Adaptation of the gill epithelium of an euryhaline fish, the guppy (*Poecilia vivipara*), to freshwater

Adaptação do epitélio branquial de peixes eurialinos, guaru (Poecilia vivipara), para água doce

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Abstract

The south-american euryhaline fish *Poecilia vivipara* (BLOCH; SNEIDER, 1801), the guppy, is found both in estuary and river waters, which suggests high adaptability to environments of different salinity. In this work we studied the adaptation of the interlamellar, bars and rakers epithelia of the gills of estuary fish to freshwater conditions. The results reveal that the gill epithelia of *Poecilia vivipara* can adjust itself to freshwater by decreasing the VP of mucous cells of the interlamellar epithelium and increase the volumetric proportion (VP) of chloride cells. However, there was no evidence of similar morphological alteration in the rakers region. The epithelia of the rakers appears to be part of a different compartment that is less sensitive to variation of salinity.

Keywords: Guppy. Gills. Salinity. Mucous cells. Morphology.

Resumo

O peixe eurihalino sul-americano *Poecilia vivipara* (BLOCH; SNEIDER, 1801), o *guppy*, é encontrado tanto em estuários quanto em águas de rios, o que sugere uma alta adaptabilidade aos diferentes ambientes de salinidade. Neste trabalho, estudamos a adaptação do epitélio interlamelar, do arco e do rastelo das brânquias dos peixes de estuário de água doce. Os resultados revelam que o epitélio branquial de *Poecilia vivipara* pode ajustar-se à água doce, diminuindo a proporção volumétrica (PV) de células mucosas do epitélio interlamelar e aumentando a PV de células clorídricas. No entanto, não houve nenhuma evidência de alteração morfológica semelhante na região do rastelo branquial. O epitélio do rastelo branquial parece ser parte de um compartimento diferente que é menos sensível a variações de salinidade.

Palavras-chave: *Guppy*. Brânquias. Salinidade. Células mucosas. Morfologia.

Introduction

Besides their respiratory function, the gills are also excretory and osmoregulatory organs, with a superficial microenvironment that is different from that of the water breathed by the fish[1,2,3], and most of their functions are directly associated with the branchial epithelium[4,5]. The superficial cells are exposed to a water flow containing several substances that elicit an adaptive response from the epithelial lining of the primary and secondary lamellae, thus altering its structure and cellular distribution[6,7,8,9].

Changes in water salinity induce easily detectable cellular reactions in the gill surface. This adaptation requires an adjustment of the whole branchial epithelium, of which the three main cellular types are the pavement, chloride and mucous cells[6,10,11,12,13,14]. Pavement cells constitute more than 80% of the filament and the lamella epithelia. Chloride (or mitochondria-rich cells) and mucous cells consist of approximately 10% and 2% of the total amount of gill cells[15]. How-

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ever, only the response of a few morphological aspects of the epithelium are described in the literature, especially those observed in the chloride and pavement cells[13,16,17,18,19,20]. Studies of the effect of salinity changes on the mucous cells are less frequent.

Investigations about the branchial mucous cells are mainly directed to their anatomical or histochemical aspect[21,22]. Some authors have studied the function of the mucus as well as the effect of several types of substances on the epithelial mucus layer[8,9,13,23,24,25,26]. Histochemical differences in the mucin contents of branchial mucous cells among several species of fish were reported[21,22,27,28,29]. Although mucous cells have been less studied than chloride cells, it is now well established that the mucus layer that covers the exposed surfaces in fish is important for several reasons. It has an effective role as a protective barrier and it contributes to ion exchanges enhancing its diffusion[13,30,31,32]. The mucus has other functions, such as reducing surface tension, decreasing the friction of water and acting as a protective barrier against microorganisms[23,33,34]. These functions are all closely related to the type of glycoprotein produced by mucous cells[22]. Changes in mucous cells are often explained as an epithelial adaptation to the process of ion absorption and secretion[19,35,36,37].

The adaptable nature of euryhaline fish to waters of wide variation in salinity made these animals an object of studies about physiological and biochemical adjustments of the fish organism to hyperosmotic and hyposmotic environments[38]. *Poecilia vivipara,* an euryhaline and eurythermal fish from the American continent, can adapt itself to different aquatic salinities and temperatures. This process normally occurs in the Capibaribe River and estuary region, where the fish migrates from estuary to river and vice-versa. In a previous work four types of branchial mucous cells were described in *P. vivipara* estuarine fish. These cells were differentiated by the morphological and histochemical properties of their mucus secretion[39]. Our purpose in this study was to investigate whether the morphology of the branchial epithelium is modified when the estuarine-adapted (hyperosmotic environment) *P. vivipara* migrates to the river, which is a freshwater environment. The effect of adaptation to freshwater in the morphology, distribution and the volumetric proportion of the cellular components of the interlamellar and rakers pavement epithelium were also described.

Material and method

Experimental procedure

Sixty *P. vivipara* adult males with 5.0 cm of length were selected among those collected from the hatcheries of the Centro de Pesquisas Agrícolas de Itamaracá - Pernambuco - Brazil located in the estuary of the Capibaribe River (07°42´S; 034°50´W). All animals were collected in the first half of the morning. Thirty animals from estuarine water were immediately sacrificed by immersion in 50 ppm of benzocaine solution in estuarine water and processed as described below. The estuarine water had the following characteristics: 25‰ salinity, 736 mOsm and 30 °C. A second group of thirty animals was transferred to tanks of 100 L with dechlorinated tap water with 1‰ salinity, 146 mOsm and 30 °C. The water was replaced daily. After 15 days they were sacrificed with 50 ppm benzocaine solution in water.

Tissue processing and microtomy

Four left holobranchs of twelve estuarine and twelve freshwater fish randomly selected among the population of sixty fish were dissected and fixed in a solution of 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer of pH 7.2, 0.1 M at 4 °C for 24 hours[40]. The tissue was then dehydrated in 95° ethanol and embedded in glycol methacrylate (GMA, Leica Historesin, Nussloc/Heidelber, Germany). Sections of 1-2 µm were made, and then stained with 1% alcoholic toluidine blue followed by 1% aqueous basic fuch-

sin for morphological observation and measurement. Neutral mucins were identified with the periodic acid-Schiff (PAS) method[41]. Acidic mucins (sialomucins and sulfomucins) were identified with Alcian blue pH 2.5 staining technique[41].

Measurement

The volumetric proportion was calculated by the following method: an eyepiece with a 360-point grid (Zeiss - 8X) was inserted in the binocular tube of a Zeiss microscope, then 1 μm sections stained by the toluidine-fuchsin method were observed with a 100X oil immersion objective. The mucous cells, pavement cells and chloride cells were easily differentiated by this staining method. The points that were coincident with the interlamellar epithelium in four non-serial microscopic slides for each fish were recorded. The point-counting method applied to filaments considered only the interlamellar region. A total of 13509 points in the lamellar epithelium of 7 estuarine fish and 14029 point in 7 freshwater fish were counted. The percentage of points that fell over mucous cells, pavement cells and chloride cells was registered separately in order to calculate the volumetric proportion (VP) of each cell type in the interlamellar epithelium. The same procedure was applied to the branchial arch, gill bars and in the gill rakers epithelia. The arithmetic means were compared employing the t -Student statistical test.

Cellular areas of several branchial filaments and rakers from each fish were measured in 2 μm hematoxylin-PAS-stained sections by the image analysis system JAVA 3.0 (Jandel Video Analyses Software - California - USA) of the University of Sao Paulo School of Medicine. Sections were observed with a 100X oil immersion objective and 25 cells from each fish were measured. Images of the PAS-stained (neutral) mucous cells from both the interlamellar space and gill bars and rakers epithelia were digitalized and each cell cytoplasm was manually outlined with a mouse cursor, in order to calculate the area employing the software. Only the cytoplasm PAS-positive area was considered, while the nuclei area was not measured. The quantity of neutral and acidic mucus in each cell was subjectively scored in both experimental groups in slides stained with either the PAS or Alcian blue pH 2.5 alone. In this case, gills of both fish groups were embedded in the same historesin block. Consequently, estuarine and freshwater tissues were present in the same histological section, and were stained at the same time and in the same conditions, in order to avoid differences in stain intensity due to section thickness, staining procedures and light variation of the microscope system.

Results

Branchial filaments are covered by two types of epithelia, the simple pavimentous respiratory epithelium and the stratified pavimentous epithelium that lines the filament and interlamellar region. The cellular response to freshwater varies according to cellular type, branchial region and parameter analysed, and we observed a significant overall difference between the histological aspect of the estuarine-adapted and freshwater-adapted epithelium filament. Initially, histological sections of freshwater-adapted animals showed a reduction in number and size of filament mucous cells (Figure 1). The population of chloride cells appeared to increase. After 15 days of freshwater adaptation the area of the mucous cells cytoplasm had a reduction of 43.4% (from 58.9 μm^2 to 33.1 μm^2) whereas the population of chloride cells remained constant (Table 1). The area of the rakers mucous cells remained constant in both experimental conditions (Table 1).

Employing the point-counting method we observed that the relative volume occupied by the mucous cells in the freshwater filament epithelium decreased 9.8 times or 89.8% (from 5.9% to 0.6%) while the chloride cells volume increased 2.5 times or 143% (from 17.7% to 43.8%). The relative volume of pavimentous cells was also reduced 1.37 times or

27.2% (from 76.4% to 55.6%). Volumetric densities of the rakers cells were not affected by exposition to freshwater (Table 2). Mucous cells of the branchial filament secrete both neutral and acidic mucus, but were less stained by the PAS method (neutral mucus) in the freshwater group when compared to estuarine group. The Alcian blue stain (acidic group), however, did not reveal differences between treatments. In the rakers region, the staining intensity of the mucous cells was similar, and such characteristic was independent of the group or the staining method employed (Table 3).

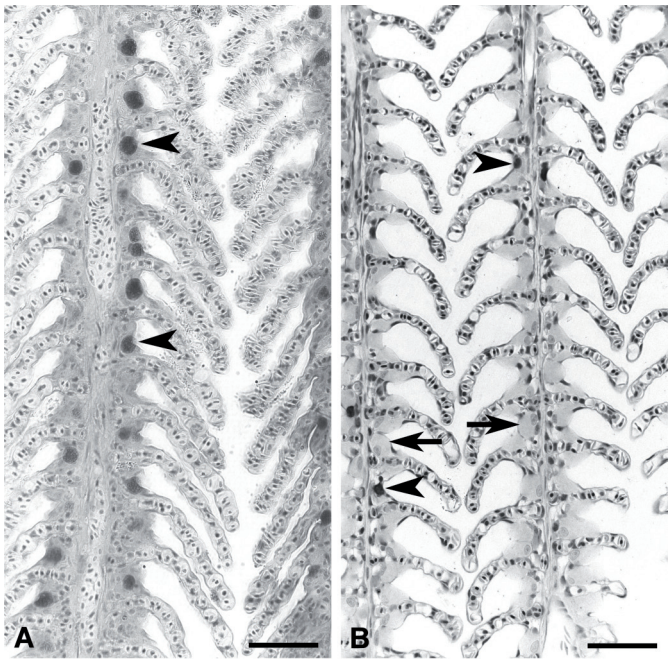

Figure 1 - Histologic sections of the lamellar region of *P. vivipara* gills by PAS-hematoxylin method in GMA resin. A) Estuarine group. The mucous cells (arrowheads) are frequently observed in the interlamellar region. B) Freshwater adapted group. The mucous cells frequency is greatly reduced (arrowheads) while the chloride cells (arrows) frequency increases. Bar = 40 µm. (FMVZ-USP - São Paulo - 2009)

Table 1 - Cytoplasmic area of mucous cells and chloride cells of the gills filaments and rakers of estuarine and freshwater-adapted fish. The numbers are the mean area of each epithelial cell type in μm^2 units. The standard deviation ($\pm$ sd) is shown in parenthesis. The means in the same column displaying different letters are significantly different to $\alpha = 0.05$ with Student t test. (FMVZ-USP - São Paulo - 2009)

Treatment	Cell Area*		Region
	M	C	
Estuarine	58.9 (11.3)[a] n[b] = 7	152.9 (24.4)[a] n = 7	Filaments
Freshwater	33.1 (6.9)[b] n = 8	138.4 (18.9)[a] n = 7	
Estuarine	81.1 (25.0)[c] n = 7	-	Rakers
Freshwater	82.5 (29.5)[c] n = 7	-	

*M = mucous cells; C = chloride cells
[b]**n** is the number of animals

Table 2 - Volumetric proportion of mucous cells (M), chloride cells (C) and pavimentous cells (P) of the gills filaments and rakers of estuarine and freshwater-adapted fish. The numbers are the mean proportion (%) of each cell type in the epithelium. The standard deviation (± sd) is shown in parenthesis. The means in the same column displaying different letters are significantly different to $\alpha = 0.05$ with Student t test. (FMVZ-USP - São Paulo - 2009)

Treatment	n*	Volumetric Proportion			Region
		M	C	P	
Estuarine	7	5.9 (1.4)[a]	17.7 (2.9)[a]	76.4 (3.2)[a]	Filaments
Freshwater	7	0.6 (0.5)[b]	43.8 (4.2)[b]	55.6 (3.9)[b]	
Estuarine	7	6.5 (4.4)[c]	29.9 (6.4)[c]	63.6 (5.5)[c]	Rakers
Freshwater	7	8.1 (3.3)[c]	32.8 (9.1)[c]	59.1 (7.2)[c]	

*n is the number of animals

Table 3 - Intensity of staining of mucous cells to PAS (neutral mucin) and Alcian Blue (acidic mucins) of the gills filaments and rakers of estuarine and freshwater-adapted fish. (FMVZ-USP - São Paulo - 2009)

Treatment	Intensity of staining*		Region
	Neutral mucin	Acidic mucin	
Estuarine	+++	+++	Filaments
Freshwater	+	+++	
Estuarine	+++	+++	Rakers
Freshwater	+++	+++	

*+++ - strong staining, + - light staining

Discussion

Euryhaline teleosts have a remarkable ability to maintain the homeostasis of their internal environment regardless of the external salinity variation. This may allow one to infer the existence of precise and efficient branchial adaptive mechanisms[42]. In this study we found that adaptation of estuarine *P. vivipara* to freshwater implies in structural modifications or the gills interlamellar epithelium that are characterized by a reduction of overall volume of mucous cells in the epithelium (Table 2) and decrease of the area of cytoplasm of secretory cells (Table 1). The consequence of such alterations probably is the reduction of the quantity of mucus secreted by the branchial filament. The measurement of the area and the VP parameters seems to indicate a lower need of mucous cells and mucus secretion in the branchial filaments of freshwater-adapted *P. vivipara*. Although the measurement of the cytoplasmic area may be useful to estimate the metabolic activity of single cells, the measurement of the volumetric proportion (VP) with the point-counting method may give a better insight of the relative contribution of a cellular type in the integrated functional activity of an epithelium. This method is based on the principle of Delesse (M. A. Delesse), a french geologist who stated in 1842 that the volume fraction of minerals could be estimated from their area profiles in rock surfaces. In 1943 a method of counting a number of points projected over an image of tissue section as an estimate of relative volume was proposed[43]. This method was later supported by

mathematic tests[44]. The principle of Delesse applied to quantitative microscopy implies that the mean area is an unbiased estimate of volume. The VP of secretory cells estimates the proportional quantity of active secretory tissue in the whole epithelium.

Adaptation of other euryhaline species to seawater increases the number of branchial and skin mucous cells[45,46,47,48,49]. Mallatt[8] found that high salt concentration acts as a branchial irritant even in euryhaline fish. This probably occurred in *P. vivipara* estuarine-adapted fish, in which branchial epithelium was stimulated by the salt in the water to produce a protective mucus barrier on the gill surface. After transfer to freshwater, the salinity stimulus ceased and the secretory volume of the epithelium decreased, as well as the secretory activity of mucous cells. Also, the thickness reduction of the lamellar mucus layer could improve the uptake of ions in freshwater, as it is known that this substance acts as a barrier to ion diffusion[50]. Additionally we observed that the quality of the mucus produced was changed. The epithelial cells of the freshwater group secreted a mucus that was depleted of neutral mucins, although the acidic mucin component was not reduced (Table 3). Roy[9] verified that the epithelial mucus changed from acidic to neutral when the opercular and gill surfaces of *Rita rita* were exposed to an irritant (detergent). The neutral mucus is apparently responsible for chemical protection (as the neutral mucus in the stomach) while the acidic mucus appears to protect from mechanical abrasion (as the acidic mucus secretion in the large intestine). The reduction of the neutral fraction thus suggests that the freshwater condition is less aggressive or less stressing to the branchial epithelium of *P. vivipara* than the salt estuarine water. The silent mucous cells do not react to the histochemical methods and are indistinguishable from the pavimentous cells by light microscopy.

Surprisingly, the rakers mucous cells did not react to freshwater as the filament mucous cells. Their cellular area, epithelial volumetric proportion and the mucus histochemistry remained unchanged. In a previous work Sabóia-Moraes, Hernandez-Blazquez, Mota e Bittencourt[39] showed that the histochemical and morphological features of the filament mucous cells (type III cells) were different from those of the rakers mucous cells (type IV cells). In that work they suggested that these differences could be due to a regional specialization in the secretion of mucus by the gills. The present results showed that there is a branchial regional specialization concerning the response of the mucous cells to the environmental salinity, and the rakers region is less reactive to salinity changes. Subpopulations and regional specialization of mucus-secreting cells are commonly found in the gastrointestinal tract of fish[51,52]. The mucous cells of the rakers region and those from the filament region may thus be regarded as different subpopulations of cells with particular responses under the same stimulus.

The volume of *P. vivipara* chloride cells correspond to 17,7% (estuarine water) and 43,8% (freshwater) of the volume fraction of the filament epithelium, with a 143% volumetric increase after freshwater adaptation (Table 2). The quantity of chloride cells and accessory cells usually changes after the readaptation of an euryhaline teleost fish to a different osmotic environment[18,24,53,54,55]. As in *P. vivipara*, several examples may be found where the chloride cells proliferate and have their NaCl-transporting capacity enhanced when fish are maintained in low NaCl concentration water[14,19,56]. In *Sparus auratus*, the frequency of chloride cells was higher in hyposaline water that in 15‰ or 25‰ salinity waters[57]. However, some species react differently and the chloride cells accumulate in the interlamellar region of some fish adapted to high salinity water[13,19]. In *P. vivipara* the increase of the chloride cells volumetric proportion may occur in order to compensate a lower concentration of chloride ions. This fact, associated with the reduction of the volume of mucous from epithelium secretory cells, may reveal a mecha-

nism to enhance the process of capture of chloride ions in freshwater in order to keep the ionic balance.

Conclusion

The volumetric proportion of the chloride cells and mucous cells of the rakers and gill bars epithelia was not modified in freshwater-adapted fish, further stressing that the rakers are regions with low sensitivity to changes in water salinity. Thus the rakers and gill bars epithelia may be considered as separate compartments in studies regarding the effects of substances on the gills surface because they may react differently to the same stimulus.

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