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Ultramorphological changes in gill rakers of *Astyanax altiparanae* (Characidae) kept in contaminated environments

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Abstract Most water bodies in Brazil, and in the world, are contaminated by some types of pollutants, ranging from sewage to metal/chemicals, carcinogenic products, and biodegradable detergents. Despite the extensive knowledge on their effects on fish biology and especially on gill morphology, research that concerns their impacts on gill rakers and implications in parameters such as food consumption cannot be found in the literature. Gill rakers are vital because, together with gills, they are responsible for the defense and protection of the organism and for selecting appropriate food for survival. When detergents, which can act as toxic chemical agents, get in contact with the body of the fish, they can cause severe effects that must be understood. Therefore, our study investigated ultramorphological changes in gill rakers of *Astyanax altiparanae* (Lambeth) caused by the exposure to biodegradable detergents. Fish were exposed to a 1 mg/L dilution of a mixture of detergents and pure water from an artesian well for 5 months. Results revealed that the first month of exposure to detergent caused dilation of chemical receptors in taste buds and the rise of a large number of orifices for mucus release among pavement cells in gill rakers, although only a small amount of mucus was found in fish exposed both to pure water and the detergent dilution. After 5 months, there was an increase in the dilation of these chemoreceptors, excess mucus on gill rakers of detergent groups, and the emergence of microbridges between microridges in pavement cells.

Keywords Mucus · Chemical receptors · Taste buds · Microbridges

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Introduction

Gill rakers are organs located in the ventral region of the right and left gill arches (Rodrigues and Bemvenuti 2001), oriented toward the pharyngeal cavity (Machado 1999). They consist of cartilaginous tissue surrounded by a multi-stratified epithelium of polygonal pavement cells, taste buds, mucous cells, mitochondria-rich cells (Fiuza et al. 2011; Fonseca Neto and Spach 1999; Kumari et al. 2005), and claviform cells. This epithelium also exhibits structures called spines (Lopes and Sampaio 2002; Sergipensel et al. 1999; Silva and Hahn 2009), or ctenii (Serra and Langeani 2006), or

denticles (Jardim 1988; Rodrigues and Menin 2006). Most fish species possess gill rakers, but some families, such as *Oligoplites perugiae* (Eiras-Stofella 2000) and *Xiphias gladius* (Vaske Jr and Castello 1998), do not. Gill rakers are a site of food retention for later ingestion (Abelha 2001; de Oliveira Ribeiro and Menin 1996; Lopes and Sampaio 2002; Sergipensel et al. 1999), preventing its escape and facilitating swallowing (de Moraes and de Freitas Bárbola 1995; de Oliveira Ribeiro and Menin 1996; Rodrigues and Menin 2006).

On the other hand, in anostomids, this organ does not have the function of retaining food. Instead, it protects gill arches, filaments, and primary lamellae. Such function is found in some species of planktonic, benthic, carnivorous (Santos 1982), and herbivorous fish (Ferretti et al. 1996), contributing to a better performance and protection of primary lamellae and gill filaments against solid elements (de Oliveira Ribeiro and Menin 1996; Machado 1999). However, if the element is soluble in water, such as detergents, this protection becomes ineffective (Machado 1999). This can happen probably because substances with detergent proprieties will interfere on the cytoplasmic membrane and consequently cause problems with ion balance and the protection of the gill.

Gill analyses, on the other hand, usually reveal morphological changes such as edemas, epithelial hyperplasia in secondary lamellae, infiltration of epithelial cells, lamellar fusion, and proliferation and hypersecretion of mucus cells and mitochondria-rich cells (Mallatt 1985). These alterations can be classified according to their nature and be related to circulatory disturbances, including hemorrhage, hyperemia, and aneurism; regressive changes, including structural changes such as plasmic alterations, the formation of cytoplasmic deposits, nuclear alterations, atrophy and necrosis; progressive changes, including hypertrophy and hyperplasia; inflammation responses, including the formation of exudate, endoplasmic reticulum activation and infiltration; and the formation of tumors (Bernet et al. 1999).

In comparison to gills, which are the most used bioindicators in fish, gill rakes can provide information directly related to food metabolism and point to possibly grave effects on a fish's feeding behavior and ecological status, which cannot be inferred through gill analyses, considering the types of morphological alterations they undergo and their nature. Thus, it constitutes an important complementary technique to assess the effects of aquatic contaminants.

Currently used detergents have been criticized due to contamination of rivers and lakes (Kumari et al. 2005; Misra et al. 1985; Mungray and Kumar 2009; Venhuis and Mehrvar 2004). The most widely used anionic surfactants in the market are LAS-based detergents (linear alkylbenzene sulphonate-based detergents), which use linear alkylbenzenesulfonate as their main component (Mungray and Kumar 2009). Several authors have explored the toxic effects and dosages of LAS in various

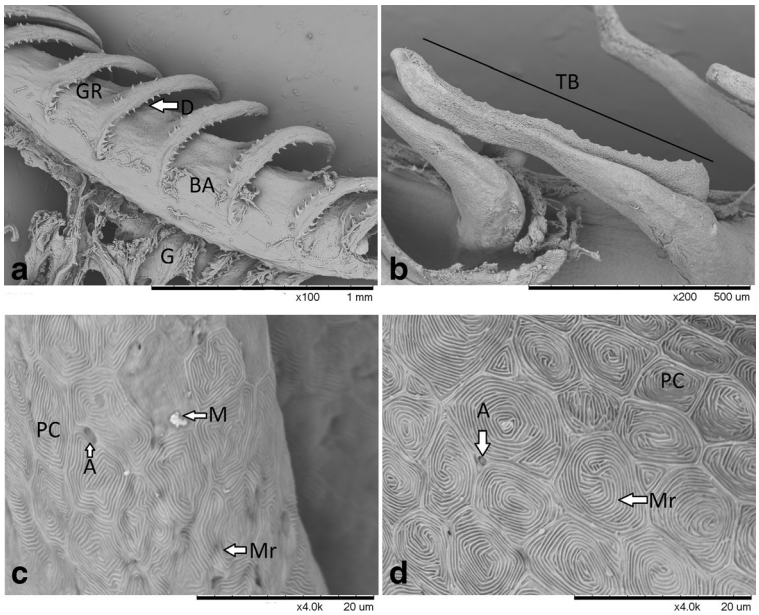

Fig. 1 Gill raker ultramorphology of *Astyanax altiparanae* after 30 days of experiment. **a** Overview of gill rakers (GR) showing denticles (D) arranged on the sides—control group. *BA* gill arch; *G* gill. **b** Overview of taste buds (TB) aligned in the ventral region of gill rakers—detergent group. **c** Overview of gill raker epidermis showing orifices for mucus release (A)—control group. *PC* pavement cell, *M* mucus, *Mr* microridge. **d** Detail of microridges (Mr) on the pavement cell's surfaces (PC)—detergent group. *A* orifice for mucus release

types of water and effluents (Mungray and Kumar 2009). Gills would be the first organ to be affected by the LAS as it is the first one to be in contact with the pollutants in the water.

The most common critiques are related to their biodegradation toxicity (Misra et al. 1985; Mungray and Kumar 2009; Venhuis and Mehrvar 2004) and high phosphate content (≤ 50%) (Allinger 1978). The latter

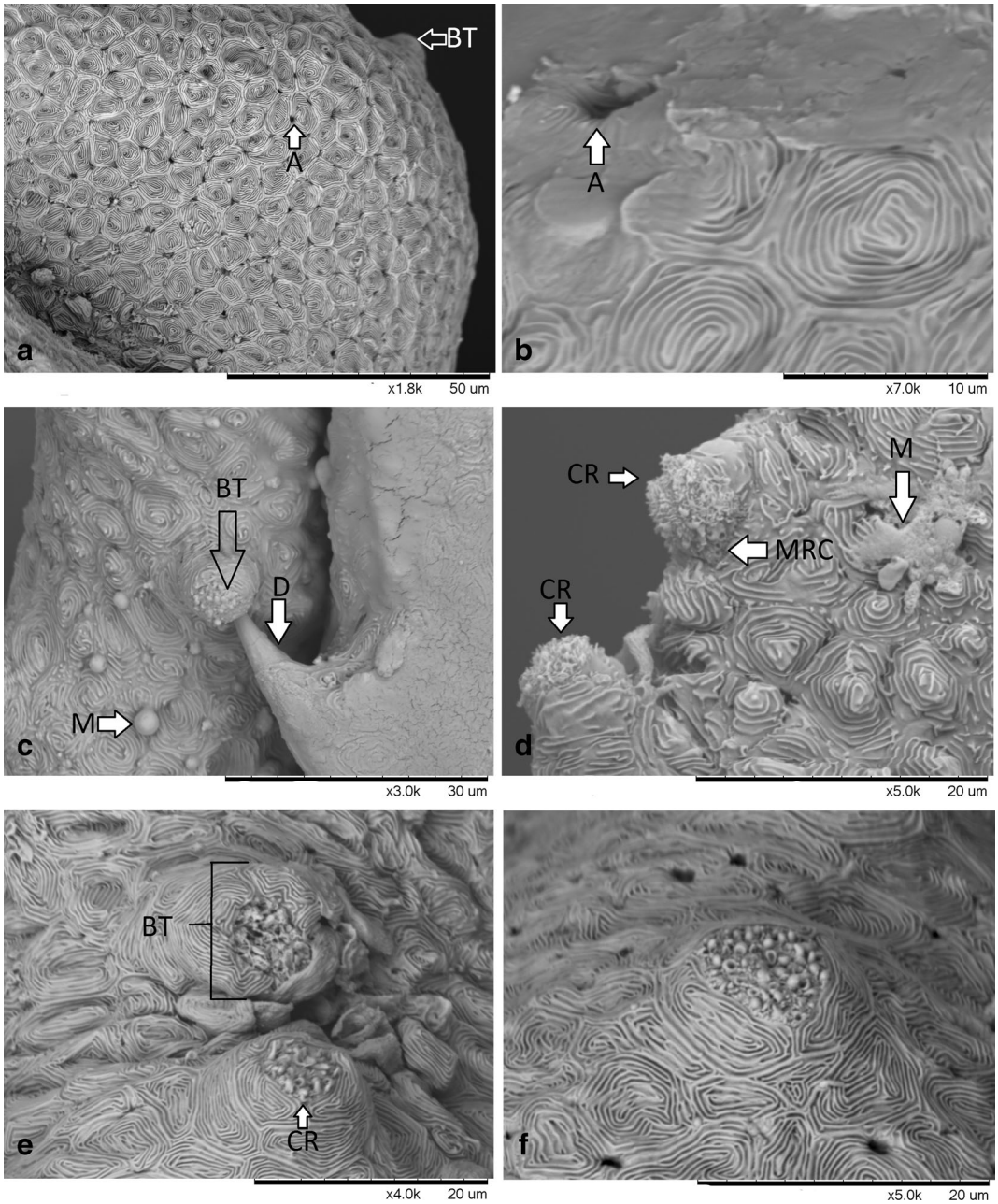

Fig. 2 Ultramorphology of the gill raker epithelium of *Astyanax altiparanae* after 30 days of experiment. **a** Overview of orifice for mucus release among pavement cells in the ventral region of gill rakers—detergent group. **b** Detail of an orifice for mucus release (A)—control group. **c** Mucus droplets (M) leaving the orifices in the gill raker epithelium—detergent group. *TB* taste buds, *D* denticles. **d** Chemical receptors (CR) in taste buds of the gill raker—control group. *MRC* mitochondria-rich cell, *M* mucus. **e** Apical pores of taste buds (TB) filled with chemical receptors (CR)—detergent group. **f** Detail of dilated chemical receptors—detergent group

have contributed to the increase in both growth velocity and reproduction of algae and weeds in a process known as eutrophication, which diminishes oxygen content in the water, causing fish mortality (Allinger 1978).

Our study aimed to identify possible changes in gill raker ultramorphology of *Astyanax altiparanae* exposed to detergent and contribute to the growth of studies in this area. The gill raker was chosen instead of the gill because as the gill, it is directly in contact with the pollutants in the water, but has a different physiological function and gills responses to surfactants and others pollutants are already well known. With this work, we aimed to not only show different physiological effects caused by pollutants, but also present a potential new biomarker (gill rakers).

Material and methods

For this experiment, 60 individuals were kept in two polyethylene tanks with 500-L capacity each. The animals were fed with the same diet used at CEPTA - Chico Mendes Institute, location where the animals were collected. Temperature and oxygen levels were controlled by a system of recirculated water in each box to make sure only the water remained as a difference between the groups. Each experimental group had 50% males and 50% females, both sexes with 6-month-old individuals; mean body size was 6 cm. Half were treated with clean water collected in a well, located at UNESP (Rio Claro - São Paulo, Brazil) and named as the control group (CG). The other half was treated with the same water used in the control group, but with the addition of 1 mg/L of a mixture of ten brands of commercial biodegradable detergents composed basically of LAS added in the water at the beginning of the experiment, and was referred to as detergent group (DG), using a semi-static exposure system. Other than the active surfactant principle, detergents were also composed of glycerin, coadjuvants, conservatives, dye, and sequesters. The water was replaced every 15 days in both groups, and the equivalent volume of detergent was included to maintain pollutant concentration. The study was approved by the Ethics Committee of Uniararas, Araras, São Paulo, Brazil (protocol number 646/2009). Water samples from both groups were analyzed as described below, at the beginning of the experiment, and on each time that the fish were sampled. Analyses were performed by the Laboratory of Water Analysis of the Department of Applied Geology at the Institute of Geosciences and Exact Sciences – UNESP Rio Claro, following the norms of the Standard Methods for the Examination of Water and Wastewater (American

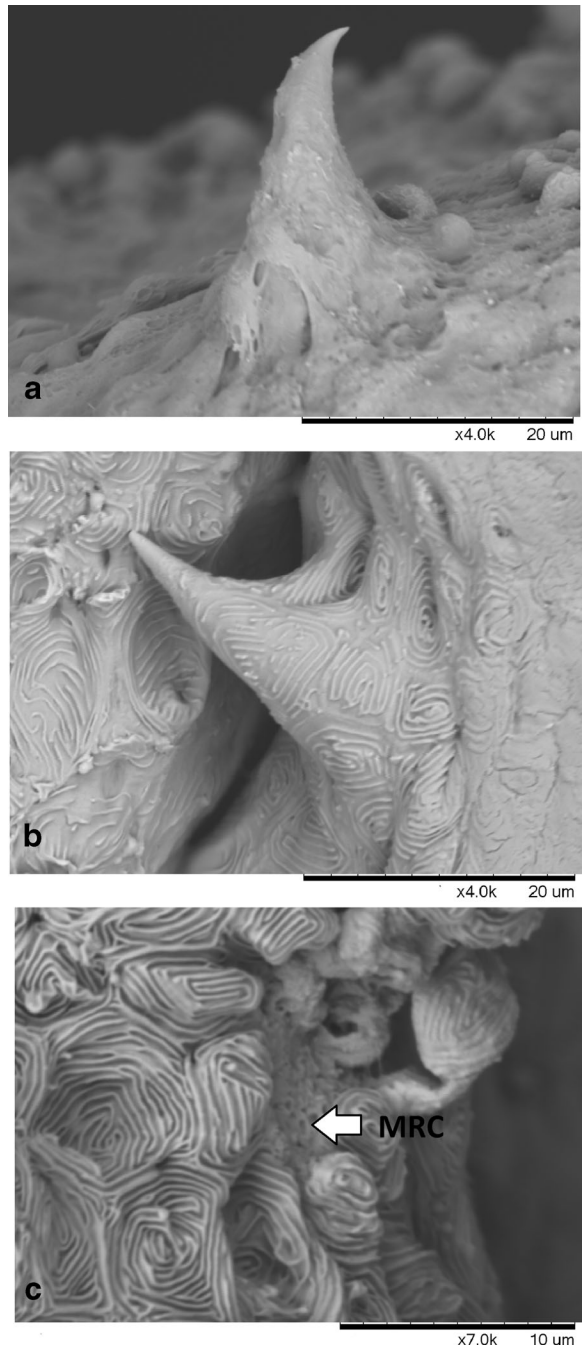

Fig. 3 Ultramorphology of denticles and mitochondria-rich cells in gill rakers of *Astyanax altiparanae* after 30 days of experiment. **a** Denticle covered by mucus—control group. **b** Detail of a denticle in the gill raker—detergent group. **c** Mitochondria-rich cell (MRC) on the gill raker surface—detergent group

Public Health et al. 2005), regarding the following parameters:

Metals for the following elements were determined by ICP-AES (equipment precision guaranteed in concentrations between 1 and 10 ppb): Ba, Ca, Cd, Co, Cr (t), Cu, Fe, Mg, Mn, Ni, P (t), Pb, Si, Sr, and Zn. The anions Cl^-, ClO_2^-, F^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, acetate, and oxalate and further cations K^+, Li^+, Na^+, and NH_4^+ were determined by ion chromatography. The analysis of pH, conductivity, total alkalinity, and carbonate was performed by potentiometric titration (Pereira et al. 2014). Liquid chromatography was performed to determine real LAS concentration using an Agilent Technologies 1200 series HPLC in collaboration with a private company (Global Análise & Consultoria©, São Carlos, SP, Brazil). In Alves et al. (2016) can be found the further information on the samples used for LAS analyses (Alves et al. 2016). The equipment used in such analyses can determine pollutant concentrations with precision in solutions with concentrations higher than 0.002%.

Exposure to the pollutant had a total duration of 5 months. During this period, there were two sampling campaigns, one at 30 days after the beginning of the experiment and another one after 5 months.

For ultramorphological analyses, 24 fishes were sacrificed, 6 from each group in every sampling campaign. The first left gill arch of every fish was removed and fixed in Karnovisky. Samples were then dehydrated in acetone (in a sequence ranging from 50% acetone up to 100% with 5 min, baths and two baths in pure acetone), glued on to stubs, metalized with gold, and observed in a Hitachi TM3000 scanning electron microscope (Electron Microscopy Center of the Institute of Biosciences of Rio Claro - UNESP).

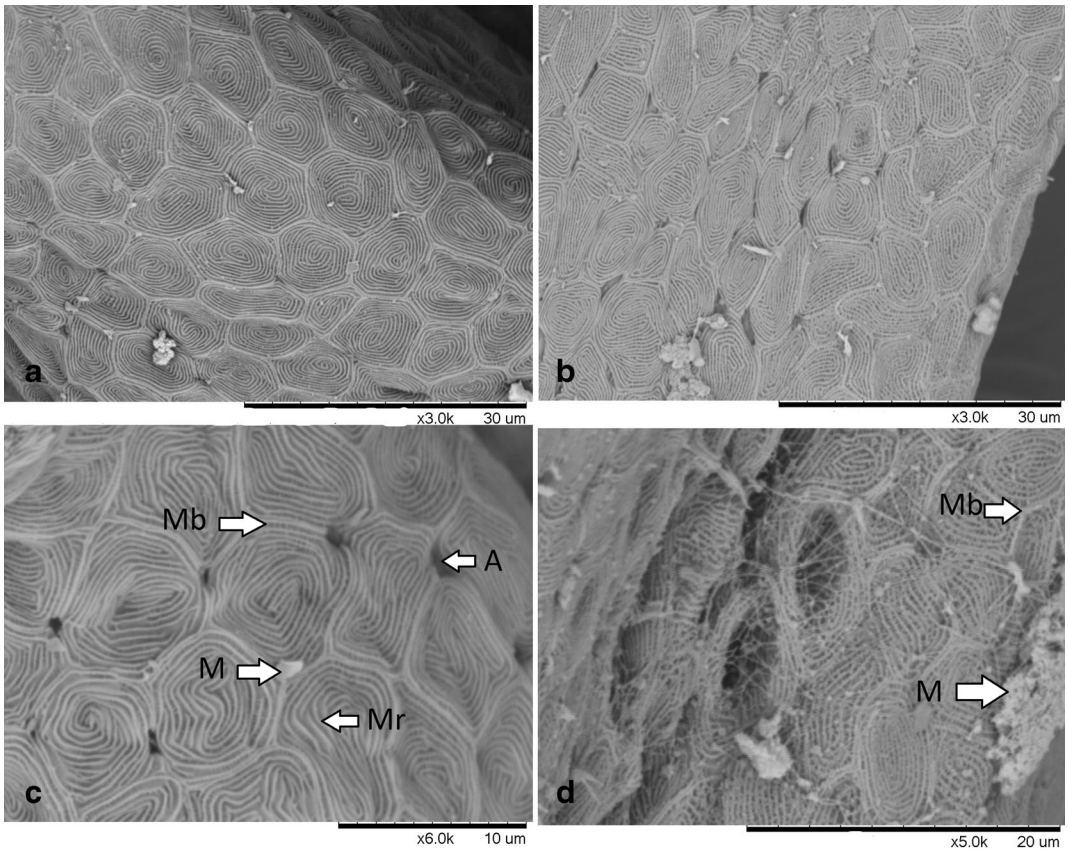

Fig. 4 Ultramorphology of gill raker epidermis of *Astyanax altiparanae* after 5 months of experiment. **a** Overview of pavement cells in the gill raker—control group. **b** Overview of pavement cells in the gill raker—detergent group. **c** Detail of pavement cells (PC) showing microridges (Mr) and microbridges (Mb)—control group. *A* orifice for mucus release. **d** Pavement cell completely filled by microbridges (Mb) and covered by mucus threads. *M* mucus

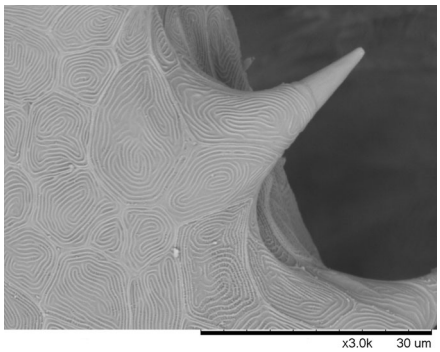

Fig. 5 Detail of denticle in a gill raker—detergent group

Results

30th day analysis

Gill rakers of the left gill arch are located opposite to gills, geared toward the oropharyngeal cavity (Fig. 1a), and contain taste buds in their ventral region (Fig. 1b) and denticles on the sides (Fig. 1a).

The epithelium is composed of polygonal cells (Fig. 1c, d) of different sizes, delimited by two rows of microridges. Epithelial cells are smooth on the dorsal region (Fig. 1c, d) and crimped on the ventral region (Fig. 2d). Within pavement cells, the concentric microprotrusions, sometimes irregular, are called microridges (Fig. 1c, d).

Mucus cells were not directly observed in gill rakers, due to pavement cells shrouding the former. Evidence of their presence was the large number of gaps between pavement cells responsible for the release of mucus (Figs. 1c, d and 2a, b, f). Secreted mucus (Fig. 2c) then adheres onto microridges (Figs. 1c and 2d) and denticles (Fig. 3a).

Each taste bud found had a taste pore on its apex (Fig. 2c–f), which was filled by chemical receptors (Fig. 2c–f) that help select food to be ingested. Chemoreceptors in gill rakers of detergent groups (Fig. 2e, f) were dilated and scarce compared to the control group (Fig. 2d).

Denticles on the sides of gill rakers (Fig. 1a) were small evaginations (Figs. 2c and 3a, b) that collaborate in food retention.

Two mitochondria-rich cells were also identified in the gill rakers, due to their shape (Figs. 2d and 3c).

5th month analysis

Pavement cell shape was not altered after 5 months of experiment; however, microbridges—bridges that interconnect one microridge to another in the same pavement cell—were found in large amounts in both groups and probably contributed to increase mucus retention efficiency (Fig. 4a–d).

There was no change in denticle structure (Fig. 5) compared to 30-day analyses.

Regarding taste buds, chemical receptors in gill rakers from the detergent group widened even further on the top of corpuscles, indicating that these receptors are very sensitive to detergent contamination (Fig. 6b, c).

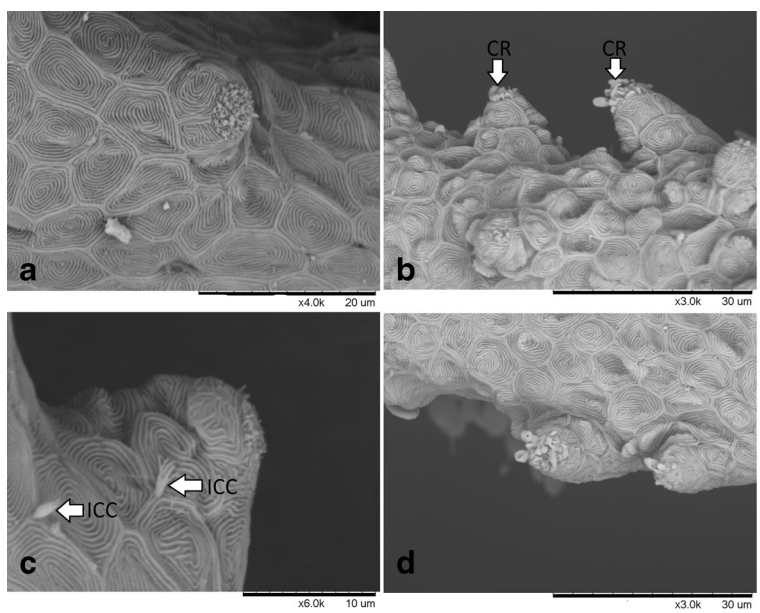

Fig. 6 Ultramorphology of taste buds in gill rakers of *Astyanax altiparanae* after 5 months of experiment. **a** Detail of chemical receptors of taste buds in the gill raker—control group. **b** Frontal view of taste buds with dilated chemical receptors (CR)—detergent group. **c** Side view of taste bud—control group. *ICC* isolated chemoreceptor cell. **d** Detail of chemoreceptors of taste buds—detergent group

Isolated chemoreceptor cells were found between pavement cells (Fig. 6c), in addition to orifices for mucus release (Fig. 4b, c).

Water results

Results are shown in Table 1. There is a variation in the concentration of some ions that can be correlated to an ion balance problem in the detergent group in such a way that fish from this group could be releasing or absorbing ions inappropriately. All the data were submitted to the Student's *t* test with Mann-Whitney as posttest ($p \leq 0.05$). Results for all experiments were the same, probably because the water volume that had to be corrected was too small to change the water parameters.

Discussion

The gill raker epithelium constitution, as it was observed in the present study (marked by the presence of mucus cells concealed by pavement cells and the release of mucus by orifices formed among these cells), has already been observed in gill filaments and lamellae of other species such as *Steindachnerina brevipina* (de Lima et al. 2011). Mucus adheres to microridges present in the epidermis of gill rakers because these structures form channels inside pavement cells (Eiras-Stofella and Charvet-Almeida 1998; Fonseca Neto and Spach 1999; Machado and Fanta 2003; Mazon et al. 2002), which help protect gills from abrasive external agents during the passage of food directed to the stomach (Eiras-Stofella and Charvet-Almeida 1998). After 5 months of experiment, another structure was observed between microridges in both experimental groups, microbridges—bridges that interconnect one microridge to another in the same pavement cell. These microbridges probably contribute to increase the efficiency of mucus retention in microridges or to increase pavement cell consistency or rigidity (Kumari et al. 2005).

After 30 days of exposure to detergent, gill rakers did not show excessive mucus production. However, after 5 months, there was an increase in mucus release, which acts as a defense mechanism against the toxicity generated by this chemical agent or one of its components (Cerqueira and Fernandes 2002; Franchini et al. 1999; Haensly et al. 1982; Leonardo et al. 2015; Reis et al. 2009).

Table 1 Water quality results from de control group (CG) and the detergent group (DG) expressed on mg/L as concentration of ions, below temperature on Celsius degree, followed by conductivity (μS/cm), hydrogen potential, and linear alkylbenzene sulphonate (LAS) concentration

Parameters	CG	DG
HCO_3^- (mg/L)	1.2	4.9[a]
Li^+ (mg/L)	<0.01	<0.01
Na^+ (mg/L)	17.5	20.1
NH_4^+ (mg/L)	10.7[a]	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[a]	5.56
NO_2^- (mg/L)	<0.04	1.15[a]
NO_3^- (mg/L)	121	106
PO_4^{3-} (mg/L)	18.9*	9.33
SO_4^{2-} (mg/L)	11.6	10.4
Acetate (mg/L)	<0.10	<0.10
$C_2O_4^{2-}$ (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	0.020*
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
Conductivity (μS/cm)	282	237
pH	5.39	6.04
Concentration of LAS (mg/L)	0	0.375[a]

[a] The most relevant differences; Student's *t* test with Mann-Whitney post-test ($p < 0.05$)

Misra et al. (1985) found points of high mucus deposition in the epidermis of *Cirrhinam rigala*, stating that they result from a molecular interaction between certain mucus components and detergents containing LAS, further suggesting that LAS-based detergents can

cause morphological changes in organs such as the epidermis and gills, even in cases of short-time exposure to low concentrations. Venhuis and Mehrvar (2004) show that concentrations between 0.02 and 1 mg/L of LAS-based detergent in water bodies may cause physiological problems in animals, damage the gills of fish, and cause hypersecretion of mucus, interfering in epithelial cell integrity.

Shape and size of chemical receptors in taste buds showed differences in the first month of experiment, indicating that these structures are highly sensitive to one or many components of detergents and result in a probable impairment of the perception of food in the environment. Water contamination studies have given little attention to taste buds, despite its importance in taste recognition (EIRAS-STOFELLA 2000; Eiras-Stofella and Charvet-Almeida 2000; Kumari et al. 2005) and how it can affect food consumption, nutritional state, and ultimately, the health status in fish.

Long-term exposure to large amounts of detergent caused inflammation of the olfactory mucosa and denaturation of receptor proteins due to solubilization of chemosensory membranes in *Ictalurus punctatus* (Misra et al. 1985). Cancalon (1983) exposed the same specie to both low (0.03–0.1%) and high (4%) concentrations of Triton X-100 and found that low dosages affected the surface of the olfactory mucosa, especially in sensory areas, once it removed membrane receptors, and generated electrophysiological malfunction in the epithelium. High concentrations caused removal of the olfactory mucosa, which hampered the ability to locate food for a period of time, even after the epithelium regenerated (Cancalon 1983). The same author also states that fishes exposed to 0.5 mg/L of LAS-based detergent lose chemosensitivity, which suggests that the chemoreceptive surface of taste buds was injured.

Even with all these data, contamination studies in gill rakers are still necessary, once we recognize detergents as very common water contaminants but still do not have a full insight into their effects in gill rakers and how they relate to broader complications such as effects in food consumption and nutritional health.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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