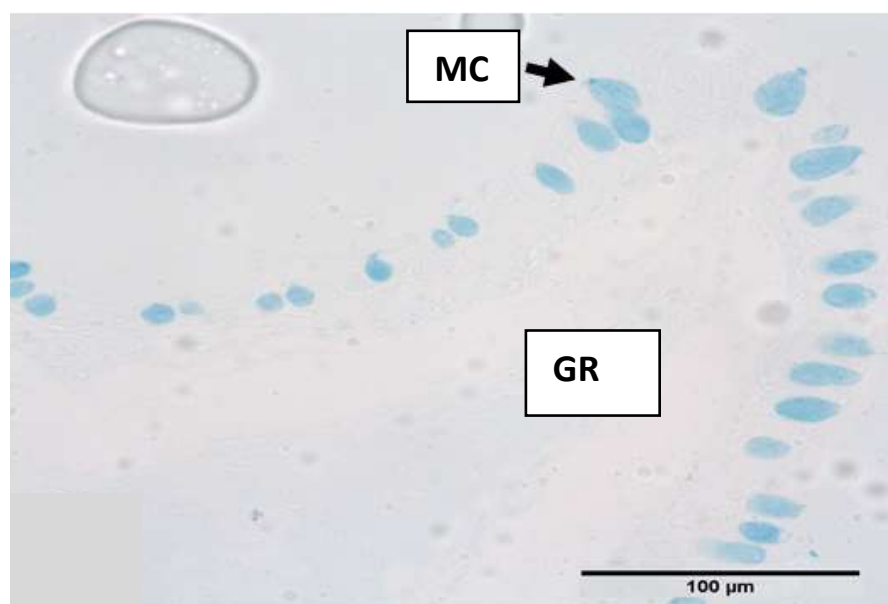


Figure 8: Gill rakers of *A. altiparanae* after 5 months of experimentation. Mucous cells (MC) in gill raker (GR) of the control group. Alcian Blue technique pH= 1,0. Scale bar = 100 μm.

were produced in an attempt to protect the animal from any damage caused by the contaminant toxicity. As the body remained in contact with the chemical agent, at the

end of five months, the neutral mucous cell production dropped, possibly due to its wide use in the protection against potential injuries caused by the detergent.

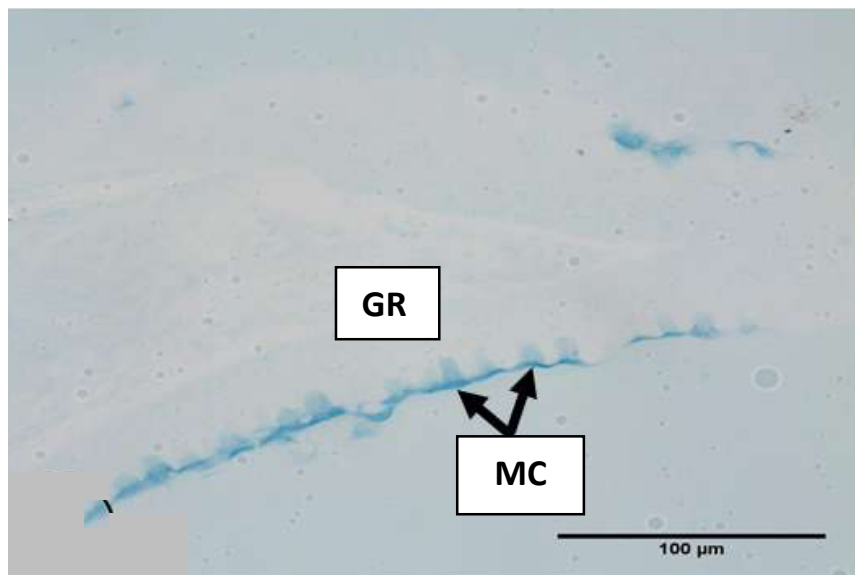


Figure 9: Gill rakers of *A. altiparanae* after 5 months of experimentation. Fewer mucous cells (MC) in gill raker (GR) of the detergent group, some releasing their content (blue borders). Alcian Blue technique pH= 1,0. Scale bar = 100 μm.

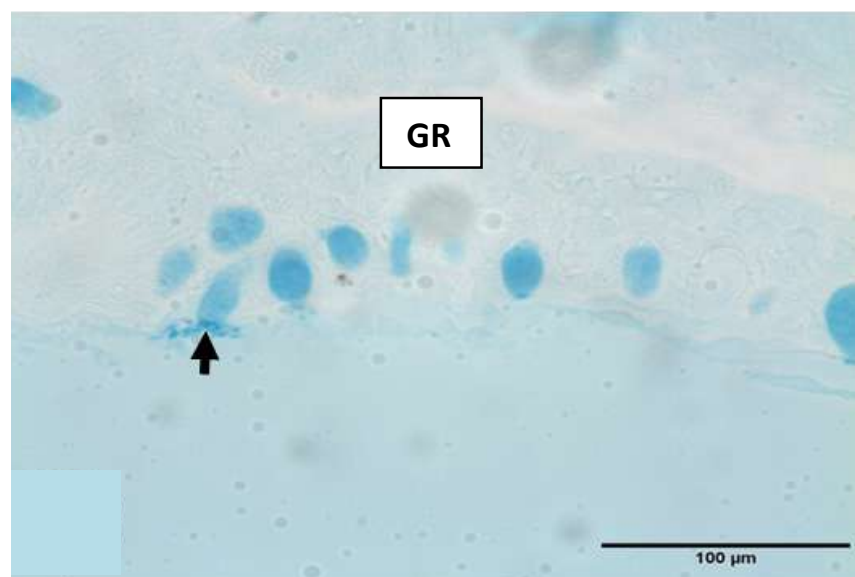


Figure 10: Gill rakers of *A. altiparanae* after 5 months of experimentation. Detail of a mucous cell releasing its content (arrow). Alcian Blue technique pH= 2,5. GR: gill raker. Scale bar = 100 μm.

One month of exposure also caused higher mucous cell production in gill rakers of DG fish as compared with CG fish (considering the sum of average mucous cell numbers of all types in gill rakers, $M_{CG} = 59.2$ and $M_{DG} = 102.9$), accompanied by low mucous secretion. However, five months exposure caused an increase in the release of mucous in DG fish and a decrease in the number of mucous cells as compared with CGs ($M_{CG} = 73.9$ and $M_{DG} = 35.1$).

Such an increase in mucous cell number at the start of experimentation, followed by a decrease due to hypersecretion, has also been observed in studies by Roy (1988b), Misra et al. (1985) and Venhuis and Mehrvar (2004) in response to detergent exposure. In the latter case, Venhuis and Mehrvar (2004) found that fish exposed to 0.02 -1 mg/L of LAS-based detergent showed mucous hypersecretion, which often hindered gas exchanges.

The role of mucous in the ionic balance between water and blood plasma is an important aspect of the mucous cell's function observed in the present study and denoted by these cells reaction to the Von Kossa technique. Other than contributing to food retention and protecting squamous cells, mucous may also assist in the diffusion of water and ions (Fonseca Neto and Spach, 1999) because its glycoprotein's electric charge attract ions and cations from the environment to be absorbed by mitochondria-rich cells (Pauline et al., 2012). Such an attraction generates an ionic gradient on the body surface which helps protect the respective organ (Paulino et al., 2012), in this study's case, gill rakers.

ACKNOWLEDGMENTS

The authors are thankful to FAPESP, process number: 2009/17118-9, for their financial support and to ICMBio/CEPTA—Instituto Chico Mendes for providing the specimens used in these experiments.

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