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Morphology of the urogenital papilla and its component ducts in *Astyanax altiparanae* Garutti & Britski, 2000 (Characiformes: Characidae)

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The histological description of the urogenital papilla is an important tool to comprehension of the reproductive mechanisms in fish, as well as a pre-requisite to germ cell transplantation in adult fish, besides to be a good biological indicator to environmental changes. Was performed the histological description of the urogenital papilla and its component ducts in the tetra *Astyanax altiparanae*. The genital and urinay ducts pass separately throughout most part of its extension, joining in a single duct before opening. In males this opening is asymmetric and seems to have double origin, being completely surrounded by striated muscle fibers, while in females it is symmetric and the muscle fibers does not surround it totally. Spermatic duct and oviduct undergo changes throughout their extension, mainly in the morphology of the surrounding epithelium. In the spermatic duct, squamous epithelial cells change to columnar and cuboid with possible secretory activity, close to testes. In the oviduct, anteriorly epithelial cells are also squamous, however, close to ovary there are lamellae composed by a pseudostratified epithelium with columnar and cuboid cells. The urinary duct is highly similar for both sexes presenting globoid cells, which description is known in mammals, however, rare in fish.

A descrição histológica da papila urogenital é uma importante ferramenta para a compreensão dos mecanismos reprodutivos em peixes, assim como um pré-requisito para a realização do transplante de células germinativas em peixes adultos, além de um bom indicador biológico de possíveis alterações ambientais. Foi realizada a investigação histológica da papila urogenital e seus ductos constituintes no lambari *Astyanax altiparanae*. Os ductos genital e urinário ocorrem separadamente ao longo de maior parte de sua extensão, entretanto, unem-se em um ducto simples antes de abrir para o meio externo. Nos machos esta abertura é assimétrica e parece ter dupla origem, sendo completamente envolvida por fibras musculares estriadas, enquanto nas fêmeas ela é simétrica e as fibras musculares não a envolve totalmente. O ducto espermático e oviducto sofrem alterações ao longo de sua extensão, principalmente na morfologia do epitélio que os envolve. No ducto espermático as células epiteliais passam de pavimentosas a colunares e cuboides, com possível atividade secretora, à medida que se aproxima dos testículos. No oviducto, anteriormente as células também são epiteliais pavimentosas, entretanto, próximo aos ovários, formam-se lamelas compostas por um epitélio pseudoestratificado composto por células cuboides e colunares. O ducto urinário é bastante similar em ambos os sexos apresentando células globosas, cuja descrição é conhecida em mamíferos, porém rara em peixes.

Keywords: Fish reproduction, Histological analysis, Oviduct, Sperm duct, Urinary duct.

Introduction

The fish adaptability in distinct aquatic habitats reflects the numerous reproductive strategies adopted by these animals (Vazzoler, 1996). As result fish show considerable anatomical, morphological and physiological differences in their reproductive systems, both in the gonads and in the genital ducts (Lopes *et al*., 2004; Batlouni *et al*., 2006; Muñoz *et al*., 2011). Therefore, examining the reproductive aspects of fish, including sexual dimorphism, fecundity, gonadal morphology and gametogenesis, is important for understanding how species have maximised their reproductive strategies in different environments (Grier & Taylor, 1998; Brown-Peterson *et al*., 2002; Cassel *et al*., 2013; Siqueira-Silva *et al*., 2013; Wildner *et al*., 2013).

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However, apart from general knowledge about the fish reproductive system little is known about the morphology of their genital ducts, particularly the histological details of the urogenital papilla of freshwater fish. Most studies only describe its macroscopic morphology and anatomical position for use identifying the sex of individuals, in fish farming and for toxicological and ecological studies (Ferreira *et al*., 2010; Kruger *et al*., 2013). Moreover, most past histological studies were focused on marine fish species (Ross, 1984; Hastings & Petersen, 1986; Kott *et al*., 1988; Rasotto & Shapiro, 1998; Suzuki & Shibata, 2004; Kobayashi *et al*., 2012).

Furthermore, histological descriptions of the urogenital papilla in freshwater fish could be valuable for biotechnological studies, such as germ cell transplantation in adult fish, whose researchers can use the studied specie as host such as Lacerda *et al*. (2010), that injected spermatogonial stem cell though *Oreochomis niloticus* (Linnaeus, 1758) urogenital papilla, after the description of this organ; it description can be also useful in systematic reviews, as an additional feature to be considered, as well as was already used by some authors (Kott *et al*., 1988; Hoshino *et al*., 2004); and in toxicological studies, since this organ can be very sensitive to environmental changes (Rodrigues *et al*., 2006).

Thus, the Characidae species *Astyanax altiparanae* Garutti & Britski, 2000*,* a typical fish from the upper rio Paraná Basin, Brazil (Garutti & Britski, 2000), seems to be a great candidate for this kind of morphological description, since it shows a great potential to be used as a host species in germ cell transplantation studies owing its reduced size, early maturation, prolificacy and management resistance (Orsi *et al*., 2004).

Moreover, this species belongs to the *Astyanax* genus assigned as *Incertae sedis,* being a frequent target of systematic studies (Reis *et al*., 2003; Orsi *et al*., 2004; Kantek *et al*., 2009; Martinez *et al*., 2012). Thus, one more described structure could be valuable in attempt to solve this phylogenetically question, and since *A. altiparanae* shows a great phenotypic plasticity, it could be useful as bio-indicator as some studies have already shown this organ can suffer changes under certain environmental conditions (Kirby *et al*., 2003; Larsen *et al*., 2009).

The present study aimed to provide a histological description of the urogenital papilla, the components ducts and the surrounding musculature in *A. altiparanae*.

Material and Methods

Animals. Ten sexually mature male and 10 sexually mature female of *A. altiparanae* of standard size ranging from 12 to 15 cm were used in this study. As mentioned before, the choice of this species was motivated by characteristics such as ease of handling, small size, sexual maturity at the fourth month of age, and ability to reproduction three to four times a year. These features are considered appropriate for the development of many different studies in laboratory conditions. Experimental procedures were carried out in strict accordance with the guide for care and use of laboratory animals of the Universidade Estadual Paulista (UNESP). The research ethics committee of UNESP approved the protocols (Permit Number: 006/2012/CEUA). All surgical procedures were performed under anesthesia with 0.5 g of benzocaine in 5 ml of absolute ethanol, and all efforts were made to minimize suffering. Fish were identified and a group of voucher individuals was deposited in the collection of fish of Laboratório de Ictiologia Departamento de Zoologia e Botânica IBILCE/UNESP (DZSJRP) under the register number DZSJRP 008999.

Sampling and analyses. The posterior part of the specimens' abdomen that contained the urogenital papilla and the distal portion of the gonads, gut and urinary duct were dissected. These samples were fixed in 4% paraformaldehyde with 2% glutaraldehyde solution in Sorensen phosphate buffer, pH 7.4, for a minimum period of 24 hours. Then, the samples were dehydrated in ethanolic solutions at increasing concentrations and were impregnated in glycol methacrylate (Technovit 7100/historesin; Heraeus Kulzer, Wehrheim, Germany). Finally, the samples were sectioned to a thickness of 3.0 μm using a microtome (LEICA RM 2145; Leica Instruments GmbH, Heidelberg Nussloch, Germany) equipped with a glass blade and stained with Haematoxylin and Eosin, Toluidine Blue and Metanil-Yellow/PAS/Harrys Haematoxylin. The photo-processing and analyses were completed with a Zeiss optical microscope equipped with an AXIOCAM-MRc5 camera (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

Results

Microscopy similarities between males and females, such as the single and folded opening and the skin that was comprised by a squamous stratified epithelial tissue covering the urogenital papilla in both sexes were observed (Figs. 1a; 2b).

However, there were significant differences in the morphology of this organ between males and females, mainly in the epithelium that borders the terminal portion of the urogenital opening (Figs. 1a; 3a; 4). In males, this epithelium was formed from the union of the epithelia stemming from both the genital and urinary ducts (Figs. 1a, c; 4b). The epithelium region formed from the genital duct was simple and composed by squamous cells (Fig. 1c; 4b), while the epithelium region formed from the urinary duct epithelium showed columnar and cuboid cells, many of which containing clusters of neutral polysaccharides in their cytoplasm beyond one brush border in their top (Fig. 2a; 4b). Mucus-producing cells were dispersed throughout the epithelium (Fig. 2a). In females, the epithelium bordering the urogenital duct opening has similar characteristics to the epithelium coming from the urinary duct described in males (Fig. 4c).

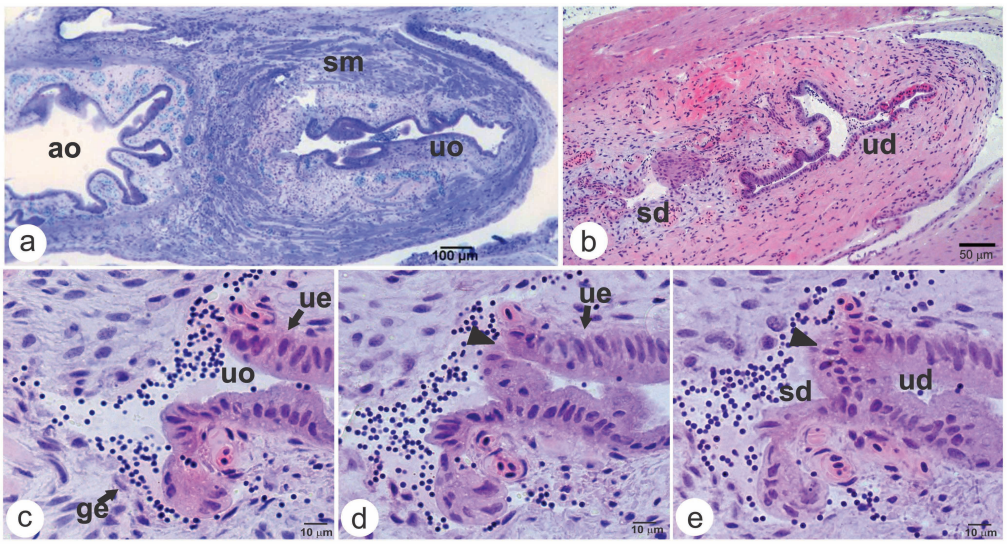

Fig. 1. Histological characteristics of the urogenital opening in male *Astyanax altiparanae*. a) Urogenital opening (uo); b) Sperm duct (sd) and urinary duct (ud) split; c-e) Sequence showing the urogenital duct splitting in the sperm duct (sd) and urinary duct (ud). Abbreviations and symbols: ao = anal opening; ge = squamous genital epithelium formed from the genital tunic; sm = striated skeletal muscle surrounding the urogenital opening; ue = urogenital epithelium formed from the urinary duct; arrowhead = epithelial separation forming the sperm and urinary duct. Label: (a) = Toluidine Blue; (b-e) = Haematoxylin/Eosin.

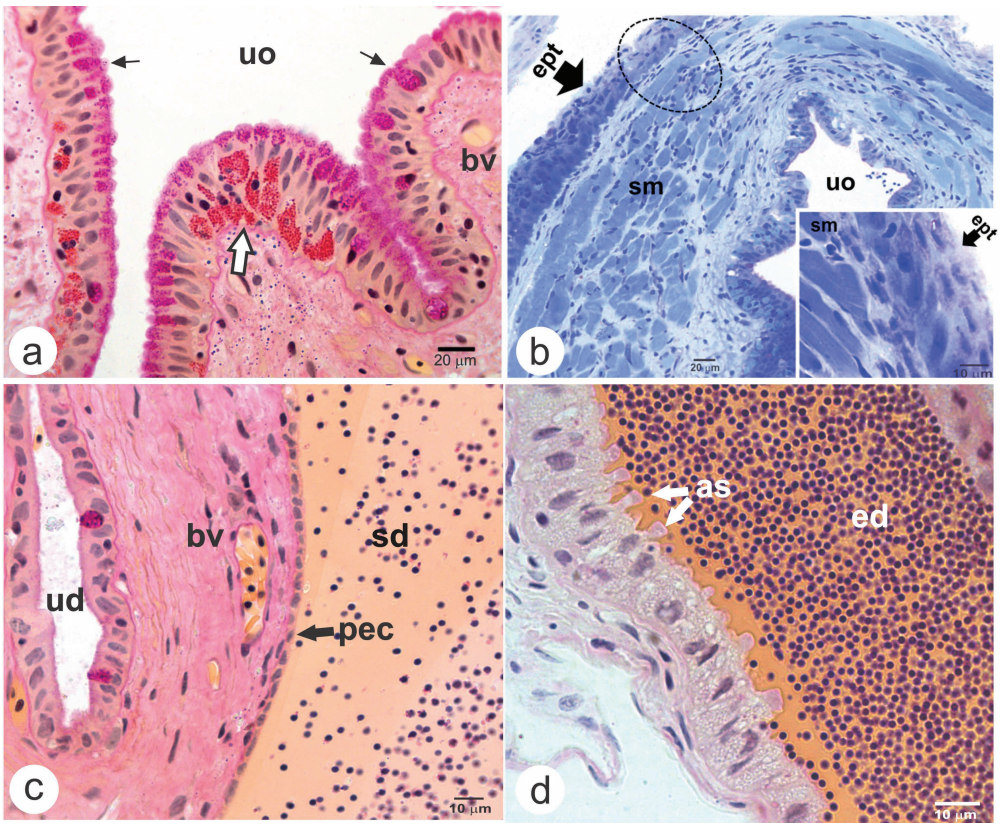

Fig. 2. Histological characteristics of the genital duct in male *Astyanax altiparanae*. a) Urogenital opening (uo) highlighting the clusters of neutral polysaccharides (white arrow), the mucous-producing cells and the brush border (thin arrow); b) detail of the striated muscle (sm) that surround the urogenital opening and the stratified skin (ept) that involve the urogenital papilla; c) sperm duct (sd) highlighting the pavimentous epithelial cells (pec) and seminal fluid labelled in orange; d) efferent duct (ed) with secretory cells with possible apocrine activity (as). Abbreviations and symbols: bv = blood vessel; ept = squamous stratified epithelium; ud = urinary duct. Label: a, c-d = Metanil-Yellow/PAS/Harris Haematoxylin; b = Toluidine Blue.

In males, the urogenital ducts were completely surrounded by a loose connective tissue containing numerous striated muscle fibers and blood vessels (Figs 1a; 2b). In females, this tissue did not involve totally the urogenital papilla, since there were no muscle fibers in the region between the anal and urogenital openings (Fig. 3a).

Posteriorly, the urogenital duct splits between the genital (positioned posterior to the rectum) and the urinary duct (caudally positioned in relation to the genital duct) (Figs. 1b-e; 3a-c; 4). In males, the genital duct (sperm duct) maintained a single, squamous epithelium until the efferent ducts, which also had a single epithelium but with cuboid and columnar secretory cells with possible apocrine activity (Figs. 2c-d). Seminal fluid was also present in this region (Figs. 2c-d; 4b). In females, a single, squamous epithelium was also observed at the beginning of the genital duct (oviduct) (Figs. 3b; 4c). However, close to the ovaries, the epithelium of the oviduct became pseudostratified with cuboid and columnar cells, forming lamellae from the tissue septa projected in the direction of the lumen (Figs. 3d; 4c). In the oviduct lumen no fluid was observed (Figs. 3a-d).

The urinary duct in both sexes had portions with both, single and stratified epithelia contained squamous, cuboid and columnar cells, many of which presented clusters of neutral polysaccharides in their cytoplasm beyond a brush border in their top (Figs. 3e-f). Furthermore, globoid cells, some of them binucleate, were observed among the epithelial cells (Fig. 3f). Smooth muscle fibers surrounded this epithelium (Fig. 3e).

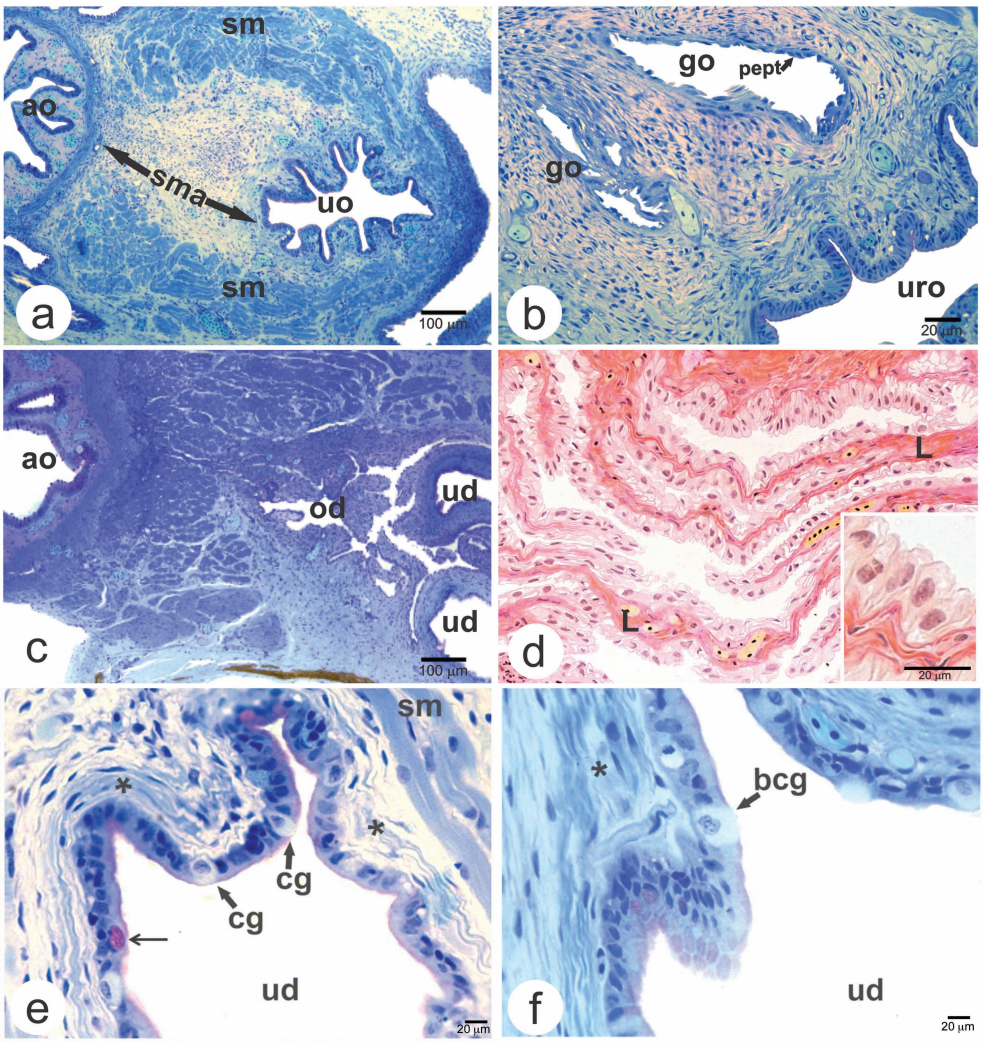

Fig. 3. Histological characteristics of the urogenital papilla in female and urinary duct in *Astyanax altiparanae*. a) General view of the urogenital opening (uo) and its position in relation to the anal opening (ao). See the absence of muscle fibers (sm) between these two openings (sma); b) The beginning of the urogenital duct separation in genital (go) and urinary ducts (uro); c) The beginning of lamellae formation in the oviduct (od); d) Oviduct lamellae (L) Insert = highlight of the epithelial cells that composes the oviduct lamellae; e) Urinary duct highlighting the globoid cells (cg), mucus-producing cells (thin arrow) and smooth muscle fibers (asterisk) surrounding it; f) In detail, a binucleated globoid cell (bcg). Abbreviations and symbols: sm = striated skeletal muscle fibers; Label: a-c, e-f = Toluidine Blue; d = Metanil-Yellow/PAS/Harris Haematoxylin.

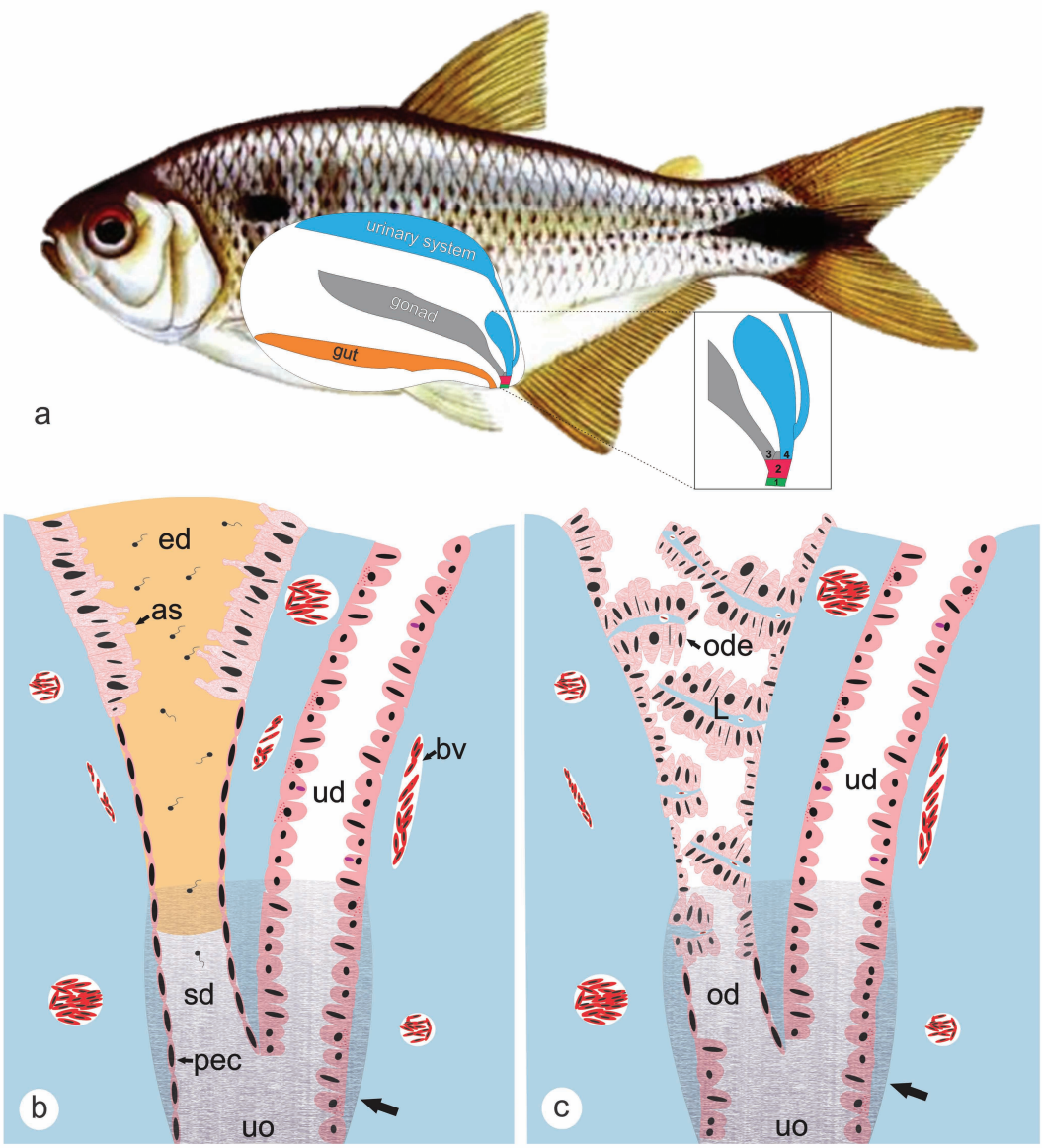

Fig. 4. Scheme of the urogenital papilla in *Astyanax altiparanae*. a) Position of the urogenital papilla in relation to fish body. 1 = urogenital opening; 2 = split of the urogenital duct in genital and urinary duct; 3 = genital duct; 4 = urinary duct. Fish image adapted from: causoeacasodepescador.blogspot.com.br: b) Structure of the urogenital papilla and its components ducts in males; c) Structure of the urogenital papilla and its components ducts in females. Abbreviations and symbols: as = cells with possible apocrine activity; bv = blood vessels; ed = efferent ducts; L = oviduct lamellae; ode = epithelial cells of the oviduct lamellae; sd = spermatic duct; ud = urinary duct; large arrow = striated skeletal muscle fibers.

Discussion

Unlike some fish species (*e.g.*, Kirby *et al*., 2003; Rodrigues *et al*., 2006; Larsen *et al*., 2009; Dumont *et al*., 2011; Kornis *et al*., 2012), the sex of *A. altiparanae* cannot be identified by macroscopic examination of the urogenital papilla. However, histological analyses showed significant differences in this organ between the sexes, most likely correlated to reproductive events. These differences were only observed between the genital ducts and in the epithelium that encloses the common urogenital opening. In males, this epithelium has a double origin.

The structure of the urogenital opening is variable among teleost species and it can be different between two species from the same order, as in Cypriniformes whose the common carp *Cyprinius carpio* (Linnaeus, 1758), as in *A. altiparanae*, has a single opening, while the goldfish *Carassius auratus* (Linnaeus, 1758) has ducts opening separately on the papillary surface (Uematsu & Hibiya, 1983). Furthermore, the structure can be different between the sexes of species from the same genus, as in the inseminating genus *Astroblepu*s Humboldt, 1805 in which the females show separate openings while males have only one opening (Spadella *et al*., 2012).

Similarities in this structure can be found among evolutionarily distinct groups, such as the Neoteleostei bluehead wrasse *Thalassoma bifasciatum* (Bloch, 1791) (Labriniformes) and the Euteleostei chum salmon *Onchorhynkus keta* (Walbaum, 1792) (Salmoniforms), whose divergence time was more than 290 million years ago and both of them show the genital and urinary ducts opening independently on the papillary surface (Uematsu & Hibiya, 1983; Ross, 1984; Hastings & Petersen, 1986; Rasotto & Shapiro, 1998; Ortí & Li, 2009; Kobayashi *et al*., 2012).

The arrangement of the muscle tissue fibers in *A. altiparanae,* may be involved in the control of gamete release during the reproductive act, working as a sphincter (Uematsu & Hibiya, 1983; Hastings & Petersen, 1986; Muñoz *et al*., 2002). This mechanism may be a strategy to ensure a more efficient fertilization, since only the males have the muscle fibers running circularly around the entire urogenital papilla, making them able to control the timing of sperm release. Other sphincter-like musculature structure was described in *T. bifasciatum*. This ligament muscle, as the authors named it, was hypothesized to prevent water reflux into the genital and urinary ducts during the swimming movement (Rasotto & Shapiro, 1998).

Another important difference observed between *A. altiparanae* males and females is in the genital ducts and it can be assigned to the functional and physiological necessities of sexes. For example, the lamellae formation by the oviduct epithelium may be related to oocyte transportation to the urogenital opening (Kobayashi *et al.,* 2012). This mechanism would be similar to the peristaltic movements of the uterine tube in mammals. By contrast, the sperm duct epithelium is related to seminal fluid production (Kobayashi *et al*., 2012; Spadella *et al*., 2012), and according to Lahnsteiner *et al*. (1998), feeds the spermatozoa and keeps them motionless.

The epithelium that covers the urinary duct of the urogenital papilla, in tetra and in the marine species *T. bifasciatum* has a similar structure (Rasotto & Shapiro, 1998). Besides this, the urinary system of mammals also presents some similarities, since like in the tetra *A. altiparanae* it also has cells with voluminous cytoplasm, known as globoid cells, showing that this system has probably been preserved among the vertebrates (Junqueira & Carneiro, 2011).

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