The Pineal Complex: A Morphological and Immunohistochemical Comparison Between a Tropical *(Paracheirodon axelrodi)* and a Subtropical *(Aphyocharax anisitsi)* Characid Species

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ABSTRACT Cardinal neon *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. In this work, we carried out an anatomical, histological and immunohistochemical study of the pineal complex of *P. axelrodi* and *A. anisitsi*. In both species, the pineal complex consisted of three components, the pineal and parapineal organs and the dorsal sac (DS). The pineal organ was composed of a short, thin pineal stalk (PS), vertically disposed with respect to the upper surface of the telencephalon, and a pineal vesicle (PV), located at the distal end of the PS and attached to the skull by connective tissue. The pineal window (PW), a site in the skull where the luminal information accesses the pineal organ, appeared just above the latter structures. In the epidermis of *P. axelrodi's* PW, club cells were identified, but were not observed in the epidermis of *A. anisitsi's* one. With respect to the DS, it appeared to be folded on itself, and was bigger and more folded in *A. anisitsi* than in *P. axelrodi*. Immunohistochemical assays revealed the presence of cone opsin-like and rod opsin-like photoreceptor cells in the PS and PV. These results provide a first insight into the morphological assembly of the pineal complex of both species, and contribute to a better understanding of the integration and transduction of light *stimuli* in characids. J. Morphol. 000:000–000, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: characiformes; opsins; pineal complex; pinealocytes

INTRODUCTION

The pineal complex is a photosensory organ that transduces photoperiodic information into neural (excitatory neurotransmitters) and hormonal (melatonin) rhythmic outputs (Falcon, 1979; Collin et al., 1989; Ekström and Meissl, 1997; Confente et al., 2008; Falcon et al., 2010; Herrera-Pérez et al., 2011; Wanger, 2011). Melatonin is synthesised through four enzymatic steps, from the amino acid L-tryptophan, following the serotonergic pathway (Klein, 2007; Herrera-Pérez et al., 2011). In some teleost fish, it was reported that melatonin reaches its highest levels in complete darkness, allowing the calibration of processes that display seasonal and daily rhythms, such as reproduction, skin pigmentation and thermoregulation (Ekström and Meissl, 1997; Confente et al., 2008; Falcon et al., 2010; Herrera-Pérez et al., 2011; Birba et al., 2014).

In teleosts, the pineal complex comprises a pineal and a parapineal organ (PP) and a dorsal sac (DS; Oksche, 1965; Omura and Oguri, 1969; Falcon, 1979; Ekström and Meissl, 1997; Wanger, 2011). The pineal organ consists of a relatively large pineal vesicle (PV) located dorsal to the forebrain, immediately below or within the skull roof and connected to the

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brain through a slender pineal stalk (PS; Oksche, 1965; Omura and Oguri, 1969; Rüdeberg, 1971; Falcon, 1979). The PP is located close to the *habenular nuclei* of the diencephalic roof, specifically projecting to the left habenula, or the left side of the brain (Concha and Wilson, 2001; Gamse et al., 2005). Both organs develop simultaneously during ontogeny, but reach different levels of differentiation in adults (Oksche, 1965; Ekström and Meissl, 1997; Wanger, 2011). The PV is located underneath a relatively translucent portion of the head, an area usually called pineal window (PW) and related to the entrance of luminal information (Omura and Oguri, 1969; Herwig, 1976; Ekström and Meissl, 1997; Confente et al., 2008; Wanger, 2011).

Cardinal tetra *Paracheirodon axelrodi* (Schultz, 1956) is a characid species endemic from South America which inhabits the Amazon, Orinoco and Río Negro basins (tropical habitat). It inhabits middle water layer's with temperatures between 23°C and 27°C, pH ranging from 5.5 to 7 and hardness oscillating between 5 dH and 12 dH (Geisler and Annibal, 1986; Anjos and Anjos, 2006). It is an omnivore species, tending to be carnivorous and, with respect to breeding, individuals spawn in shaded areas (Walker, 2004; Anjos and Anjos, 2006; Marshall et al., 2007).

Bloodfin tetra *Aphyocharax anisitsi* (Eigenmann and 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 (Lima, 2003; 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).

Both species, cardinal and bloodfin tetra, exhibit high market value and an increasing importance for South American ornamental aquaculture, as the correspond to the fish order of greatest export interest in countries such as Brazil, Colombia, Venezuela and Argentina (Esquivel *et al.*, 2014; Panné, 2014). Some studies have focused on understanding the basic biology of both species; however, some structures related to the reproduction, such as the pineal complex, have never been explored in depth. Here, for the first time, an anatomical, morphological and immunohistochemical analysis was performed on the pineal complex of aforementioned species with special emphasis on their differences to better understand about the *stimuli* integration and transduction in Characidae.

MATERIALS AND METHODS

Animals

Adult individuals of *Paracheirodon axelrodi* (Schultz, 1956) and *Aphyocharax anisitsi* (Eigenmann and Kennedy, 1903) were obtained from commercial aquaria. In both cases, animals were housed in aquaria mimicking their natural conditions: 25 ± 2°C for both species, pH of 6.0–7.0 for *A. anisitsi* and pH 5.0–6.0 for *P. axelrodi*. Fish were fed twice daily with commercial fish pellets (Tetra®) and acclimatized for at least 1 month before analysis. 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, Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio*).

In total, nine adult *P. axelrodi* specimens, standard length (L_S) of 2.52 ± 0.11 cm, a total length (L_T) of 2.88 ± 0.13 cm and a weigh of 0.36 ± 0.05 g, were used. Five of them were used for histological procedures, and four for immunohistochemical assays. For *A. anisitsi*, eight adult were processed, with a L_S of 3.08 ± 0.04 cm, a L_T of 3.71 ± 0.06 cm and a weigh of 0.57 ± 0.03 g, four of them used for histological studies and the rest for immunohistochemical procedures.

Histological Study of the Pineal Complex of *P. axelrodi* and *A. anisitsi*

Animals were anesthetized with (0.1 g/l) benzocaine and killed by decapitation. In order to preserve the pineal position, whole heads were processed so that the brain and the pineal complex maintained their original anatomical relationship.

Heads were decalcified and fixed in a Bouiń's solution (5% acetic acid, 70% saturated picric acid and 25% formaldehyde) for 24 h at 4°C in the dark. Afterwards, heads were dehydrated through a descending series of alcohols (100%, 96%, 90% and 70%), clarified with xylene and embedded in Paraplast® (Sigma). Embedded heads were sectioned at 7 μm thick (sagitally and transversally orientated), and mounted on gelatine-coated glass slides. Then, sections were deparaffinized in xylene, rehydrated through a graded ethanol series and stained with Masson trichrome (Carazzi haematoxylin: haematoxylin 0·125%, potassium alum 6·25%, potassium iodide 0·025%, glycerol 25%; ponceau acid fuchsin: xylidine ponceau 0·67%, orange G 0·67%, acid fuchsin 0·33%, acetic acid 0·2%; 1% fosfomolibdic acid; aniline blue: aniline blue 0·45%, acetic acid 2·5%). Finally, the slides were mounted in DPX (Sigma), examined using a Nikon Microphot FX microscope and digitally photographed (Coolpix 4500, Nikon; Japan).

Immunohistochemical Characterization of Pineal Complex Cells

Photoreceptive cells within the pineal complex were inmunohistochemically studied for both species, using anti-cone opsins and anti-rod opsins antisera (generously donated by W. De Grip), as described by Birba et al. (2014). Briefly, the specimens were processed as described for the histological analysis. Heads were serial sectioned (7 μm thick), each mounted on a different slide; one slide incubated with anti-rod opsins and the other one with anti-cone opsins. Then, the slides were deparaffinized in xylene, and rehydrated through a graded ethanol series to phosphate-buffered saline (PBS, pH 7.4). Transverse sections were treated with 3% H_2O_2 for ten minutes to saturate endogenous peroxidase activity, followed by the blocking of unspecific binding sites with 5% non-fat dry milk. Afterwards, samples were incubated overnight at room temperature in a moist chamber with either a rabbit anti-human long wavelength (LW) cone opsin (1:500 dilution, CERN-874) or a rabbit anti-bovine rod opsin (1:500 dilution, CERN-922). Both antisera have been previously well characterized and successfully used in teleost fish to identify opsins (García-Fernández et al., 1997; Confente et al., 2008; Herrera-Pérez et al., 2011; Birba et al., 2014).

On the next day, sections were washed in PBS and incubated for 45 min with a biotinylated anti-rabbit IgG diluted 1:600 (Dako). Amplification of the signal was achieved by incubating the sections with peroxidase-conjugated streptavidin (STRP-HRP) (Dako) diluted 1:700, and visualized with 0·1% 3,3′-diaminobenzidine with 0·03% H_2O_2. Sections were slightly counterstained with haematoxylin (haematoxylin 0·125%, potassium alum 6·25%, potassium iodide 0·025% and glycerol 25%), mounted in DPX and examined. Retinal sections were used as positive controls. Negative controls were achieved by omission of the primary antisera. For *P. axelrodi*

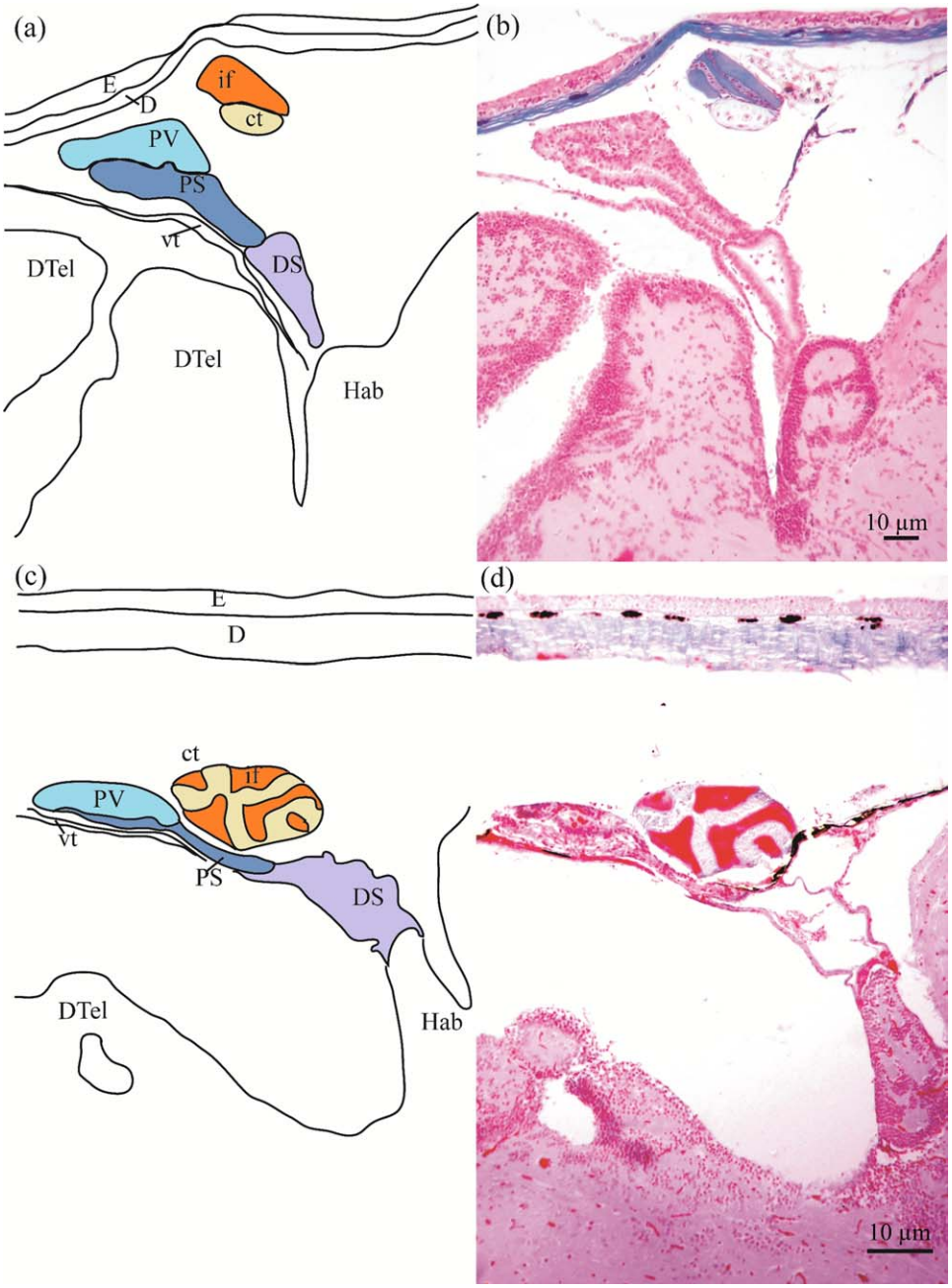

Fig. 1. Sagittal section of the pineal complex of the *P. axelrodi* (**a**, **b**) and *A. anisitsi* (**c**, **d**). (**a** and **c**) *Camera lucida* drawings of the pineal complex (parapineal organ not seen here) located between the telencephalic hemispheres and the habenula. (**b** and **d**) Photomicrographs showing the location of the components of the pineal complex and its relation to the different regions of the brain. ct, cartilage; E, epidermis; D, dermis; DS, dorsal sac; DTel, dorsal telencephalon; Hab, habenula; if, interfrontal junction; PS, pineal stalk; PV, pineal vesicle; vt, velum transversum.

and *A. anisitsi*, three randomly chosen sections form the four specimens where sampled to asses the number of immunostained photoreceptor cells from the PV and PS.

RESULTS

Anatomical and Histological Description of the Pineal Complex

The pineal complex of *P. axelrodi* and *A. anisitsi* was composed of a thin and short PS, a PV located on the distal end of the PS and a DS that appeared to be folded on itself (Fig. 1). In both species, the pineal organ (composed of the PS and the PV) emerged tangentially to the brain, at the midline of the junction of the telencephalic hemispheres with the optic tectum. The PV was located below a translucent, elliptic space, the PW (Fig. 2), which exhibited scattered melanophores, with unpigmented intervening spaces. These unpigmented cells seemed to be in a lesser density in

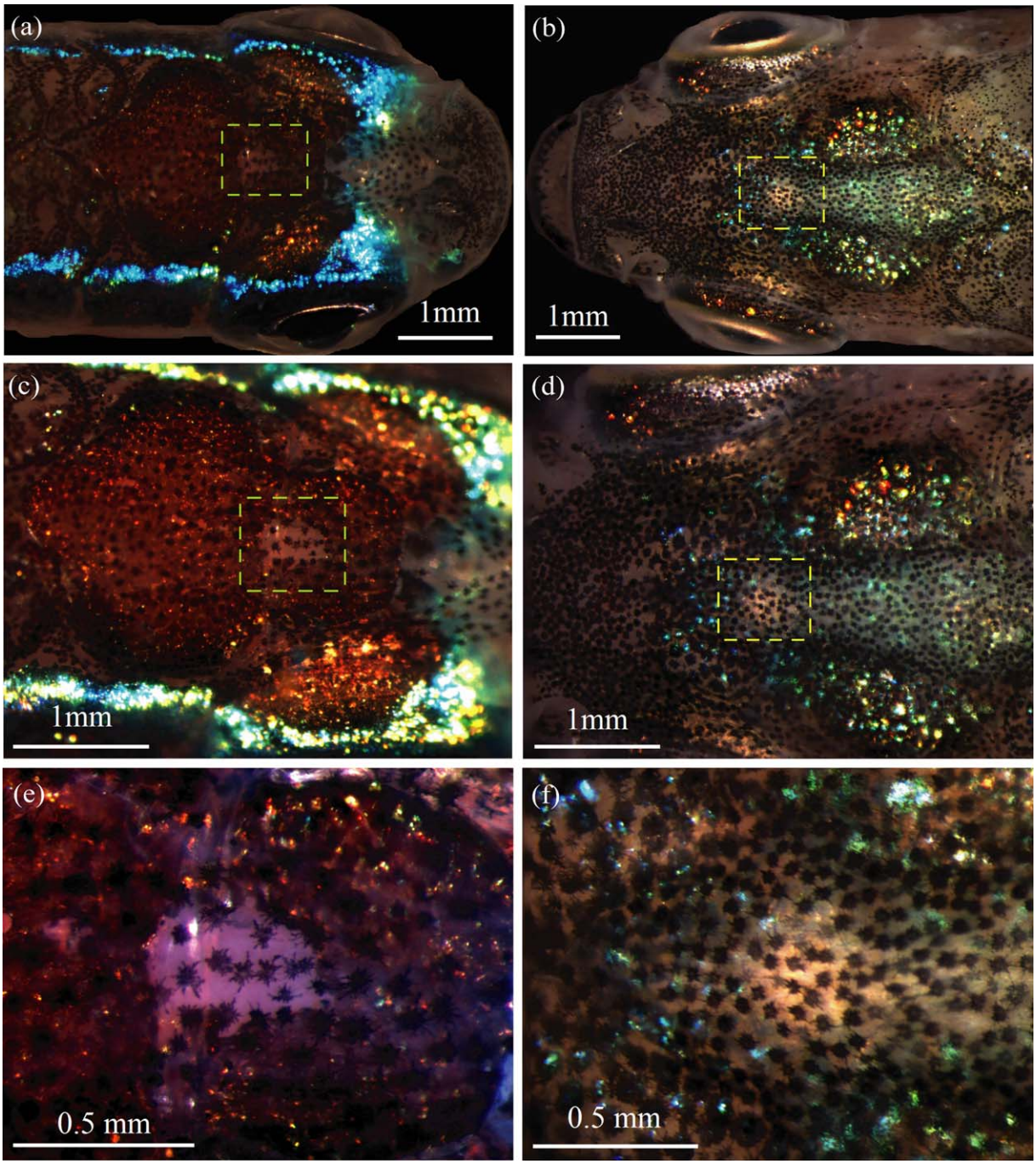

Fig. 2. In vivo photos of *P. axelrodi's* (**a**, **c**, **e**) and *A. anisitsi's* (**b**, **d**, **f**) dorsal surface of the head showing the translucent pineal window (PW). The area enclosed within the dashed squares corresponds to the PW. Detail of dashed squares, where scattered melanophores with unpigmented intervening spaces at the PW of the *P. axelrodi* (**d**) and *A. anisitsi* (**f**) can be observed.

P. axelrodi (Fig. 2A,C,E) compared with *A. anisitsi* (Fig. 2B,D,F). At the level of the PW, a conspicuous fissure allows the light to penetrate (namely front fontanel). In this sense, an ample intracranial space could be appreciated between the dorsal surface of the brain and the roof of the skin (Figs. 3A,B and 4A,B).

In both species, at the level of the PW, it was possible to observe a stratified epidermis, containing Malpighian cells (Mal), dermal collagenic bundles and an hypodermal thick layer with loose connective tissue and some melanocytes at superficial and deep margins (Figs. 5G,H and 7A,C,G). In

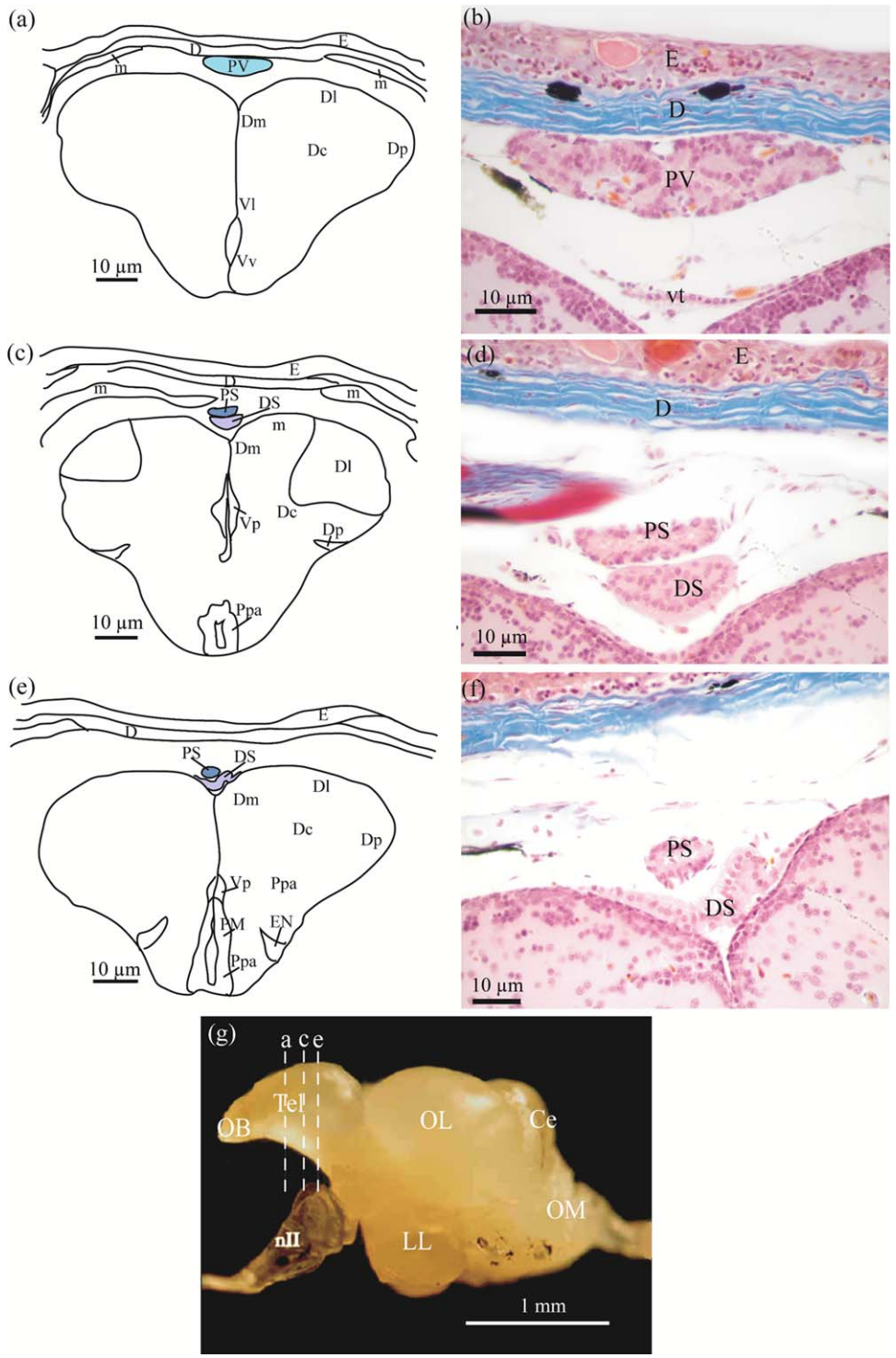

Fig. 3. (**a**) *Camera lucida* drawing of transverse sections of *P. axelrodi* at the level of the pineal vesicle (PV). (**b**) Microphotograph of transverse sections from PV. (**c** and **e**) *Camera lucida* drawing from transverse sections at the level of the pineal stalk (PS) and dorsal sac (DS) (**d** and **f**). Microphotograph of transverse sections from PS and DS. (**g**) Lateral view of *P. axelrodi*'s brain, where the position of the respective transverse sections (**a–f**) is shown. Ce, cerebellum; D, dermis; Dc, central zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Dm, medial zone of the dorsal telencephalic area; Dp, posterior zone of the dorsal telencephalic area; DS, dorsal sac; E, epidermis; EN, entopeduncular nucleus, Hab, habenula; IL, inferior lobes; m, muscle; nII, optic nerve; OB, Olfactory bulbs; OL, optic lobes; OM, oblongata medulla; PM, magnocellular preoptic nucleus; Ppa, anterior part of the parvocellular preoptic nucleus; PS, pineal stalk; PV, pineal vesicle; Tel, telencephalic hemispheres; Vl, nucleo lateral del área telencefálica ventral; Vp, post commissural nucleus of the ventral telencephalic area; vt, velum transversum; Vv, ventral nucleus of the ventral telencephalic area.

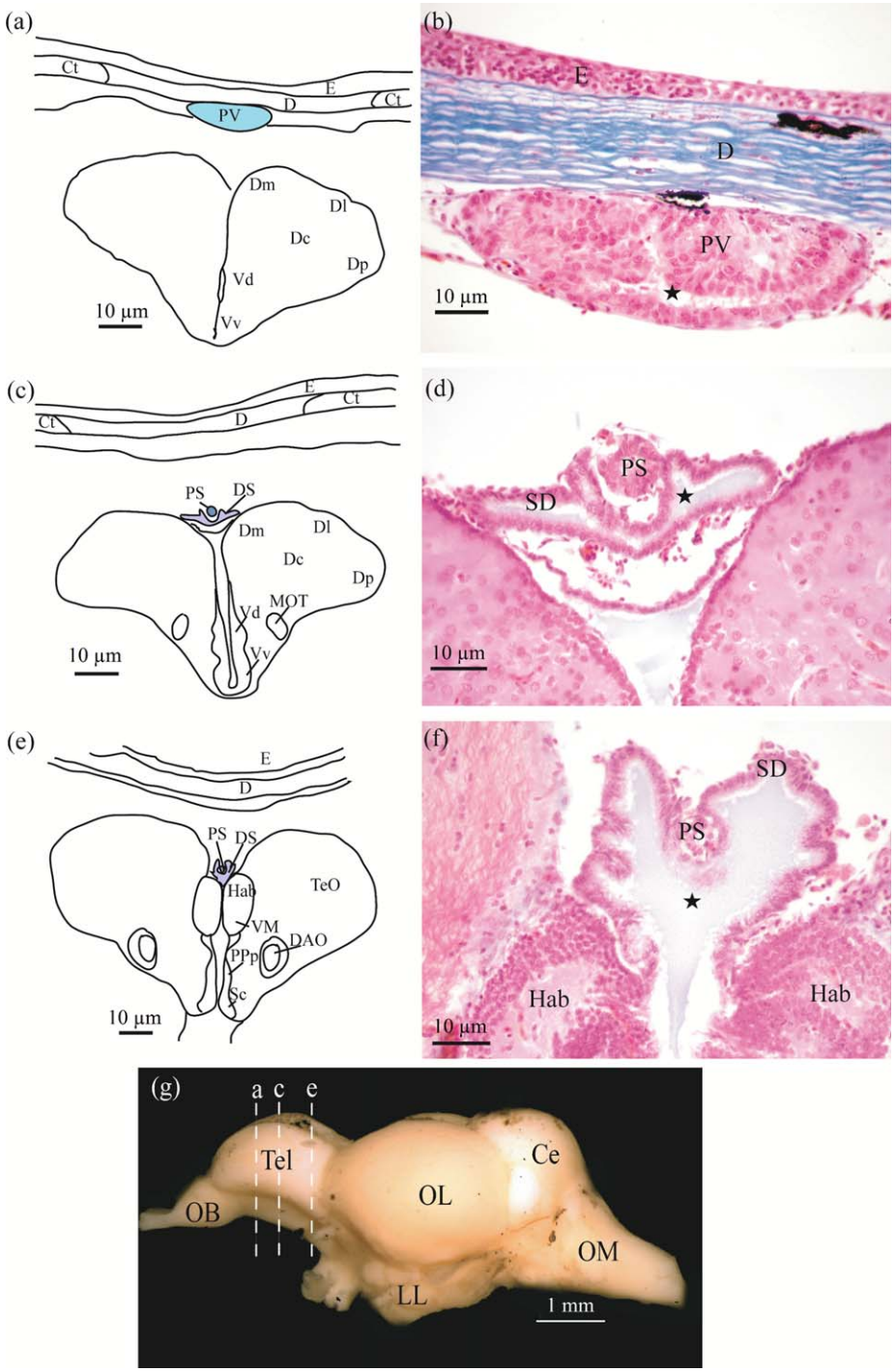

Fig. 4. (**a**) *Camera lucida* drawing of transverse a section of *A. anisitsi* at the level of the pineal vesicle (PV). (**b**) Microphotograph of a transverse sections from the PV. (**c** and **e**) *Camera lucida* drawings from transverse sections at the level of the pineal stalk (PS) and dorsal sac (DS). (**d** and **f**) Microphotograph of transverse sections from PS and DS. (**g**) Lateral view of *A. anisitsi*'s brain. Vertical dashed lines indicate the position of the transverse sections shown on (**a–f**). Ce, cerebellum; Ct, cartilage; D, dermis; DAO, dorsal accessory optic nucleus; Dc, central zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Dm, medial zone of the dorsal telencephalic area; Dp, posterior zone of the dorsal telencephalic area; DS, dorsal sac; E, epidermis; EN, entopeduncular nucleus, Hab, habenula; LL, lower lobes; m, muscle; OM, oblongata medulla; MOT, medial olfactory tract; nII, optic nerve; OB, Olfactory bulbs; OL, optic lobes; PM, magnocellular preoptic nucleus; PPa, anterior part of the parvocellular preoptic nucleus; PPp posterior part of the parvocellular preoptic nucleus; PS, pineal stalk; PV, pineal vesicle; Sc, suprachiasmatic nucleus; Tel, telencephalic hemispheres; TeO, optic tectum; Vl, nucleo lateral del área telencefálica ventral; VM, ventromedial thalamic nucleus; Vp, post commissural nucleus of the ventral telencephalic area; vt, velum transversum; Vv, ventral nucleus of the ventral telencephalic area.

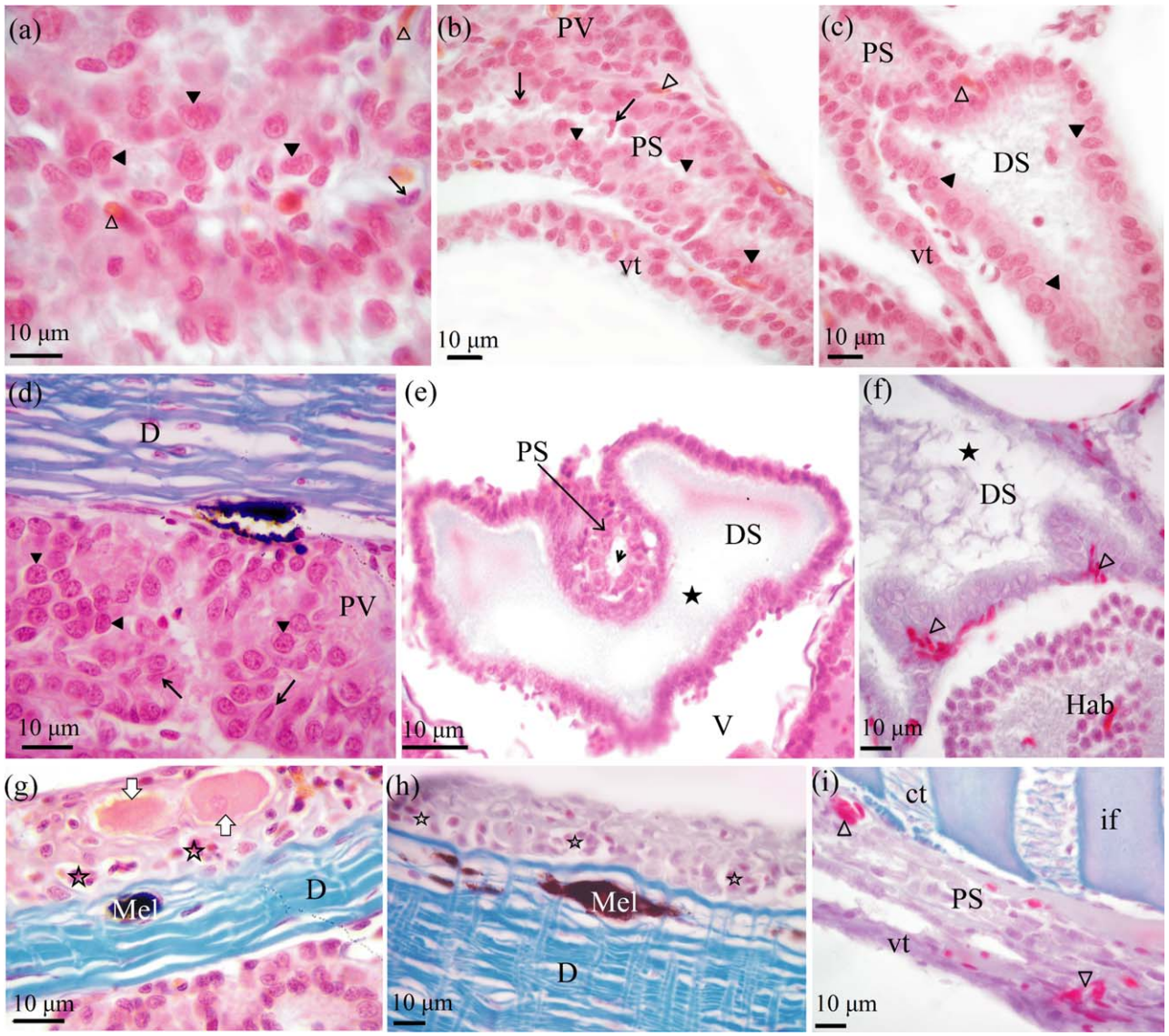

Fig. 5. Details of various components of the pineal complex. Photomicrographs of sagittal sections of the pineal vesicle (PV) (**a**, **b**), dorsal sac (DS) (**b**, **c**) and pineal stalk (PS) (**c**) of *P. axelrodi*. Brain cross-section photomicrographs of the pineal vesicle (PV) (**d**), dorsal sac (DS) and pineal stalk (PS) (**e**) of *A. anisitsi*. Photomicrographs of the DS (**f**) and PS (**i**) of *A. anisitsi* in sagittal sections. Photomicrographs of the epidermis and dermis of *P. axelrodi* (**g**) and *A. anisitsi* (**h**); black arrows, glial-like cell; (▲) pinealocyte; black star, lumen of DS; ct, cartilage; white arrows, club cells; D, dermis; DS, dorsal sac; Hab, habenula; if, interfrontal junction; Mel, melanocytes; PV, pineal vesicle; PS, pineal stalk; Malpighian cells; unfilled stars, (△), blood vessels; V, ventricle; vt, velum transversum; black arrowhead, lumen of the PS.

particular, the PW of *P. axelrodi's* epidermis exhibited eosinophilic (acidophilic) epidermal cells, that is, named club cells (Cb), with a centrally located nucleus (Fig. 7A,C). These cells were not observed in the PW of *A. anisitsi* (Fig. 7G).

In both species, the PV appeared firmly attached to the ventral portion of the PW by a thick layer of connective tissue. The PV of *P. axelrodi* (Figs. 3A,B and 5A) had an antero-posterior length of 15.90 ± 0.15 μm, a dorso-ventral extent of 20.63 ± 1.00 μm at its middle portion part and 59.13 ± 1.53 μm in transverse length. The PV of *A. anisitsi* (Figs. 4A,B and 5D) had an antero-posterior length of 16.53 ± 0.42 μm, a dorso-ventral extent of 26.57 ± 1.95 μm at its middle portion and 76.93 ± 6.73 μm of transverse length. *P. axelrodi's* PV lumen (Figs. 3B and 5A) was almost indistinguishable, whereas that of *A. anisitsi* (Figs. 4B and 5D), although it showed narrow at the lateral and antero-posterior ends, became much more conspicuous in the central region.

The PV parenchyma of *P. axelrodi* and *A. anisitsi* was composed of a stratified folded epithelium that contained a high density of pinealocytes (Fig. 5A,B). Two kinds of cells were observed in this portion: 1) large cells with spherical and violet

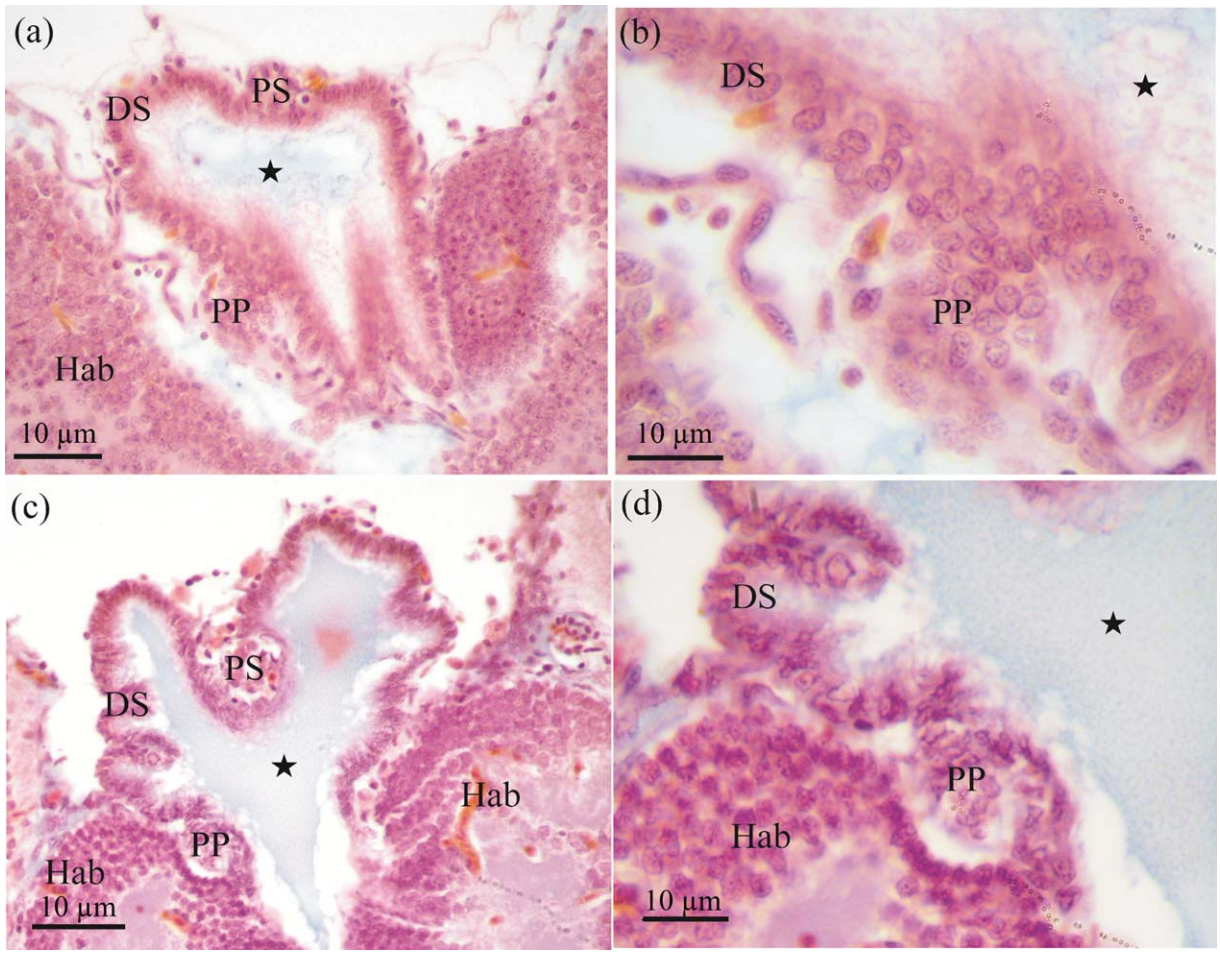

Fig. 6. Detail of the parapineal (PP) of (**a**, **b**) *P. axelrodi* and (**c**, **d**) *A. anisitsi*, located above the habenula (Hab) on the left hemisphere. Black star, lumen of DS; DS, dorsal sac; Hab, habenula; PS, pineal stalk.

coloured nuclei (Fig. 5A,D), that exhibited apical cytoplasmic projections oriented towards the pineal lumen, and 2) smaller cells that showed dark purple and spherical to ovoid nuclei (Fig. 5A,D). The latter cells adopted a more internal position in the pineal parenchyma, probably representing interstitial or glial-like supporting cells.

With respect to *P. axelrodi's* (Figs. 3D,F and 5B) and *A. anisitsi's* (Figs. 4D,F and 5E,I) PS, its rostral portion opened into the PV, whereas the proximal end reached the habenular commissure (Hab), entering the brain at the level of the posterior commissure (Cpost). The PS measured 20.54 ± 3.63 μm in length for *P. axelrodi* and 31.47 ± 8.61 μm for *A. anisitsi*. The parenchyma of the rostral portion was thicker and exhibited a multilayered epithelium, in which an internal, tiny and nonfolded lumen became evident (Fig. 5B,E). The proximal portion was slender and showed simple layers of pineal cells that delimitated a small lumen.

The DS of *P. axelrodi* (Figs. 3D,F and 5C) and *A. anisitsi* (Figs. 4D,F and 5E,F) appeared to be a hollow and folded structure settled at the base of the PS. It consisted of a highly folded simple epithelium composed of closely packed columnar cells, which showed spherical dark violet nuclei. A well-developed vascular system within the epithelial walls of the DS was also observed (Fig. 5C,F).

In both species, the PP appeared in few sections, disposed immediately rostral to the place where the PS enters the habenula (Fig. 6A,C). The PP's lumen was small and appeared to be restricted to the ventromedial margins. It showed surrounded by cells with spherical nuclei that lay closely packed in the dorso-lateral pole of the organ (Fig. 6B,D). The PP adopted an asymmetric position, as was only present in the left hemisphere, and appeared to be connected to the left habenula.

Immunohistochemical Study

The antisera raised against cone opsins (CERN-874) and rod opsins (CERN-922) provided a positive staining in the pineal organ of *P. axelrodi* and

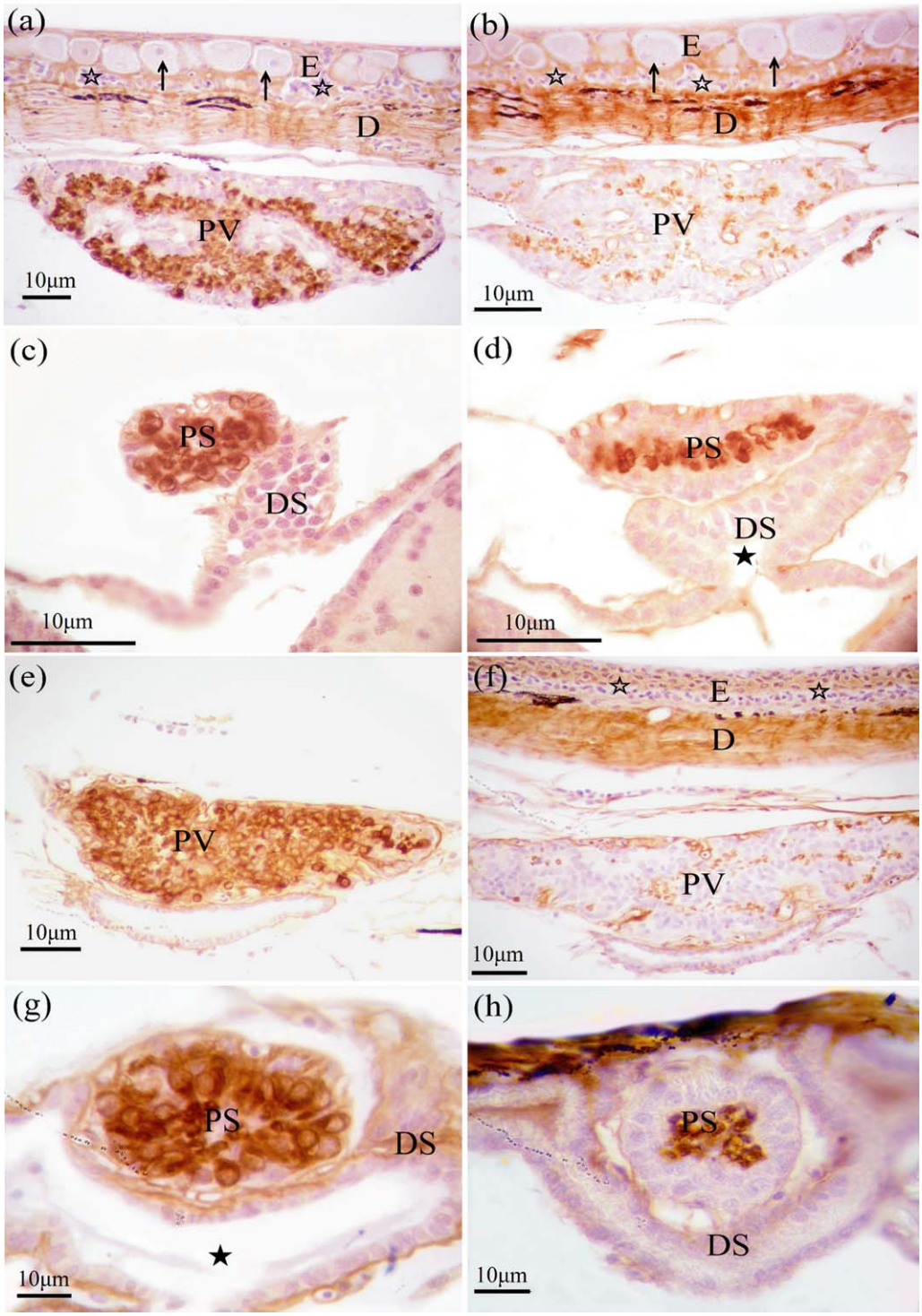

Fig. 7. Localization of immunoreactive cells for visual pigments in the pineal complex of *P. axelrodi* and *A. anisitsi* using rabbit anti-bovine rod opsin (1:500 dilution, CERN-922) and rabbit anti-human LW cone opsin (1:500 dilution, CERN-874). Immunostaining was observed for (**a**) rod opsins and (**b**) cone opsins in cells surrounding the lumen of the PV, and (**c**) rod opsins and (**d**) cone opsins in cells surrounding the PS of *P. axelrodi*. Immunostaining was observed for (**e**) rod opsins and (**f**) cone opsins in cells surrounding the lumen of the PV, and (**g**) rod opsins and (**h**) cone opsins in cells surrounding the PS of *A. anisitsi*. Black arrows, club cells; black star, lumen of the DS; D, dermis; DS, dorsal sac; E, epidermis; Hab, habenula; PV, pineal vesicle; white star, Malpighian cells.

A. anisitsi, showing the photosensitive capacity of pinealocyte cells (Fig. 8C,D,E,F). Rod opsin-like immunostaining was detected in cells from both the PS and PV, with a more evident immunostaining at the thickened ventral wall of the PV and in the anterior and posterior margins of the PS in *P.*

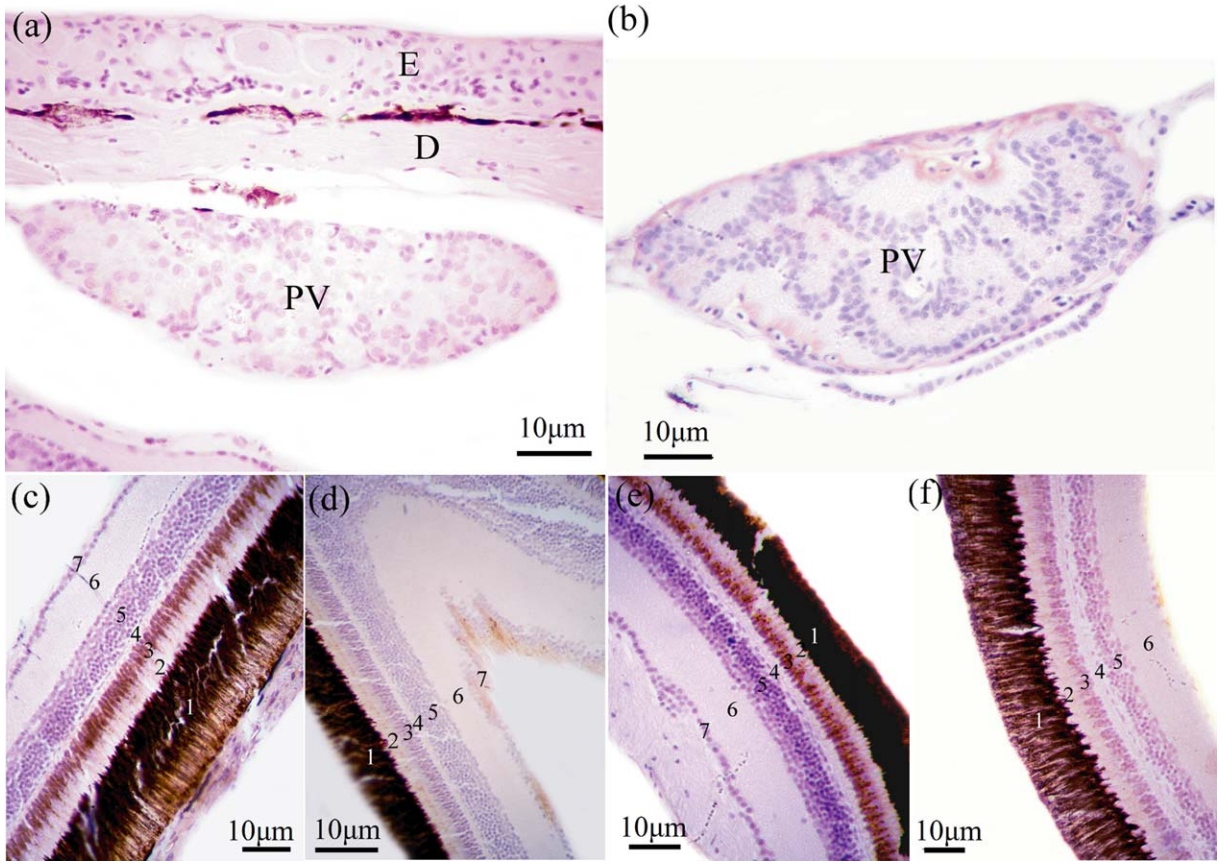

Fig. 8. Negative control (omission of primary antiserum) of (**a**) *P. axelrodi* and (**b**) *A. anisitsi*, showing the absence of immunolabel. Immunostaining for rod opsin (1:500 dilution, CERN-922) of (**c**) *P. axelrodi* and (**e**) *A. anisitsi* and cone opsin (1:500 dilution, CERN-874) of (**d**) *P. axelrodi* and (**f**) *A. anisitsi*, was also observed in the photoreceptor cells of the retina. 1, pigment epithelium; 2, photoreceptor layer (cone and rods processes); 3, outer nuclear layer (photoreceptor cell bodies); 4, outer plexiform layer; 5, inner nuclear layer; 6, inner plexiform layer; 7, ganglion cell layer; D, dermis; E, epidermis; PV, pineal vesicle.

axelrodi (Fig. 7A,C) and *A. anisitsi* (Fig. 7E,G). In *P. axelrodi*, rod opsins ir-cells where on average 57 ± 10 in the PV and 16 ± 6 in the PS for the three randomly chosen sections analysed. For *A. anisitsi*, results showed on average 65 ± 19 rod ospin-ir cells in the PV and 14 ± 3 in the PS. Cone opsin-like ir-cells were also detected in the PV and PS, particularly surrounding the pineal lumen, but were less evident than rod opsin-like immunostained cells in both *P. axelrodi* (Fig. 7B,D) and *A. anisitsi* (Fig. 7F,H). In most of the cases, cone opsin-like antisera labelled apical portions of those cells in contact with the lumen. In *P. axelrodi* the averaged number of cone opsin ir-cells was 43 ± 19 for the PV and 13 ± 4 PS. Whereas for *A. anisitsi* it was of 46 ± 12 and 11 ± 4 for the PV and PS, respectively. Ir-cells were neither detected in the PP or the DS, nor within the negative control (Fig. 8A,B), in both species.

DISCUSSION

The present study described for the first time the anatomical, histological and immunohistochemical characteristics of the pineal complex of two species of Characiformes, cardinal tetra and bloodfin tetra. These species occupy middle water layers, but inhabit different environments (tropical for cardinal tetra and subtropical for bloodfin tetra; Geisler and Annibal, 1986; López et al., 2005; Anjos and Anjos, 2006). In teleosts, the pineal complex is a photosensory organ that contains photoreceptor cells similar to those of the retina, which transmit the photoperiodic information to the brain by the release of indoleamines (melatonin) into the circulation (Ekström and Meissl, 1997). These photoreceptor cells respond to changes in ambient illumination, leading to the modulation of second-order neurons that innervate various brain centres, and indoleamine synthesis (Falcon, 1979; Ekström and Meissl, 1997; Confente et al., 2008; Herrera-Pérez et al., 2011). Photic information occupy a central point in the circadian organization, as they hold the input, clock and output machinery of the circadian system (Falcon, 1999; Migaud et al., 2007; Falcon et al., 2010).

By means of whole decalcified heads, the pineal's complex anatomical position was successfully established. As a result, the latter complex was

shown to emerge tangentially to the brain, at the midline of the junction of the telencephalic hemispheres with the optic tectum. In this area, the dorsal surface overlaying the complex is translucent and so, is called the PW (Omura and Oguri, 1969; McNulty and Nafpaktitis, 1977; Agha and Joy, 1986). In both species, at the level of the PW, a conspicuous fissure is displayed, known as front fontanelle, which probably improves the passage of light, as has been reported for *Astyanax mexicanus* (Herwig, 1976), *Solea senegalensis* (Confente et al., 2008) and *Dicentrarchus labrax* (Herrera-Pérez et al., 2011).

As was reported for *S. senegalensis* (Confente et al., 2008) and *D. labrax* (Herrera-Pérez et al., 2011), differences in the sizes of the organ (for example, antero-posterior length, dorso-ventral extent, transverse length and total length) were associated with the habitat of each species. In the present study, no differences were found between both species.

In the epidermis of the PW of *P. axelrodi*, club cells were identified, which are known to be associated with the release of alarm substances (Genten et al., 2009) that alert the presence of predators. However, these cells were not observed in the epidermis of the PW of *A. anisitsi*. Further studies must be conducted to address these hypotheses in the species natural environment.

In broad terms, when comparing the histological and immunohistochemical characteristics of the pineal complex in *P. axelrodi* and *A. anisitsi* with those of other teleost species, no significant differences were found. Some of these species are, for example, *Astyanax mexicanus* (Herwig, 1976), *Oryzias latipes* (Takahashi and Kasuga, 1971), *Danio rerio* (Laurá et al., 2012; Magnoli et al., 2012), *S. senegalensis* (Confente et al., 2008), *D. labrax* (Herrera-Pérez et al., 2011) and *Cichlasoma dimerus* (Birba et al., 2014). In all of the latter, the pineal organ was composed of two well-defined and continuous structures: a long and thin PS, vertically positioned with respect to the telencephalon, and an hypertrophied PV with a scant lumen, a "small space type" according to Omura and Oguri (1969), located at the distal end of the stalk and attached to the skull by connective tissue (Oksche, 1965; Rüdeberg, 1971; Takahashi and Kasuga, 1971; Falcon, 1979; Ekström and Meissl, 1997; Wanger, 2011). Additional structures of the pineal complex were the DS and the PP. The DS appeared to be folded on itself, and was bigger and more folded in *A. anisitsi* than *P. axelrodi*.

In teleosts, the PP is a simple and small structure dorsally located with respect to the habenular commissure (Ekström and Meissl, 1997; Guglielmotti and Cristino, 2006; Birba et al., 2014), and may present a small lumen (Confente et al., 2008) or non-lumen at all (Herrera-Pérez et al., 2011). The PP of the species studied herein was a small and asymmetrical structure with a little lumen, situated dorsally to the habenula and on the left hemisphere of the brain, as described in other species (Rüdeberg, 1969; Rüdeberg, 1971; Borg et al., 1983; Concha and Wilson, 2001). The role of the PP remains unknown, but some authors had proposed a photoreceptive function (Vigh-Teichmann et al., 1991; García-Fernández et al., 1997). In the present study, the PP of none of the species presented cone opsin or rod opsin immunoreactive cells. This fact does not necessary neglect the presence of other photoreceptors in the PP.

In both *P. axelrodi* and *A. anisitsi*, the pineal organ are distinguishable light microscopically two cell types in the PV and PS, as reported for *Oryzias latipes* (Takahashi and Kasuga, 1971). However, many studies in other teleost fish have reported, through both light and electron microscopy, the presence of three cell types in the PV and PS (Herwig, 1976; Confente et al., 2008; Herrera-Pérez et al., 2011). One of the two types found in the present work, The first, type A cells, were photosensitive pinealocytes, and exhibited big apical nuclei and short cytoplasmic projections towards the vesicle's lumen. The second, type B cells were smaller and had irregular and dark-purple nuclei occupying a basal position. These cells could presumably be glia-like cells, as described for other teleosts (Omura and Oguri, 1969; Takahashi and Kasuga, 1971; Herwig, 1976; Falcon, 1979; Ekström and Meissl, 1997; Confente et al., 2008; Herrera-Pérez et al., 2011; Birba et al., 2014). In general, the PV and PS are reported to contain a high number of photoreceptor cells, particularly in benthonic species as *D. labrax* (Herrera-Pérez et al., 2011) and *Petromyzon marinus* (Cole and Youson, 1982). These characteristics seem to represent adaptive mechanisms to enhance light sensitivity in species living in middle water layer habitats, as is the case for *P. axelrodi* and *A. anisitsi*, generally found in densely vegetated environments, or with marginal vegetation covering their environment. In this sense, *P. axelrodi* is said to live in humic "black waters" and *A. anisitsi* is frequently, but not exclusively, found in "white" muddy waters (Lima, 2003; Mikolji, 2009).

The pineal organ of both *P. axelrodi* and *A. anisitsi* exhibited cone opsin and rod opsin immunoreactive cells. The presence of different photoreceptors in the pineal organ of both species could improve light sensitivity in nektonic habitats where light access is markedly reduced, as mentioned above. Rod-like and cone-like photoreceptors have been localized within the pineal organ of different teleost species using a huge variety of rod and cone opsin specific antisera (Ekström and Meissl, 1997; García-Fernández et al., 1997; Foster and Hankins, 2002; Vigh et al., 2002).

The pattern of immunostaining of cone opsin and rod opsin antisera in the pineal organ was very similar in both species, and there was a clear

overlapping of the two photoreceptor cell types, confirming that those are putative photosensitive pinealocytes. However, the rod-opsin antisera stained the perikaryon in addition to the classical photosensitive region (outer segment). Accordingly, as stated by Herrera-Pérez et al. (2011), these patterns could be explained essentially by the following: on the one hand, it is possible that antigens recognised by this antiserum were located in different compartments of the same cell (i.e., perikaryon, inner segment, outer segment), or, on the other hand, daily differences could be present in the levels of pineal rod and cone opsin photoreceptors in different cellular compartments. The latter possibility is reinforced by the fact that opsin photoreceptors present in the retina have been reported to exhibit different levels of diurnal expression (Halstenberg et al., 2005; Grone et al., 2007).

Overall, the pineal complex of *P. axelrodi* and *A. anisitsi* presented very similar histological and immunohistochemical characteristics. Considering that both species occupy the middle layers of water bodies but inhabit environments with different photoperiods, some morphological differences could have been expected. However, since both species are frequently found in environments with reduced lighting, as explained above, this could be related to the presence of a well-developed pineal complex in both species. This has already been hypothesized in other nektonic species, such as *Esox lucius* L. 1758 (Falcon, 1979), and benthonic teleost species, such as *Catla catla* Hamilton 1822 (Dey et al., 2003) and *S. senegalensis* (Confente et al., 2008). Thus, the diversity of responses to light among teleost fish seem to reflect specific adaptations to their aquatic environment, where light may vary in terms of intensity, spectral content and duration (Falcon et al., 2010).

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