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Morphological and immunohistochemical comparison of the pituitary gland between a tropical *Paracheirodon axelrodi* and a subtropical *Aphyocharax anisitsi* characids (Characiformes: Characidae)

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Cardinal tetra *Paracheirodon axelrodi* and bloodfin tetra *Aphyocharax anisitsi* are two species of characids with high trade value as ornamental fish in South America. Although both species inhabit middle water layers, cardinal neon exhibits a tropical distribution and bloodfin tetra a subtropical one. Generally, these species are difficult to grow, so it becomes essential to know some key components of the neuroendocrine system to achieve their reproduction in captivity. Considering the importance of deepening the knowledge of the reproductive physiology through functional morphology, for the first time in this work we performed an anatomical, morphological and immunohistochemical analysis of the pituitary gland of these two species. In both species, a leptobasic type pituitary is found in the ventral zone of the hypothalamus and it is characterized by a neurohypophysis which has a well-developed pituitary stalk and a globular adenohypophysis. The pituitary components, characterized by histochemistry and immunohistochemistry, shows a distribution pattern of cells types similar to other teleost species, with only slight differences in the distribution of βFSH and βLH for *P. axelrodi*.

Keywords: Adenohypophysis, Histology, Neurohypophysis, Ornamental fish.

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El cardenal tetra *Paracheirodon axelrodi* y el tetra *Aphyocharax anisitsi* son dos especies de carácidos con alto valor comercial como peces ornamentales en América del Sur. Aunque ambas especies habitan en las capas medias de agua, el neón cardenal exhibe una distribución tropical, mientras que el tetra cola roja una distribución subtropical. En general estas especies son difíciles de cultivar, por lo que es esencial conocer algunos componentes clave de los sistemas neuroendocrinos para lograr su reproducción en cautiverio. Considerando la importancia de profundizar en el conocimiento de la fisiología reproductiva a través de la morfología funcional, en este trabajo realizamos, por primera vez, un análisis anatómico, morfológico e inmunohistoquímico de la glándula pituitaria de estas dos especies. En ambas especies, la hipófisis, del tipo leptobásica, se encontró en la zona ventral del hipotálamo y se caracteriza por una neurohipófisis con un tallo hipofisario bien desarrollado y una adenohipófisis globular. Los componentes hipofisarios, caracterizados por la histoquímica y la inmunohistoquímica, mostraron un patrón de distribución de tipos de células similares a otras especies de teleósteos, con solo pequeñas diferencias en la distribución de βFSH y βLH para *P. axelrodi.*

Palabras clave: Adenohipófisis, Histología, Neurohipófisis, Peces ornamentales.

INTRODUCTION

The understanding of the reproductive function, especially focused on the brain-pituitary-gonadal axis, is essential to acquire knowledge about the reproductive biology of different species and fundamental for the support of commercial aquaculture. In captivity, reproduction can be modulated by manipulating environmental parameters, such as water temperature, conductivity and pH, among others (Fiszbein *et al*., 2010; Mylonas *et al*., 2010). However, the biological aspects of several teleost species are not well-known, and thus it results very difficult to simulate the environmental conditions required for their reproduction in captivity.

The global trade in ornamental fish is dominated (90%) by freshwater fishes, most of which are sourced from breeding facilities located in developing countries, typically in Asia or South America. However, some fish are still obtained from natural (wild) sources and the exact percentage of wild-caught fish is difficult to quantify given a lack of reliable data (Evers *et al*., 2019). Generally, these species, collected from their natural environment, are difficult to grow, so it is important to know the morphological and physiological key components of neuroendocrine systems to better regulate the reproduction of these species.

In some commercial species, for example, reproduction can be enhanced by several strategies which can modify the synthesis and release of pituitary hormones (Mylonas *et al*., 2010; Zohar *et al*., 2010). In this sense, identifying and characterizing cell types that produce pituitary hormones will enhance our knowledge on fish reproductive physiology and will constitute a solid basis for future reproductive induction with hormonal treatments. Authors said that heterologous antisera can be used in these kind

of studies, since their specificity and absence of cross reactivity can be demonstrated (Laiz-Carrión *et al*., 2003; Pandolfi *et al*., 2005; Honji *et al*., 2015).

The pituitary gland is an important endocrine organ that presents two distinctive regions: the neurohypophysis, consisting of neurosecretory terminals principally from the hypothalamus, and the adenohypophysis, constituted mainly by secretory cells. In teleost fish, these endocrine cells are arranged in groups that produce different hormones and are located at specific regions of the adenohypophysis (Van Oordt, Peute, 1983; Laiz-Carrión *et al*., 2003; Pandolfi *et al*., 2005).

Cardinal tetra *Paracheirodon axelrodi* (Schultz, 1956) is a characid species endemic from South America, which inhabits the Amazon, Orinoco and Rio Negro basins (tropical habitat). It inhabits middle water layers with temperatures between 23°C and 27°C, pH ranging from 5.5 to 7, hardness oscillating between 5 dH and 12 dH (Anjos, Anjos, 2006) and it is an omnivore species (Walker, 2004; Marshall *et al*., 2008). Female spawning occurs in shaded territories and each spawning event can range from 154 to 562 oocytes. It is worth mentioning that this species has an asynchronous oogenesis, since more than two oocyte stages can be present simultaneously (Anjos, Anjos, 2006; Brito, Bazzoli, 2009).

Bloodfin tetra *Aphyocharax anisitsi* Eigenmann, Kennedy, 1903 is a characid species that inhabits the basin of La Plata's river in South America (subtropical habitat). In this habitat, the water temperature range is between 18°C and 28°C, pH ranges from 5.4 to 7.9 and hardness of 30 dH (Burgess, 2004; Casciotta *et al*., 2005). The reproductive biology and the development of sexual dimorphic structures in this species have been already described (Gonçalves *et al*., 2005). Other works on its biodiversity and distribution in Argentina report that this is a vulnerable species in the Mesopotamia (López *et al*., 2005), but it is not a threatened species in La Plata basin (Zayas, Cordiviola, 2007), suggesting that their conservation status may differ among different habitats.

Both species, cardinal and bloodfin tetra, exhibit high market value and an increasing global importance for ornamental aquaculture (Evers *et al*., 2019) as they correspond to the fish order of greatest export interest in countries such as Brazil, Colombia, Venezuela and Argentina (Mancera-Rodríguez, Álvarez-León, 2008; Panné-Huidobro, Ferino, 2017). Some studies have focused on understanding the basic biology of both species, *P. axelrodi* (Burton *et al*., 1998; Walker, 2004; Anjos, Anjos, 2006; Oliveira *et al*., 2008; Brito, Bazzoli, 2009; Tovar-Bohorquez *et al*., 2009; Gómez-Ramírez *et al*., 2011; Obando-Bulla *et al*., 2013) and *A. anisitsi* (Gonçalves *et al.,* 2005; López *et al*., 2005; Zayas, Cordiviola, 2007). However, some structures related to reproduction, such as the pituitary gland, have never been explored.

Taking into account the importance of deepening the knowledge of structures related to reproduction, the brain atlas (Obando-Bulla *et al*., 2013; Rincón *et al*., 2016a, 2017) and also a histological and anatomical comparison of the pineal complex in *P. axelrodi* and *A. anisitsi* (Rincón *et al*., 2016b) have been recently published. In this context, considering that we intend to continue assessing the brain-pituitary-gonadal axis, the present study assesses a morphological, anatomical and immunohistochemical characterization of the pituitary gland of both species for the first time.

MATERIAL AND METHODS

Animals. Adults of *Paracheirodon axelrodi* and *Aphyocharax anisitsi* were obtained from commercial aquaria. In both cases, animals were housed in community aquaria mimicking their natural conditions: 25°C for both species, pH 6.0 to 7.0 for *A. anisitsi* and pH 5.0 to 6.0 for *P. axelrodi*. Fish were fed twice a day with commercial fish pellets (Tetra ®) and acclimatized for at least one month before being used. All procedures described in the following sections were conducted in accordance with international standards, Guide for Care and Use of Laboratory Animals (NRC, 2011) on animal welfare as well as being compliant with local regulations (CICUAL, Comision Institucional para el Cuidado y Uso de Animales de Laboratorio).

Eight adult specimens, 5 females and 3 males of *P. axelrodi*, of standard length (L_S) of 2.86 ± 0.10 cm, total length (L_T) of 3.36 ± 0.38 cm and weight of 0.42 ± 0.17 g were used. Two females and two males were used for classical histological procedures and three females and one male for immunohistochemical assays. Four females and four adult males of *A. anisitsi* of L_S 3.08 ± 0.04 cm, L_T of 3.71 ± 0.06 cm and weight of 0.57 ± 0.03 g were processed. Two females and two males were used for histological studies and two females and two males for immunohistochemical procedures. Voucher specimens are deposited at the Ichthyological collection of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN-Ict 12710), Buenos Aires, Argentina.

Histological analysis of the pituitary gland. Animals were anesthetized with benzocaine (0.1 g/l) and killed by decapitation. To preserve both pituitary´s histology and its anatomical relationship with the brain, whole heads were dissected and fixed in Bouin's solution for 24h at 4°C in the dark. Afterwards, heads were dehydrated through an ascending series of ethanol, clarified with xylene and embedded in Paraplast ® (Sigma). Embedded heads were sagitally and transversally sectioned at 7 µm and mounted on gelatine-coated glass slides. Then, these sections were deparaffinized in xylene, rehydrated through a descending ethanol series and stained with haematoxylin eosin (H-E), Masson trichrome (MT) and periodic acid-Schiff (PAS). Finally, slides were mounted in DPX (Sigma), examined using a Nikon Microphot FX microscope and digitally photographed (Coolpix 4500, Nikon; Japan).

Immunohistochemical analysis of the pituitary gland. For this study, the specimens were processed as described for the histological analysis. Briefly, heads (with pituitaries) were sagittal sectioned at 7 µm and mounted on different slides. They were deparaffinized in xylene, and rehydrated through a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4). Sections were treated with 3% H_2O_2 for ten minutes to saturate endogenous peroxidase activity, after which they were incubated with 5% non-fat dry milk to block unspecific binding sites. Then, slides were incubated overnight at 4°C, in a moist chamber, with different specific primary antisera (antiserum and dilutions used are detailed on Tab. 1). After washing with PBS, sections were incubated for 45 min with a biotinylated anti-rabbit IgG diluted 1:600 (Dako), at room temperature in a moist chamber. Amplification of the signal was achieved by incubating the sections with peroxidase-conjugated streptavidin (STRP-HRP) (Dako) diluted 1:600, at room temperature in a moist chamber, and visualized with 0.1% 3.3'-diaminobenzidine with

TABLE 1 | Primary antisera dilutions used in the immunohistochemical analysis. All the antisera used in this study were kindly provided by 1 Dr. A. Shimizu. National Research Institute of Fisheries Science, Fisheries Research Agency, Kanazawa, Yokohama, Japan. 2NIDDK. The National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Marylan. ACTH: adrenocorticotropic hormone; FSH: follicle-stimulating hormone; GH: growth hormone; LH: luteinizing hormone; NS, not supplied; PRL: prolactin; SL: somatolactin.

Antiserum raised against	Code	Made in	Dilution	Provided by
Chum salmon β-FSH	2687	Rabbit	1:200	Dr. A. Shimizu[1]
Chum salmon β-LH	2685	Rabbit	1:200	Dr. A. Shimizu[1]
Mummichog β-FSH	003	Rabbit	1:2000	Dr. A. Shimizu[1]
Chum salmon PRL	8206	Rabbit	1:1000	Dr. A. Shimizu[1]
Chum salmon SL	8906	Rabbit	1:2000	Dr. A. Shimizu[1]
Chum salmon GH	8502	Rabbit	1:1000	Dr. A. Shimizu[1]
Human ACTH+	NS	Rabbit	1:500	NIDDK[2]
Human TSH	M3503	Mouse	1:100	Dako

0.03% H_2O_2. Sections were slightly counterstained with haematoxylin, mounted in DPX and examined.

For β-FSH and β-LH antigen immunoreactivity was recovered. Before the block of endogenous peroxidase activity, samples were heated for 10 min at 90°C with citrate buffer (10mM, pH=6) for epitope unmasking, cooled at room temperature (RT) and washed with PBS. Finally, the slides continued with the immunohistochemical method described above. Negative controls (data not shown) were achieved by omission of the primary antisera and preabsorption tests performed on other species showed antisera specificity (see Pandolfi *et al*., 2006; Honji *et al*., 2013). Furthermore, the specificity of the antisera could also be inferred from the localization of the immunostained cells, which agree with previously reports in other neotropical fish species (Borella *et al*., 2009; Honji *et al*., 2013; Nóbrega *et al*., 2017).

RESULTS

Anatomical description of the pituitary gland. The pituitary glands of both *P. axelrodi* and *A. anisitsi* appears attached to the ventral region of the hypothalamus (H) by a thick infundibular stalk and consists of two components: the neurohypophysis (NH) and the adenohypophysis (ADH). In both species, the NH consists of a well-developed infundibular stem, and the ADH is globular with its three areas located in an anterior-posterior position. According to these characteristics, the pituitary gland of both species is of the leptobasic type (Fig. 1C-E). The ADH is subdivided in three different areas: a rostral part of the gland, *rostral pars distalis* (RPD), a central part, the *proximal pars distalis* (PPD), and a posterior part, *pars intermedia* (PI). Additionally, each

of these areas are invaded by the NH, in a greater proportion in the PPD and PI than RPD, and present different cell types with distinct staining characteristics (Figs. 1, 2). Most of the individuals used for this study were mature males and females. Ovaries had vitellogenic oocytes in greater proportion than earlier oogenetic stages, while testis presented mature spermatozoa in their lumen. No differences were observed in the distribution of pituitary cells with respect to the different phases of the reproductive cycle of individuals (data not shown).

Histochemistry and immunohistochemistry. Rostral pars distalis (RPD). In both species, PRL and ACTH cells are spherical, acidophilic and chromophobic (with H-E and MT) (Figs. 1A–D, 2B, C), and PAS-negative (Figs. 1E, F; 2E, G). Immunoreactive (ir) PRL and ACTH cells are observed in the dorsal and ventral portions of *A. anisitsi* (Fig. 3A–D) and *P. axelrodi* (Fig. 4A–D). In the most dorsal portion, ir-ACTH cells are close to the neurohypophysis, while in the more ventral region they are isolated or grouped where PRL cells are settled. Particularly, in *P. axelrodi,* ir-βFSH and ir-βLH cells are observed in the dorsal part of the RPD. These cells are spindle-shaped, intermingled with PRL cells and they are in close relationship with the interdigitations of the neurohypophysis (Figs. 4A, B; 6A–D). In the negative control, no ir-PRL or ir-ACTH cells were detected (data not show).

Proximal pars distalis (PPD). In both species, one group of cells are ovoid with large central nucleus, larger, acidophilic (H-E), blue with MT (Figs. 1A–D; 2B, C) and PAS-negative (Figs. 2E, G). These cells correspond with the location of ir-GH cells (Figs. 5E, F; 6E, F). Another group of cells are basophilic (H–E), violet with MT (Figs. 1A–D; 2B, C) and PAS-positive (Figs. 2E, G). These cells overlap with ir-βFSH and ir-βLH cells (Figs. 5A-D; 6A-D). The ir-βFSH and ir-βLH cells show a dispersed distribution, closer to the interdigitations of the NH and interspersed with ir-GH cells that are dorso-ventrally located, more distant to the NH (Figs. 5, 6). Moreover, in *P. axelrodi,* ir-βFSH and ir-βLH fibers are also observed in the NH (Figs. 5A, C). In the negative control, no ir-GH, ir- βFSH or ir-βLH cells were detected (data not show).

Pars intermedia (PI). In both species, ir-SL cells (Figs. 3F, G; 4F, G) are spherical, acidophilic (Figs. 1A, B; 2D), PAS-positive (Figs. 1E, F; 2F, H), and are close to the neurohypophyseal fibers that penetrate deeply into the PI. In *P. axelrodi* specifically, ir-SL cells are observed in the PPD too. Ir-MSH cells (Figs. 3C, E; 4C, E) are basophilic with H-E (Figs. 1A, B; 2D) and PAS-negative (Figs. 1E, F; 2F, H). Ir-ACTH and ir-MSH cells were recognized by the antibody against ACTH, since it recognizes a common region shared by both peptides, which derive from the same precursor molecule, proopiomelanocortin (POMC). These MSH cells show an elongated shape and are close to the neurohypophyseal fibers interspersed with ir-SL cells. In the negative control, no ir-SL or ir-ACTH cells in the negative control were detected.

Finally, Fig. 7 shows a *camera lucida* drawings of sagittal sections of the pituitary gland, obtained by histological and immunohistochemical analysis, showing the distribution of adenohypophyseal cells of *P. axelrodi* (**A**) and *A. anisitsi* (**B**).

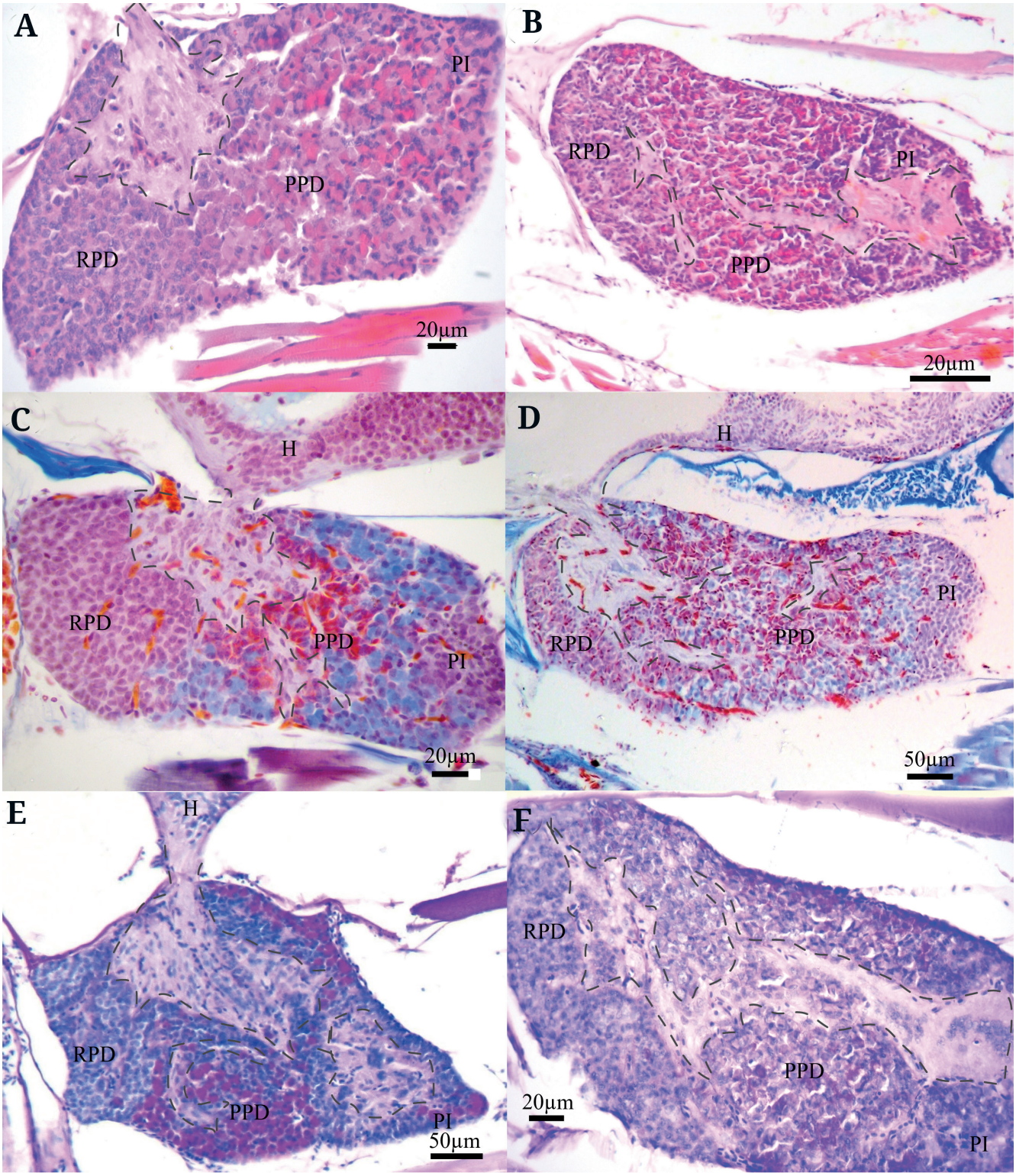

FIGURE 1 | Microphotographs of sagittal sections of the pituitary gland of *Paracheirodon axelrodi*. **A**. Stained with Haematoxylin-Eosin (H-E), **C**. Masson trichrome (MT), and **E**. Periodic acid-Schiff (PAS). Microphotographs of sagittal sections of the pituitary gland of *Aphyocharax anisitsi*. **B**. Stained with H-E, **D**. MT, and **F**. PAS. RPD: *rostral pars distalis*; PPD: *proximal pars distalis*; PI: *pars intermedia*; NH: neurohypophysis.

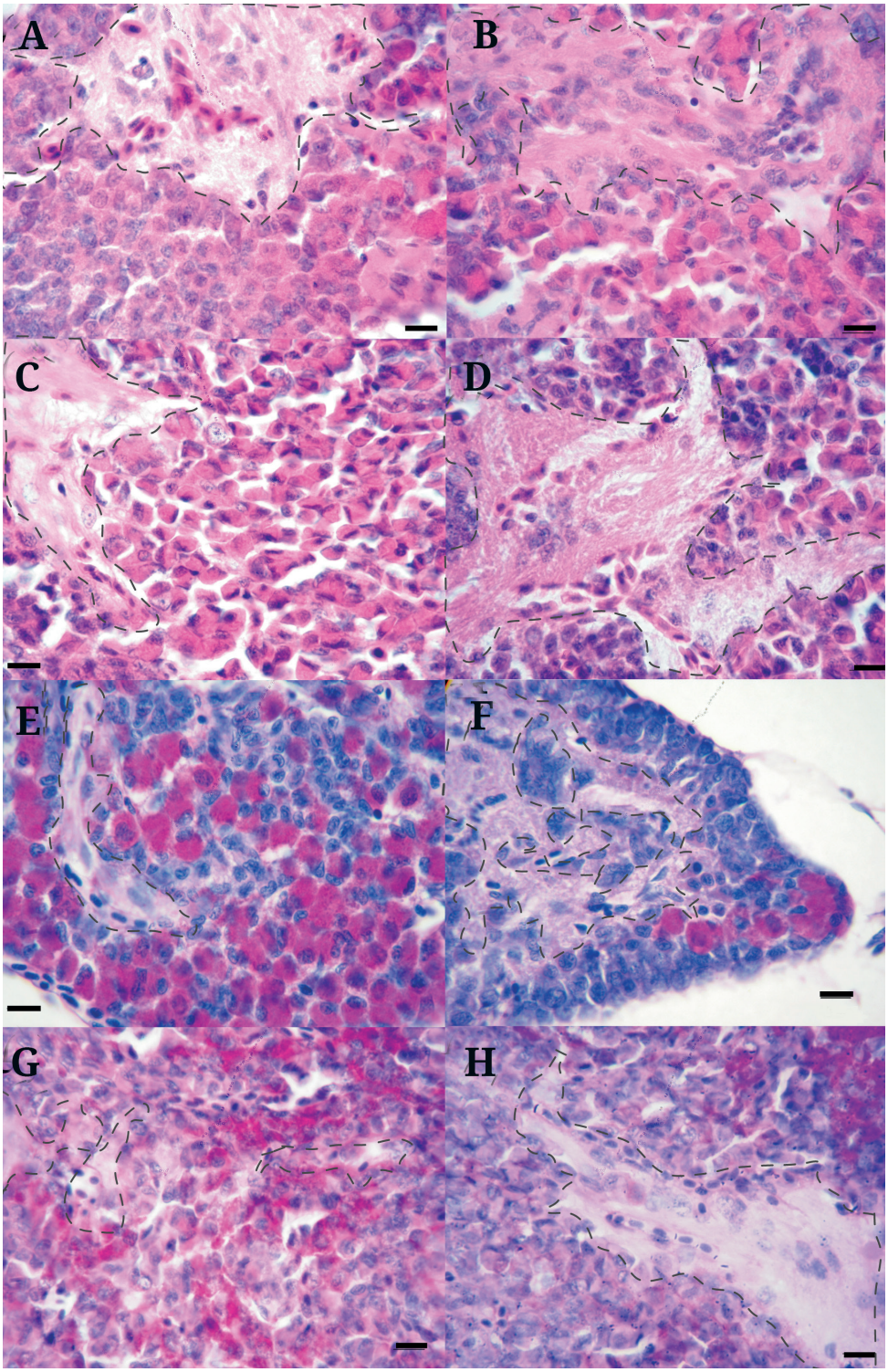

FIGURE 2 | Details of various components of the pituitary gland of *Paracheirodon axelrodi* and *Aphyocharax anisitsi*. Microphotographs of sagittal sections of the **A.** RPD, **B.** PPD in *P. axelrodi* and **C.** PPD and **D.** PI in *A. anisitsi* stained with Haematoxylin-Eosin (H-E). Microphotographs of sagittal sections of the **E.** PPD and **F.** PI of *P. axelrodi* and **G.** PPD and **H.** PI in *A. anisitsi* stained with periodic acid-Schiff (PAS). The delimited gray area corresponds to the neurohypophysis. Black arrowhead: blood vessel; RPD: *rostral pars distalis*; PPD: *proximal pars disalis*; PI: *pars intermedia*. Bars = 10µm.

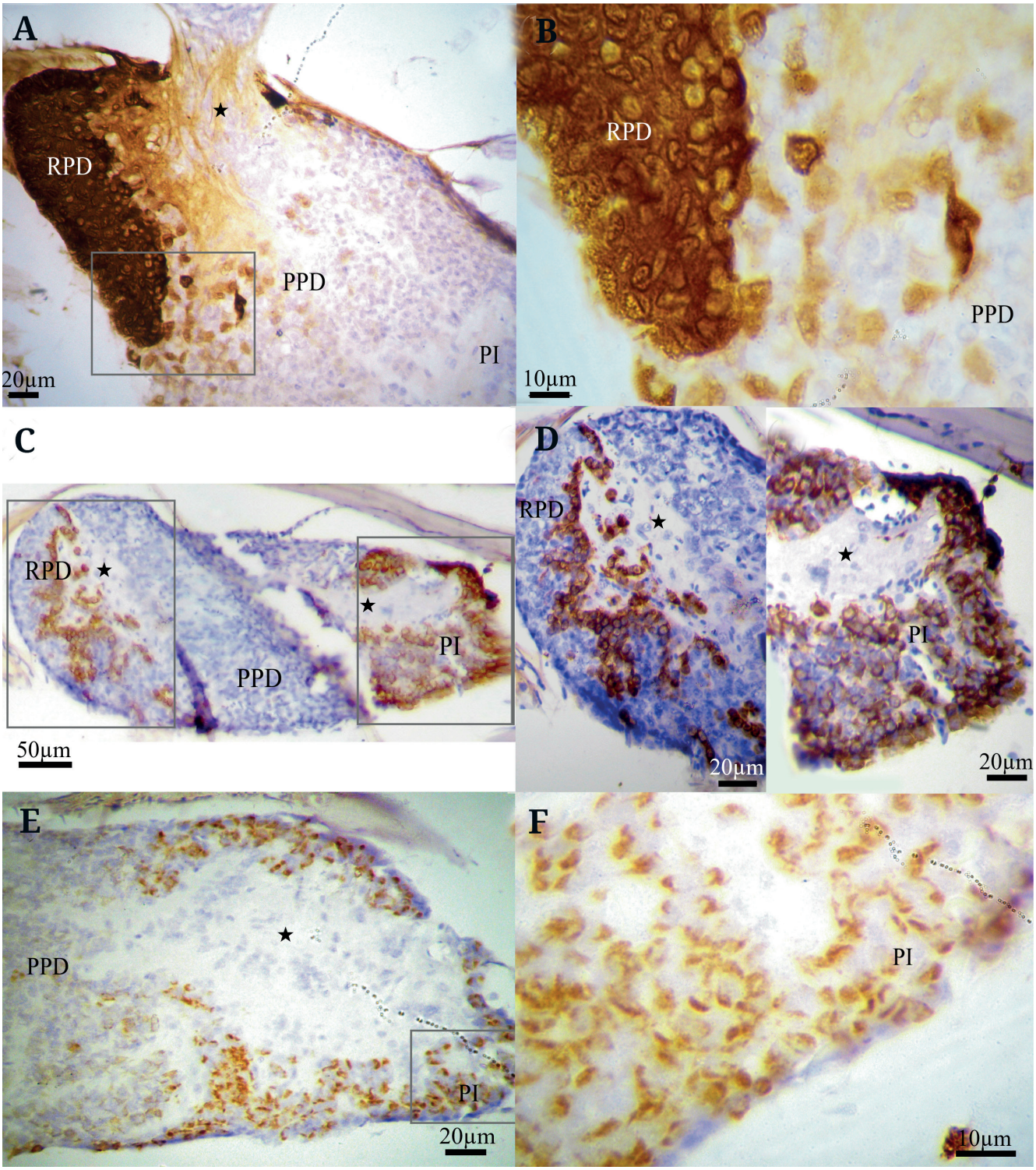

FIGURE 3 | Microphotographs of sagittal sections in the pituitary gland of *Aphyocharax anisitsi* showing the location of PRL- **A.** and ACTH- **C.** ir from the RPD and MSH- **C.** and SL- **F.** ir from the PI. Gray box shows the area of detail microphotographs. Details of PRL- **B.** and ACTH- **D.** ir from the RPD and MSH- **E.** and SL- **G.** ir from the PI. ACTH: adrenocorticotropic hormone; Black star: neurohypophysis; MSH: melanocyte-stimulating hormone; PI: *pars intermedia*; PPD: *proximal pars distalis*; PRL: prolactin; RPD: *rostral pars distalis*; SL: somatolactin.

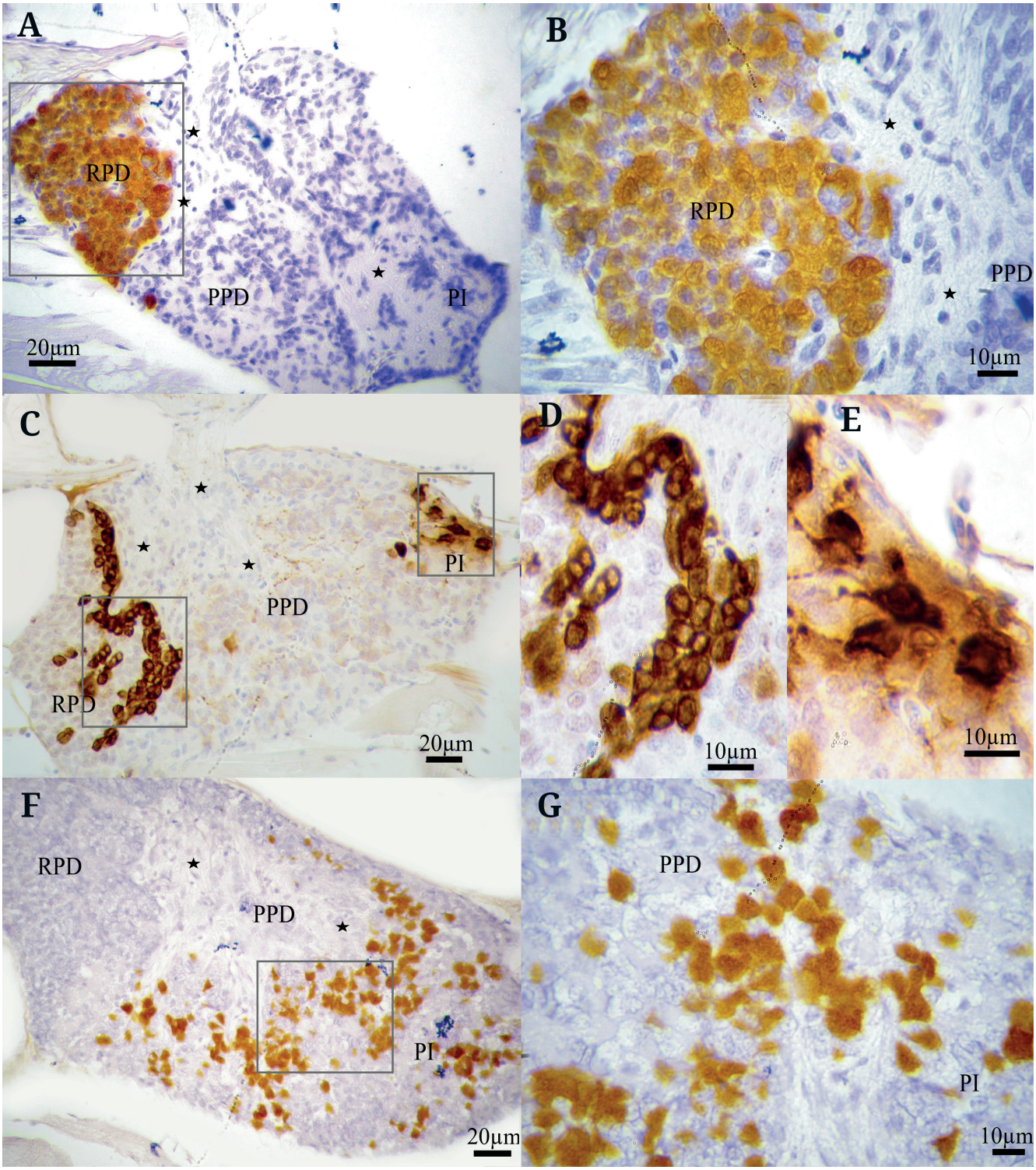

FIGURE 4 | Microphotographs of sagittal sections in the pituitary gland of *Paracheirodon axelrodi* showing the location of PRL- **A.** and ACTH- **C.** ir from the RPD and MSH- **C.** and SL- **F.** ir from the PI. Gray box shows the area of detail microphotographs. Details of PRL- **B.** and ACTH- **D.** ir from the RPD and MSH- **E.** and SL- **G.** ir from the PI. ACTH: adrenocorticotropic hormone; Black star: neurohypophysis; MSH: melanocyte-stimulating hormone; PI: *pars intermedia*; PPD: *proximal pars distalis*; PRL: prolactin; RPD: *rostral pars distalis*; SL: somatolactin.

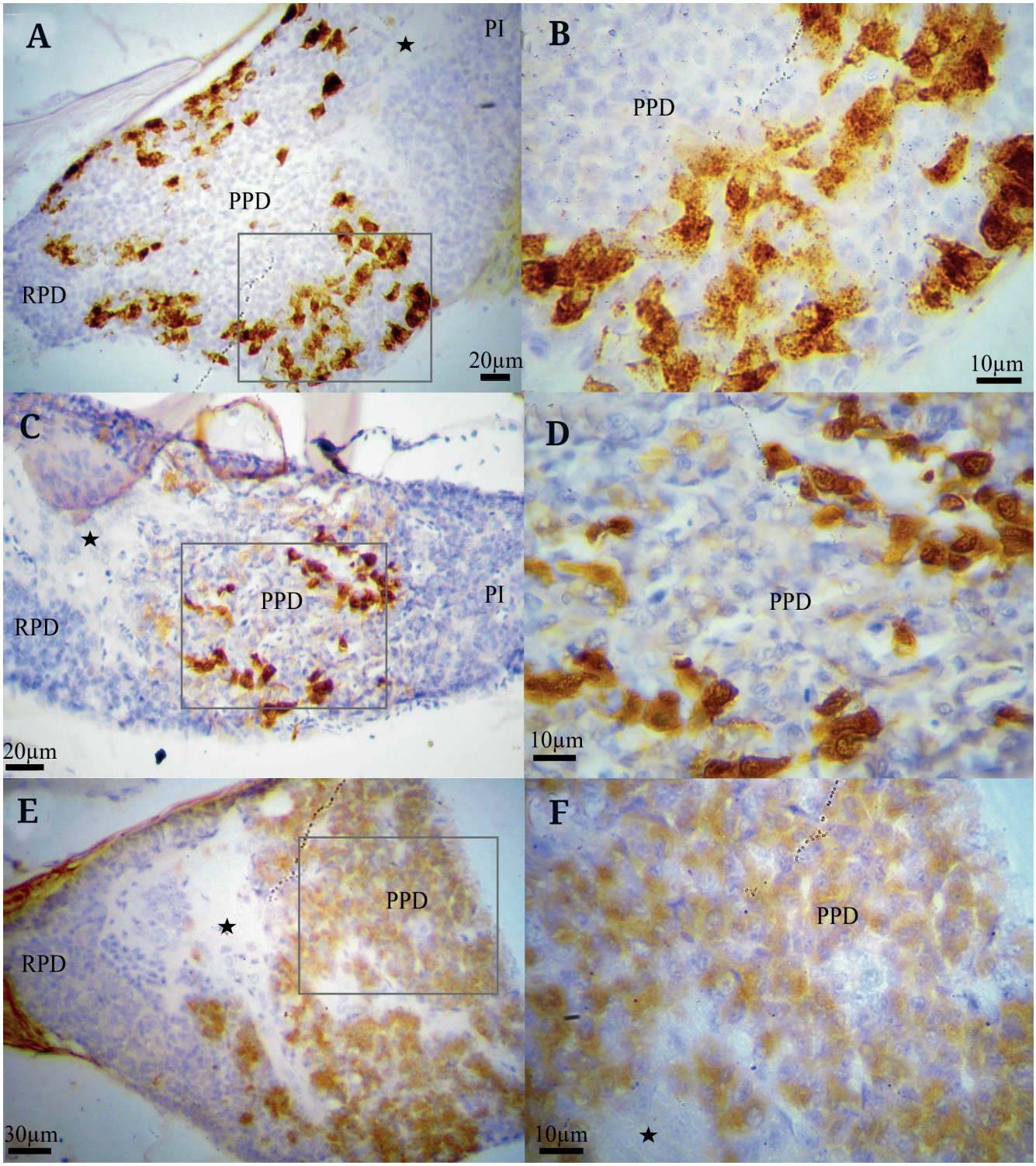

FIGURE 5 | Microphotographs of sagittal sections in the pituitary gland of *Aphyocharax anisitsi* showing the location of βFSH- **A.**, βLH- **C.** and GH- **E.** ir cells of the PPD. Gray box shows the area of detail microphotographs. Details of βFSH- **B.**, βLH- **D.** and GH- **F.** ir cells of the PPD. Black star: neurohypophysis; FSH: follicle-stimulating hormone; GH: growth hormone; LH: luteinizing hormone; PI: *pars intermedia*; PPD: *proximal pars distalis*; RPD: *rostral pars distalis*.

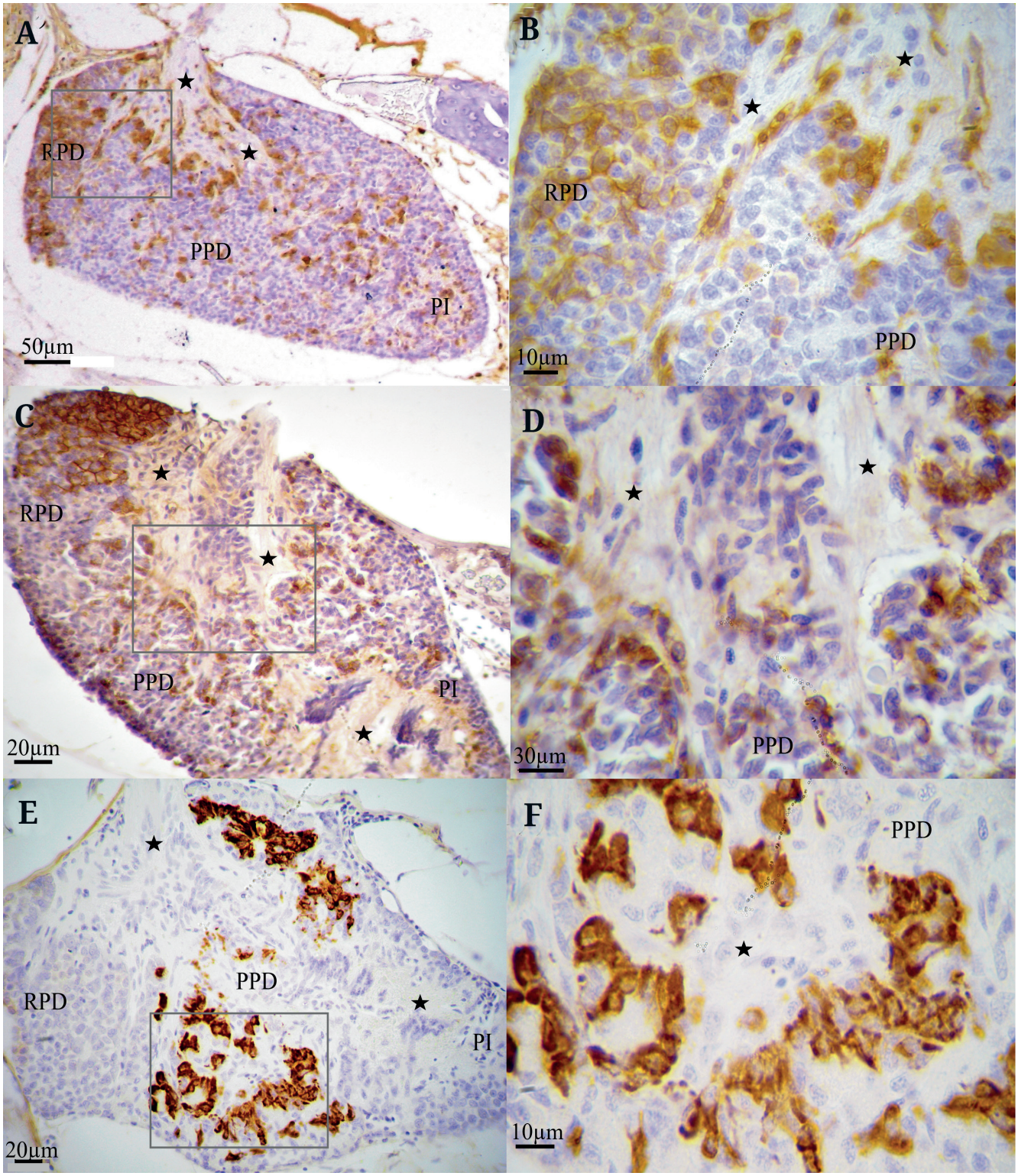

FIGURE 6 | Microphotographs of sagittal sections in the pituitary gland of *Paracheirodon axelrodi* showing the location of βLH- **A.** and βFSH- **C.** ir cells of RPD and PPD and GH- **E.** ir cells of PPD. Gray box shows the area of detail microphotographs. Details of βLH- **B.** and βFSH- **D.** ir cells of RPD and PPD and GH- **F.** ir cells of the PPD. Black star: neurohypophysis; FSH: follicle-stimulating hormone; GH: growth hormone; LH: luteinizing hormone; PI: *pars intermedia*; PPD: *proximal pars distalis*; RPD: *rostral pars distalis*.

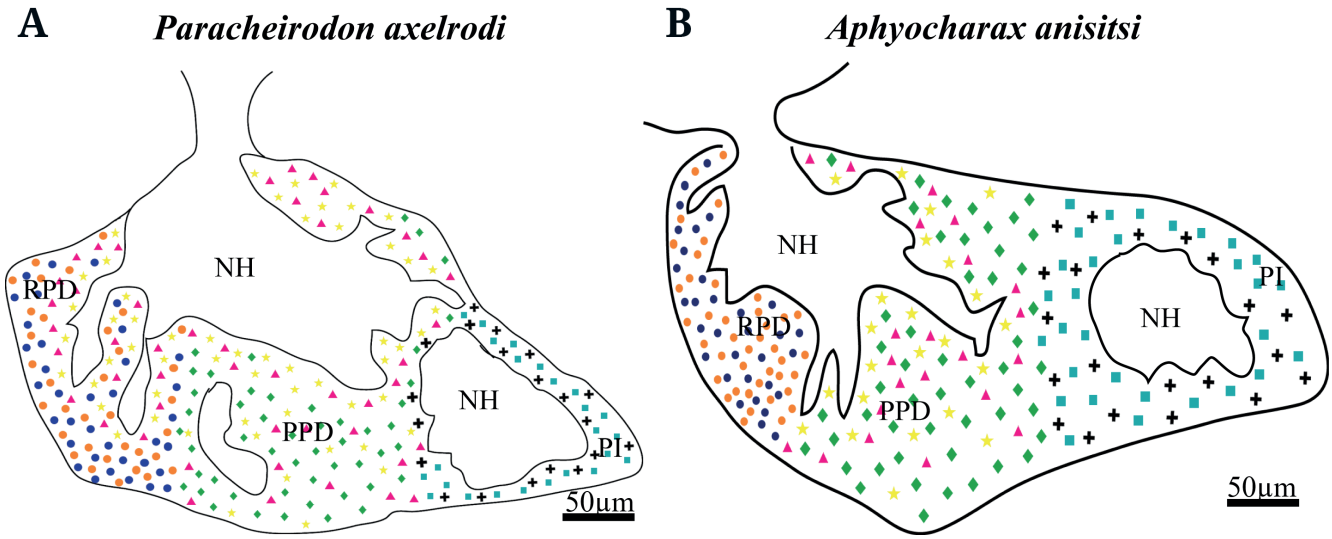

FIGURE 7 | *Camera lucida* drawings of sagittal sections of the pituitary gland, obtained by histological and immunohistochemical analysis, showing the distribution of adenohypophyseal cells of *P. axelrodi* **A.** and *A. anisitsi* **B.**. RPD: *rostral pars distalis*; PPD: *proximal pars distalis*; PI: *pars intermedia*; NH: neurohypophysis. (●) prolactin cells; (●) adrenocorticotropin cells (♦) growth hormone; (▲★) gonadotropin cells; (■) somatolactin cells; (+) melanotropin cells.

DISCUSSION

The pituitary of *P. axelrodi* and *A. anisitsi*, is in the ventral zone of the hypothalamus, and its structural and cellular characteristics coincide with that reported in other teleost species (Pandolfi *et al*., 2001; Borella *et al*., 2009; Kawauchi *et al*., 2009; Zohar *et al*., 2010; Honji *et al*., 2013; Norris, Carr, 2013). According to the scientific literature, the pituitary of both species is the leptobasic type, which is characterized by a neurohypophysis which has a well-developed pituitary stalk, and a globular adenohypophysis (Van Oordt, Peute, 1983).

Although the distribution of adenohypophyseal cells in *P. axelrodi* and *A. anisitsi* is mostly like that described in many teleost species, in general, the identification and detailed distribution of the various types of ADH cells has been investigated in a few reophilic species of South America as: the silver-plated, *Salminus hilarii* Valenciennes, 1850 (Honji *et al*., 2013), the golden, *Salminus brasiliensis* (Cuvier, 1816) (Jesus *et al*., 2014) both of the Characiform order and the tropical catfish, *Steindachneridion parahybae* (Steindachner, 1877) (Honji *et al*., 2015) from the Siluriform order. Generally, the morphological aspects, obtained by histological and immunochemical analyzes, are like those described for *S. hilarii* (Honji *et al*., 2013), chanchita, *Cichlasoma dimerus* (Heckel, 1840) (Pandolfi *et al*., 2001; 2006) and arapaima, *Arapaima gigas* (Schinz, 1822) (Borella *et al*., 2009) among others (Cerdá-Reverter, Canosa, 2009; Kawauchi *et al*., 2009), with some slight differences that we will detail below.

In both species, ir-ACTH and ir-PRL cells were observed disposed in compact groups in the *rostral pars distalis* (RPD). In *P. axelrodi*, particularly, ir-βFSH and ir-βLH cells were also found in the RPD. Ir-PRL cells have previously been demonstrated in the RPD

of many other teleosts (Batten, 1986; Parhar *et al*., 1998; Segura-Noguera *et al*., 2000; Rodríguez-Gómez *et al*., 2001; Sánchez Cala *et al*., 2003), where they were arranged as a compact mass, like in *P. axelrodi* and *A. anisitsi.* This is different than described for *A. gigas* when the ir-PRL cells are scarce, arranged in thin strands, and weakly immunostained with the employed antiserum (Borella *et al*., 2009). PRL is important for the survival of freshwater teleost fishes, since it causes an increase in the concentration of ions and maintains sodium levels in plasma (Pandolfi *et al*., 2001; Kawauchi *et al*., 2009; Zohar *et al*., 2010; Honji *et al*., 2013; Norris, Carr, 2013). Several studies have shown that PRL also influences fish reproductive cycling, as PRL may be involved in spermatogenesis, vitellogenesis and ovulation, beyond its important osmoregulatory function in fish (Edery *et al*., 1984; Sandra *et al*., 2000; Cavaco *et al*., 2003; Whittington, Wilson, 2013). In teleosts, ir-ACTH cells are typically distributed in palisade at the RPD, between PRL cells and branches of neurohypophysial tissue (Schreibman *et al*., 1973; Van Oordt, Peute, 1983; Borella *et al*., 2009), as seen in *P. axelrodi* and *A. anisitsi*. ACTH stimulates the interrenal gland to produce cortisol that is related with several physiological processes: stress response, metabolism, osmoregulation, among others (Pandolfi *et al*., 2001; Laiz-Carrión *et al*., 2003; Borella *et al*., 2009; Kawauchi *et al*., 2009; Honji *et al*., 2013).

Within the *proximal pars distalis* (PPD), ir-GH, ir-βFSH and ir-βLH cells were found dorsoventrally distributed, for both species. Ir- βFSH and ir-βLH cells abundant in an area close to the fibers of the neurohypophysis with a round or oval shape, these characteristics agree with the typical described for several teleosts (Van Oordt, Peute, 1983; Agulleiro *et al*., 2006). βFSH cells were characterized with both heterologous antibodies, anti-chum salmon and anti-mummichog βFSH, obtaining a weaker immunostaining with the first one. The processes of ovulation and secretion of gonadal steroids, depend mostly βLH, although βFSH in fish has also a steroidogenic function (Levavi-Sivan *et al*., 2010). Likewise, βFSH stimulates the early follicular development and the preparation of the gonads for the posterior actions of βLH (Pandolfi *et al*., 2006; Borella *et al*., 2009; Zohar *et al*., 2010; Norris, Carr, 2013).

Even if in *A. anisitsi* no immunostaining was observed in RPD, in *P. axelrodi* ir-βFSH and ir-βLH cells are observed in the dorsal part of this region, as already reported in *A. gigas* (Borella *et al*., 2009). This localization in the RPD constitutes a novel result, since in other teleost species such as *C. dimerus* and *Bagrus bayad* (Fabricius, 1775) gonadotropins are found both in the dorsal and ventral portions of PPD, as well as around the PI (Pandolfi *et al*., 2006; Mousa *et al*., 2012). Moreover, in *P. axelrodi*, ir-βFSH and ir-βLH fibers are also observed in the NH. This coincides with the reported distribution in *C. dimerus, C. dimerus* suggested that, even if the precise role of brain-derived FSH and LH is not clear, the abundance of immunopositive fibers in the NH could imply a neuromodulatory function together with a possible role in the control of pituitary cells activity (Pandolfi *et al*., 2006). More detailed studies are necessary to confirm the pattern of distribution of βFSH and ir-βLH cells in *P. axelrodi*.

On the other hand, previous studies demonstrated that antiserum raised against human-β-TSH selectively cross-react with the TSH-producing cells of several teleost species (García-Hernández *et al*., 1996; Segura-Noguera *et al*., 2000), although a weak immunoreactivity to this antiserum was observed in some teleosts too (Nozaki *et al*., 1990; Yan, Thomas, 1991). In this work, we could not detect ir-TSH cells with the

antibody used (data not shown). Considering that TSH has been shown in other teleost species before, the lack of immunolabelling in our experiments is probably due to the specific antibody chosen for this study and it will be interesting to describe the localization of TSH in *P. axelrodi* and *A. anisitsi* using other antibodies in future studies.

Finally, ir-GH cells were larger than the former with oval shape, some of them with vacuoles in the cytoplasm, this feature agree with other teleosts (Rendon *et al*., 1997; Vissio *et al*., 1997; Parhar *et al*., 1998; Segura-Noguera *et al*., 2000; Rodríguez-Gómez *et al*., 2001; Sánchez Cala *et al*., 2003). These cells were frequently found in teleosts surrounding the neurohypophysial processes of the PPD (Van Oordt, Peute, 1983), as seen in *P. axelrodi* and *A. anisitsi*. For example, this arrangement was not observed in *A. gigas* pituitary, as there are no NH branches penetrating the *pars distalis* (Borella *et al*., 2009). GH has the effect of increasing cellular metabolic rates, and it also induces the liver cells to produce somatomedins. These, in turn stimulate the mitotic indexes of chondrocytes of the epiphyseal plate and therefore promotes the lengthening of the bones and, consequently, growth (Pandolfi *et al*., 2001; Borella *et al*., 2009; Honji *et al*., 2015).

In the *pars intermedia* (PI), ir-MSH and ir-SL cells were found surrounding the neurohypophyseal branches. In the *pars intermedia*, the identified ADH cells release somatolactin (SL) and melanocyte stimulating hormone (MSH). On both species, the ir-SL cells present in the PI of pituitary gland were distributed mainly in the peripheral areas of this region, forming cords or clusters surrounding the neurohypophysial tissue and blood capillaries, apparently intermingled with the MSH cells (Batten, 1986; Cambré *et al*., 1986; Quesada *et al*., 1988; Vissio *et al*., 1997; Rodríguez-Gómez *et al*., 2001). However, in *P. axelrodi* specifically, ir-SL cells are observed in the PPD too. Similar to results showed for *S. hilarii* (Honji *et al*., 2013), where is observed a cross-reaction of antisera against chum salmon SL with some cells in PPD region. According to Margolis-Kazan *et al.* (1981) and Batten (1986) the SL and gonadotropins (βFSH and βLH) hormone can contain antigenically similarity, which might be difficult to remove during biochemically purification.

The SL function in teleost fishes is not yet fully established, since mode of action seems to vary between species. For example, in salmonids, SL has been reported to be involved in sexual maturity and smoltification (Rand-Weaver *et al*., 1992; Bhandari *et al*., 2003; Borella *et al*., 2009; Onuma *et al*., 2010). In other species, however, it has been associated with in the adaptation to background and photoperiod changes, in metabolism, stress response and reproductive physiology (Zhu, Thomas, 1997; Mousa, Mousa, 1999; Rand-Weaver *et al*., 1992; Vissio *et al*., 2002; Honji *et al*., 2013). On the other hand, α-MSH participates in the adaptation to skin color and in the stimulation of ACTH release during stress (Lamers *et al*., 1992; Laiz-Carrión *et al*., 2003; Borella *et al*., 2009).

There are three reproductive dysfunctions in teleost fish are frequently observed in many species when breeding in captivity, according to Zohar, Mylonas (2001) and Mylonas *et al*. (2010). First, female vitellogenesis and male spermatogenesis fail completely when broodstocks are maintained in captivity. Second, cultured females fail to spawn at the end of the reproductive cycle (oocytes undergo normal vitellogenesis, final maturation, and ovulation; however, ovulated eggs are not released to the water). Third, vitellogenesis appears to progress normally in cultured females, but oocytes fail to reach final maturation, resulting in neither ovulation nor spawning. The second case of dysfunction could be the case of *P. axelrodi* specifically, since gonadally mature females and males are normally

found, however, ovulated eggs or sperm are not released to the water.

Nevertheless, before planning the manipulation of ADH hormone secretion for this species, which will enhance fish reproduction in captivity, it is first necessary to establish the morphophysiological parameters of cell types the pituitary gland and specially that involved in the reproductive process. So, this is the first morphological and immunohistochemical study of the pituitary of *P. axelrodi* and *A. anisitsi*. Overall, the pituitary components of *P. axelrodi* and *A. anisitsi* presented very similar histological and immunohistochemical characteristics. The results showed a pattern of distribution of pituitary cells similar to that of other teleost species, with only slight differences in the distribution of βFSH and SL for *P. axelrodi*.

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Laura Rincón Camacho: Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing (original draft), Writing (review & editing).

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ETHICAL STATEMENTS

All procedures were conducted in accordance with international standards, Guide for Care and Use of Laboratory Animals (NRC, 2011) on animal welfare as well as being compliant with local regulations (CICUAL, Comision Institucional para el Cuidado y Uso de Animales de Laboratorio), Protocol #75b.

COMPETING INTERESTS

Not applicable.

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