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The pineal complex in the cichlid *Cichlasoma dimerus*: effect of different photoperiods on its cell morphology

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This research describes the pineal complex histology in juvenile and adult *Cichlasoma dimerus*, and the effect of different photoperiods on its cell morphology. In both juveniles and adults, the pineal complex of *C. dimerus* has three components: the pineal organ, consisting of a pineal vesicle (PV) and a pineal stalk, the parapineal organ and the dorsal sac. Although a strong morphological resemblance exists between the two stages, different synthesis patterns of cone and rod opsins were detected in the two life stages. An effect of the photoperiod length was observed on putative pinealocytes' activity from the PV, measured indirectly through nuclear area morphometry. Individuals exposed to a natural photoperiod (14L:10D) had smaller nuclear areas (mean ± s.e. = 13·82 ± 1·52 μm^2) than those exposed to a short photoperiod (8:16) (21·45 ± 2·67 μm^2; $P < 0{\cdot}001$). Eventually, the nuclear area of pinealocytes could be used as a putative indicator of melatonin synthesis in fishes where it is difficult to obtain plasma samples, *e.g.* due to its small size or age. This work constitutes one of the few comparative descriptions of the pineal complex of juvenile and adult teleost and suggests potential approaches for the study of melatonin synthesis in fish larvae or small adult fishes.

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Key words: histology; karyometry; opsins; pineaolocytes.

INTRODUCTION

Most living organisms inhabit a highly dynamic environment characterized by daily and annual photoperiod and light rhythms driven by the Earth's rotation on its axis and around the sun. The evolution of biological clocks or circadian systems allows animals to keep track of time, synchronize and anticipate periodic events, such as sunrise, and entrain rhythmic physiology and behaviour (Villamizar *et al.*, 2011). A circadian system comprises all the different components by which light enters the organism and is transformed into a timed nervous or hormonal signal. In fishes, this circadian system is organized as a network of tightly interconnected circadian units, where the pineal organ occupies a central position (Falcón *et al.*, 2007).

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In all teleost species, the pineal organ is a non-image forming, photoreceptive structure that traduces photoperiodic information into neural and neurohormonal messages (Confente *et al.*, 2008). The hormonal signal is melatonin, an amine-derived molecule, and its synthesis is regulated by environmental light intensity, reaching its highest levels in complete darkness. This expression pattern appears to be a feature of all vertebrates and one of the most widespread (Ekström & Meissl, 2003). The photosensitivity of melatonin synthesis enables the calibration of processes that display seasonal and daily rhythms, such as reproduction, skin pigmentation and thermoregulation (Ekström & Meissl, 1997; Confente *et al.*, 2008).

The pineal complex of many teleosts comprises a pineal organ, which consists of a proximal slender pineal stalk (PS) and a distal expanded end-vesicle (Ekström & Meissl, 1997), and two other structures: the parapineal (PP) organ and the dorsal sac (DS) (Ariëns Kappers, 1965).

The histology and structure of the pineal complex has been studied in many species of fishes such as lampreys, chondrichthyans and teleosts (Takahashi, 1969; Hafeez, 1971; Vigh-Teichmann *et al.*, 1991; Joy & Agha, 1993; Jiménez *et al.*, 1995; Yáñez & Anadón, 1998; Pombal *et al.*, 1999; Wagner & Mattheus, 2002; Dey *et al.*, 2003; Bowmaker & Wagner, 2004; Laurá *et al.*, 2012). Those investigations, however, mostly focused on adult specimens. So far, there are relatively few morphological studies on larvae or juvenile fishes (Joy & Agha, 1993; Porter *et al.*, 2001; Meléndez-Ferro *et al.*, 2002; Herrera-Pérez *et al.*, 2011; Magnoli *et al.*, 2012). Hence, a histological evaluation of the pineal complex of juveniles of the cichlid *Cichlasoma dimerus* (Heckel 1840) was performed, and the effect of different photoperiods on its cell morphology was analysed. To further extend the findings of this work and contribute to the existing literature, a description of the general structure and histology of adult *C. dimerus* pineal complex was performed.

The South American *C. dimerus* inhabits quiet, shallow waters of tributaries and lagoons in the Parana and Paraguay Rivers' basin, from Brazil southwards to the north of Buenos Aires, Argentina. This highly social species easily adapts and spawns in captivity, providing an appropriate model for reproductive, neuroendocrinology and behavioural studies (Pandolfi *et al.*, 2009; Fiszbein *et al.*, 2010; Alonso *et al.*, 2011, 2012; Ramallo *et al.*, 2012; Tubert *et al.*, 2012; Morandini *et al.*, 2014).

The first aim of this work was to describe the structural composition and histology of juvenile and adult *C. dimerus* pineal complexes. Secondly, it aimed to analyse the effect of different photoperiods on the morphometry of cells from the pineal complex, in particular, in juveniles reared under a natural or short photoperiod. It is important to note that this is the first histological description of the pineal complex of a juvenile cichlid.

MATERIALS AND METHODS

ANIMALS

Adult specimens of *C. dimerus* were captured in Esteros del Riachuelo, Corrientes, Argentina (27° 25′ S; 58° 15′ W). In all cases, animals were housed in community aquaria mimicking natural conditions (temperature: 25° C, range ± 2° C; photoperiod: 14L:10D cycle with full spectrum illumination). Fish were acclimatized for at least 1 month before their employment in any of the experiments. Once pre-spawning pairs were detected, they were transferred into 75

l tanks with a layer of gravel (*c*.4 cm), aquatic plants and flat slabs where fish laid their eggs. On the 10th day post-fertilization (dpf) (free-swimming stage; Meijide & Guerrero, 2000), each offspring was isolated in 15 l tanks. Aquaria were well aerated and provided with external filtration. Photoperiod and light conditions were maintained constant (14L:10D with full spectrum illumination; temperature 25° C, range ± 2° C). Larvae were fed daily with *Artemia* sp. (during the first 3 weeks) and then with commercial food (Tetra; www.tetra-fish.com). Larval stages of *C. dimerus* range from egg hatching, after embryonic development, to 40–42 dpf when the juvenile stage begins (Meijide & Guerrero, 2000). All juvenile animals were processed at 59 dpf between March and April at 1400 hours (local time, GMT). Appropriate actions were taken to minimize pain or discomfort of the animals, and all the experiments were conducted in accordance with international standards on animal welfare as well as being compliant with local and national regulations. All procedures are in compliance with the *Guide for Care and Use of Laboratory Animals* (NRC, 2011).

HISTOLOGICAL DESCRIPTION OF *C. DIMERUS* JUVENILES' PINEAL COMPLEX

Juvenile specimens were anaesthetized with 0·1% benzocaine and killed by decapitation. In order to preserve the pineal position, whole heads were processed so that the brain and the pineal organ maintained their anatomical relationship. At this stage, cranial cartilage calcification has not yet begun but the external and internal morphology resembles that of adults (Meijide & Guerrero, 2000); therefore, the use of a decalcification protocol was not necessary, and heads were directly fixed in Bouin's solution for 24 h at 4° C in the dark, dehydrated and embedded in paraplast (www.sigmaaldrich.com). Samples were then sagitally ($n = 8$) and transversally ($n = 8$) sectioned at 7 μm intervals and mounted on gelatine-coated slides. Afterwards, sections were deparaffinized in xylene, rehydrated through a graded ethanol series, 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%), mounted in DPX (www.sigmaaldrich.com) and examined under a Nikon Microphot FX microscope and digitally photographed (Coolpix 4500, Nikon; www.nikon.com).

HISTOLOGICAL STUDY OF *C. DIMERUS* ADULTS' PINEAL COMPLEX

Four adult specimens (at least 365 dpf) from community tanks (75 l) were anaesthetized, killed by decapitation and whole heads were fixed in Bouin's solution as previously described for juveniles. In order to preserve the pineal position, the brain and pineal organ were not excised and whole heads were processed using a decalcification protocol for the cranium bones. Heads were immersed in a solution containing sodium citrate (50%, w/v) and formic acid (50%, v/v) at a 1:1 proportion. Decalcifying solution was changed every 24 h until a precipitate was no longer observed in a chemical test for residual calcium (5% ammonium hydroxide and 5% ammonium oxalate solution at a 1:1 proportion). When the decalcification was completed, the heads were rinsed in water and then transferred to an ammonia solution for 30 min to neutralize acids left in the specimens. Afterwards, samples were washed in running tap water for 24 h, dehydrated and embedded in paraplast. Samples were then sagitally ($n = 2$) and transversally ($n = 2$) sectioned at 7 μm intervals and mounted on gelatine-coated slides. Finally, sections were stained with Masson trichrome, mounted in DPX and examined.

IMMUNOHISTOCHEMICAL DESCRIPTION OF *C. DIMERUS* PINEAL COMPLEX

For the correct identification of photoreceptive cells comprising the pineal complex, an immunohistochemical study was performed both in juveniles and adults with anti-cone opsins

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and anti-rod opsins (light-sensitive membrane-bound receptors, typical of photoreceptor cells) antisera. In addition, to assess if both photoreceptors were present in the same cell, alternate sections were compared, one incubated with anti-rod opsins and the other with anti-cone opsins. The retina was used as a positive control, and a negative one was performed by omission of primary antisera.

Three juvenile and four adult specimens were processed as formerly described for the histological study, but after rehydration through a graded ethanol series to phosphate-buffered saline (PBS, pH 7·4), sections on the transverse plane were treated for 30 min with PBS containing 5% non-fat dry milk. Then, samples were incubated overnight at room temperature in a moist chamber with either a rabbit anti-human long wavelength (LW) cone opsin (1:250 dilution, CERN-874) or a rabbit anti-bovine rod opsin (1:250 dilution, CERN-922). Both antisera have been previously well characterized and successfully used in fishes to identify opsins (García-Fernández *et al.,* 1997; Confente *et al.,* 2008). Later, sections were washed in PBS and incubated for 45 min with a biotinylated anti-rabbit IgG diluted 1:500. Amplification of the signal was achieved by incubating the sections with peroxidase-conjugated streptavidin (STRP-HRP) (Dako; www.dako.com) diluted 1:500 and visualized with 0·1% 3,3′-diaminobenzidine (DAB) in Tris buffer (pH 7·6) and 0·03% H_2O_2. Sections were lightly counterstained with haematoxylin (haematoxylin 0·125%, potassium alum 6·25%, potassium iodide 0·025% and glycerol 25%), mounted in DPX and examined.

EFFECT OF DIFFERENT PHOTOPERIODS ON THE PINEAL COMPLEX CELL MORPHOMETRY OF *C. DIMERUS* JUVENILES

In order to assess the responsiveness of the pineal complex to different photoperiods, a different set of offsprings ($n = 10$, on average five juveniles per spawning) were transferred to new aquaria at 10 dpf. After 1 week of acclimation, larvae were exposed to either a short photoperiod (8L:16D cycle; short photoperiod exposed animals: SP; $n = 4$) or kept in the natural photoperiod (14L:10D cycle; natural photoperiod exposed animals: NP; $n = 6$). Temperature (25° C, range ± 2° C) was maintained constant in both cases. After 6 weeks of treatment (59 dpf), juveniles were anaesthetized and processed as previously described for staining with Masson trichrome.

Nuclear profile area was selected as an indirect measurement of overall cell activity (Brown & Bertke, 1974). Nuclear size (μm^2) was measured as the cross-sectional area of a group of pineal complex cells, by tracing the nucleus profile with a digitizing pen on digital images of cross-sections at ×600 (ImagePro Plus; Media Cybernetics; www.mediacy.com). From each juvenile, 10 randomly chosen cells were measured from the pineal vesicle (PV) and DS, while seven randomly chosen cells were measured from the PS due to the reduced cell number in this area. In the case of the PV and PS, only putative pinealocytes were included in the analysis based on their histological properties.

STATISTICAL ANALYSIS

The effect of different photoperiods on cells' morphometry was analysed by means of one-way analysis of variance (ANOVA). Statistical significance was established at $\alpha = 0{\cdot}017$, as data for each animal were collected from the PV, the PS and the DS, thus lacking independency (Bonferroni's correction). Normality and homoscedasticity assumptions were met in all cases. Data are presented as means ± S.E.

RESULTS

The anatomical, histological and immunohistochemical results obtained in this study were consistent among all analysed specimens and can be considered as representative of this species.

ANATOMICAL AND HISTOLOGICAL DESCRIPTION OF THE PINEAL COMPLEX IN JUVENILES OF *C. DIMERUS*

The analysis of the whole heads of juveniles allowed the characterization of the anatomical and histological attributes of *C. dimerus* pineal complex, which is formed by a pineal organ, a PP organ and a DS (Figs 1 and 2).

The pineal organ emerges from the roof of the diencephalon at a medial position, between the telencephalic hemispheres and the optic tecta (dorsal mesencephalon) (Fig. 1). It consists of an expanded PV at the end of a narrow and long PS, extending from the posterior commissure. Both the PS and PV exhibit a lumen that opens into the third ventricle [Figs 1(c), (e) and 3(a)–(c)]. The PV is lodged underneath a specialized region of the skull, the pineal window, where pigmentation is comparatively scant, allowing a better light penetration. The PV parenchyma contains at least two types of cells, distinguished by virtue of their histological characteristics: type A cells, probably pinealocytes, located at the core of the PV surrounding its lumen, with large spherical nuclei and pink-stained cytoplasm, with occasional short cytoplasmic projections towards the vesicle lumen and type B cells, with smaller, dark-stained nuclei that were restricted to the basal region of the vesicle, probably corresponding to interstitial or glial-like cells. The PV also has a thin capsule of connective tissue which corresponds to the pineal arachnoid [Figs 2(c) and 3(a)].

The PS is a tubular structure with a distal (rostral) portion, which opens into the PV and a proximal (caudal) portion, where the PS extends downwards in close proximity to the habenular commissure [Figs 1(e, iv.), 2(b) and 3(d)] and finally enters the brain at the level of the posterior commissure. The PS parenchyma contains cells which exhibit either large spherical or small irregular nuclei as in the PV [Fig. 3(a)].

The DS is a saccular and folded structure that surrounds the PS in its most posterior (proximal) region. It also shows an open communication with the third ventricle [Figs 1(e) and 3(b), (c)]. It is formed by a simple columnar epithelium. The cells composing the DS are ciliated with elongated nucleus, located on the basal cellular domain and exhibit a dark stained cytoplasm [Fig. 3(b), (c)].

The PP organ showed an asymmetrical position, located on the left side of the brain immediately above the habenular commissure [Fig. 1(e)]. It has a small and central lumen surrounded by cells with spherical nuclei [Fig. 3(d)].

ANATOMICAL ANALYSIS OF THE PINEAL COMPLEX IN ADULTS OF *C. DIMERUS*

The analysis of the decalcified whole heads of *C. dimerus* adults showed that, at an anatomical level, all structures of the pineal complex were similar between both stages (juvenile and adult); however, some were more developed in the adult stage.

As in juveniles, the pineal complex in adults is composed of a pineal organ, a PP organ and a DS. The pineal organ surfaces from the roof of the diencephalon at a medial position between the telencephalic hemispheres and the optic tecta [Fig. 4(a)].

The parenchyma of the PV consists of a pseudostratified epithelium. In contrast to juveniles, adults' PV has a conspicuous lumen occupying a larger percentage of the vesicle in comparison with that of juveniles [Fig. 4(f)]. The PS is continuous to the PV and appears to be composed of the same cell types from the PV. At its distal pole, the PV exhibited a folded and lobular epithelium [Fig. 4(a)]. The caudal portion of the PS projects near the habenular commissure and enters the brain at the level of the posterior

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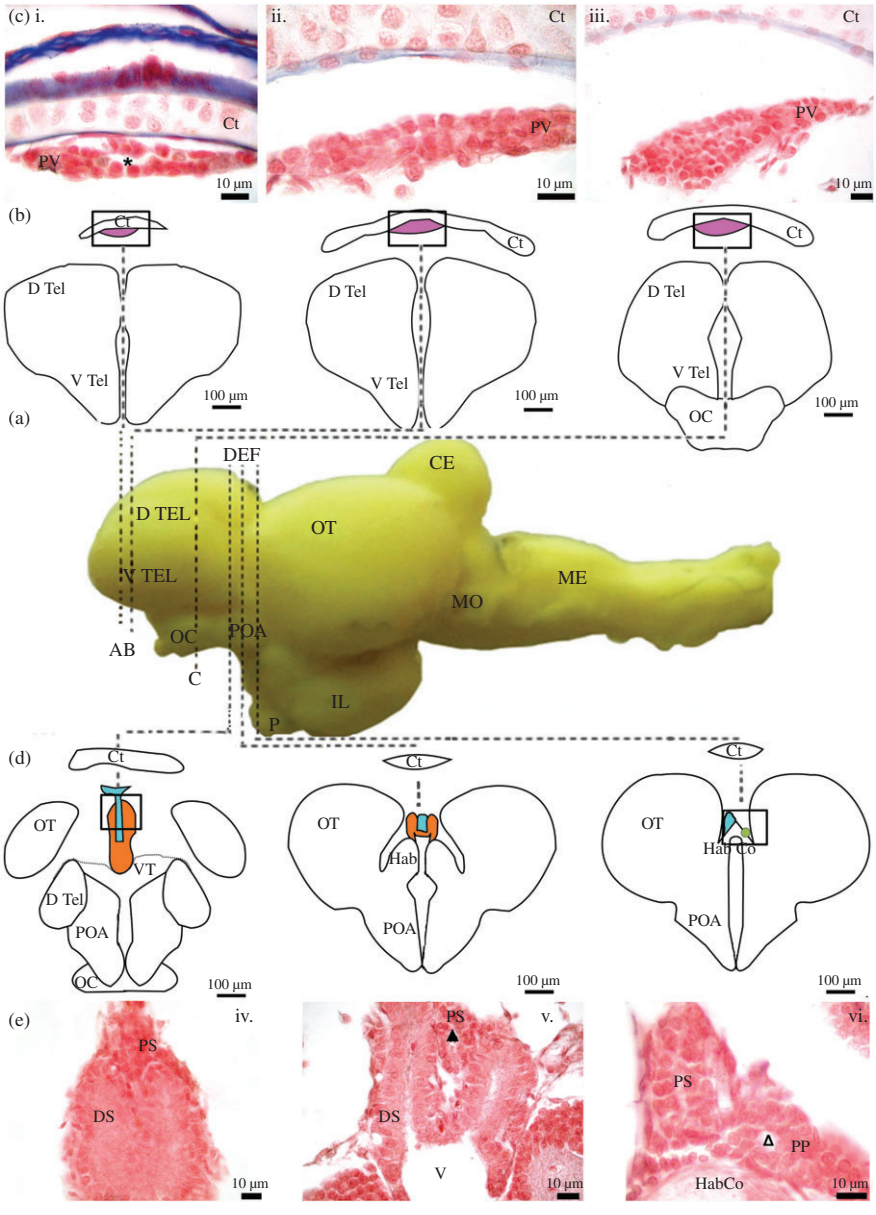

FIG. 1. (a) Lateral view of *Cichlasoma dimerus*' brain. ....., the position of the transverse sections shown on (b–e). The sections A, B and C show the position of the anterior, central and posterior regions of the PV, and the sections D, E and F correspond to the PS, DS and PP organ. (b) Camera lucida drawings of transverse sections at the level of the PV (▬). (c) (i., ii. and iii.) Microphotograph of transverse sections from PV. (d) Camera lucida drawings from transverse sections at the level of the PS (▬), the DS (▬) and the PP organ (▬) transverse sections. (e) Microphotograph of transverse sections from: (iv.) PS, (v.) DS and (vi.) PP organ. CE, cerebellum; Ct, cartilage; D Tel, dorsal telencephalon; Hab, habenula; HabCo, habenular commissure; IL, inferior lobe; ME, medulla; MO, medulla oblongata; OC, optic chiasm; OT, optic tectum; P, pituitary; POA, preoptic area; PP, parapineal organ; PS, pineal stalk; DS, dorsal sac; PV, pineal vesicle; V, third ventricle; VT, velum transversum; V Tel, ventral telencephalon; ⋆, lumen of the PV; ▲, lumen of PS; △, lumen of PP organ.

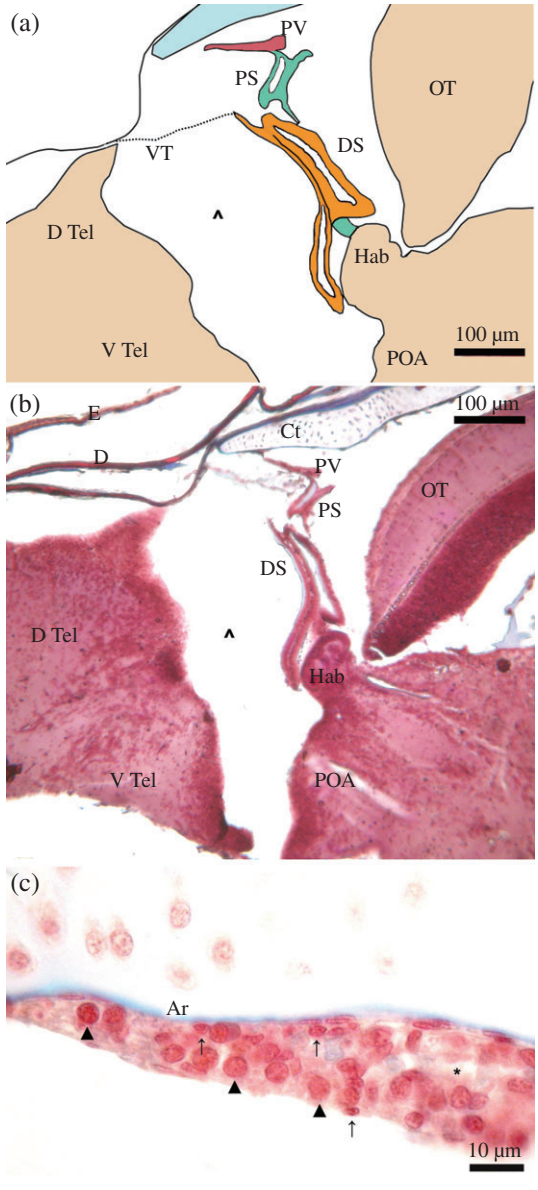

FIG. 2. Sagittal section of the pineal complex of *Cichlasoma dimerus* juveniles. (a) Camera lucida drawing from pineal complex located between the telencephalic hemispheres and the optic tecta. The presumable position of the VT is depicted (.----). (b) Photomicrograph of the location of the components of the pineal complex and its relation to the different regions of the brain. (c) Photomicrograph of the PV in a sagittal section where its cellular composition can be observed. ▲, pinealocyte; →, glial-like cell; ⋆, lumen of the PV; ∧, ventricle; Ar, arachnoid; Ct, cartilage; E, epidermis; D, dermis; D Tel, dorsal telencephalon; DS, dorsal sac; Hab, habenula; PV, pineal vesicle; POA, preoptic area; OT, optic tecta; VT, velum transversum; V Tel, ventral telencephalon.

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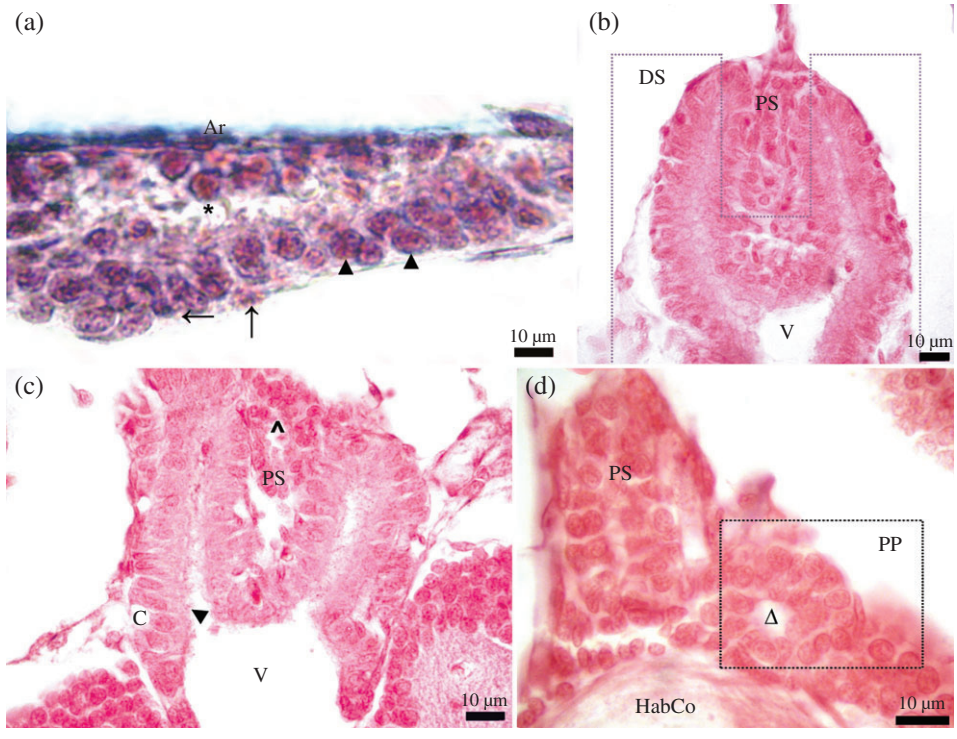

FIG. 3. Details of the various components of the pineal complex. (a) Cross section of the pineal vesicle. Both putative pinealocytes (▲) and glial-like cells (⟶) can be distinguished. (b) Cross sections of the DS (enclosed within .....) surrounding the PS. (c) Higher magnification of the DS epithelium, composed of ciliated columnar cells (▲). (d) Detail of the PP organ (enclosed within a ⋯⋯), located above the HabCo, on the left hemisphere. Ar, arachnoid; C, blood capillary; HabCo, habenular commissure; PP, parapineal organ; PS, pineal stalk; DS, dorsal sac; V, third ventricle; ⋆, lumen of pineal vesicle; ∧, lumen of the PS; △, lumen of PP organ.

commissure [Fig. 4(b), (e)]. The cells types present in the juveniles were also observed in adults.

The PP organ of adult *C. dimerus* is situated immediately above the habenular commissure on the left side of the brain. It shows a small and central lumen surrounded by cells with spherical nuclei [Fig. 4(e)].

Finally, as in juveniles, the DS partially surrounds the PS and consists of a folded monolayer epithelium [Fig. 4(b)–(e)]. The DS enters the brain at the level of the habenular commissure [Fig. 4(e)]. A well-developed vascular system can be observed next to the epithelial walls of the DS [Fig. 4(d)].

IMMUNOHISTOCHEMICAL ANALYSIS OF THE PINEAL COMPLEX

In juveniles, the pattern of immunostaining of both antisera was very similar, showing a clear overlapping of the labelled cells. These antisera labelled numerous photosensitive cells in the central region of the PV surrounding the lumen [Fig. 5(a), (b)]. No immunolabelled cells were detected in the other structures of the pineal complex of

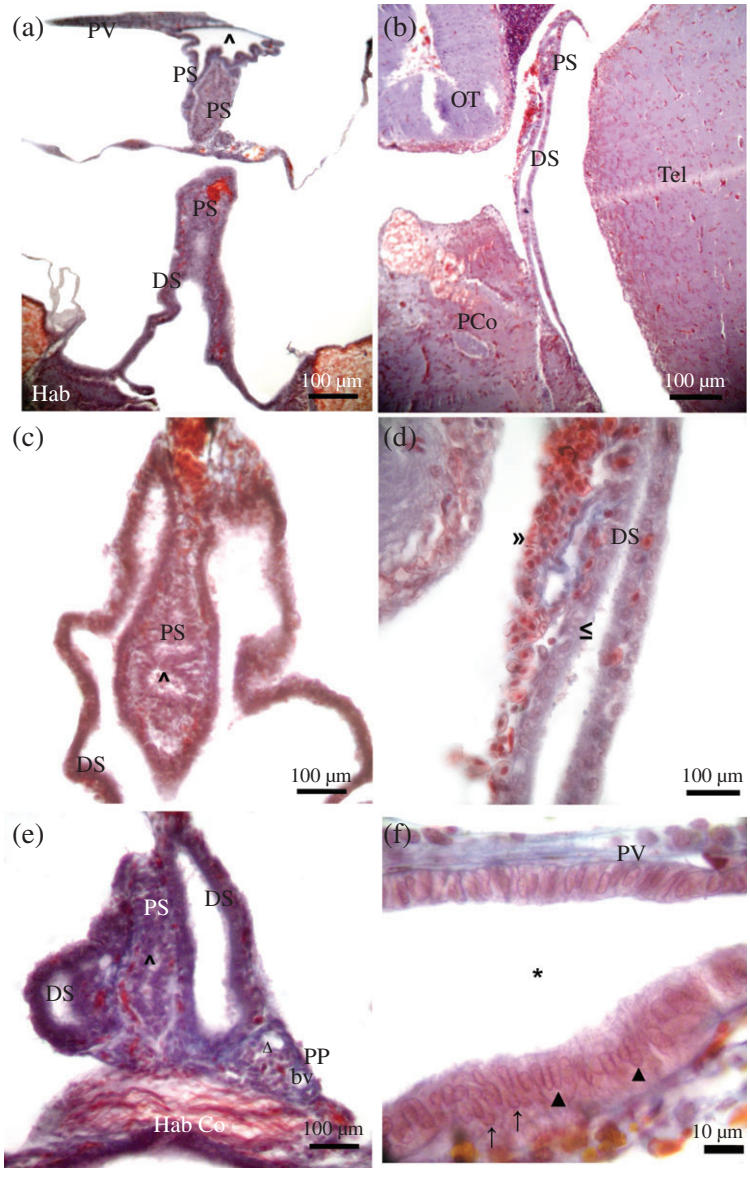

FIG. 4. Histological sections of the pineal complex of adult *Cichlasoma dimerus*. (a) Transverse section of *C. dimerus* pineal complex. Note the PV at the distal end of the pineal complex. (b) Sagittal section showing the PS and DS. The PS enters the brain at the level of the P Co. (c) Transverse section showing how the DS partially surrounds the PS. ∧, the PS lumen. (d) Detail of the monolayer epithelium (≤) of the DS ⟶ and the well-developed vascular system (»). (e) Transverse section of the PS, DS and PP organ. The PP (△) and PS (∧) lumens can be identified. (f) Detail of the PV, where putative pinealocyte (▲) and glial-like cell (⟶) nuclei can be distinguished, as well as the lumen of the PV (⋆). bv, blood vessel; DS, dorsal sac; Hab, habenula; HabCo, habenular commissure; PCo, posterior commissure; PS, pineal stalk; PV, pineal vesicle; PP, parapineal; Tel, telencephalon.

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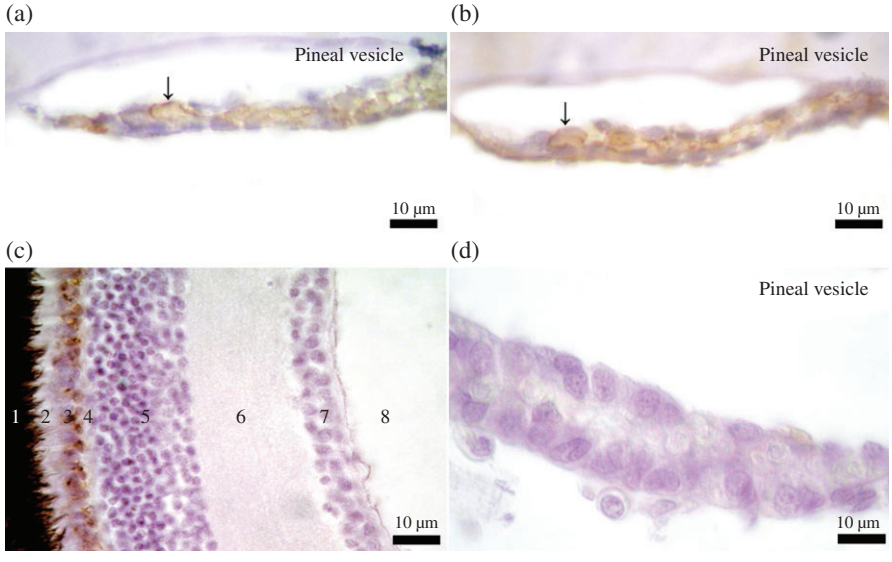

FIG. 5. Localization of immunoreactive cells for visual pigments in the pineal complex of juvenile *Cichlasoma dimerus* using rabbit anti-human LW cone opsin (1:250 dilution, CERN-874), and rabbit anti-bovine rod opsin (1:250 dilution, CERN-922). Immunostaining was observed for (a) cone opsins and (b) rod opsins in cells surrounding the lumen of the pineal vesicle. ⟶, the same cell immunolabelled by the two antisera. (c) Immunostaining for cone opsins was also observed in the photoreceptor cells of the retina of *C. dimerus* (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; 8, ganglion cell axons). (d) Negative control (omission of primary antiserum). Note the absence of immunolabel.

the *C. dimerus* juveniles. When adults were analysed, immunoreactive cells appeared in the pineal organ [anti-rod opsin, Fig. 6(a), and anti-cone opsin, Fig. 6(b)]. As in juveniles, immunoreactive cells were not detected in the PP and the DS [Fig. 6(a), inset].

EFFECT OF DIFFERENT PHOTOPERIODS ON THE MORPHOLOGY OF THE PINEAL COMPLEX CELLS IN *C. DIMERUS* JUVENILES

An effect of different photoperiods on the morphology of the cells from the PV was observed. SP fish putative pinealocytes nuclear area was 55·3% larger than that observed in NP juveniles ($21{\cdot}45 \pm 2{\cdot}67$ *v.* $13{\cdot}82 \pm 1{\cdot}52$ µm^2, $P < 0{\cdot}001$) [Fig. 7]. There was no effect of photoperiod on the nuclear area of cells from the PS (SP *v.* NP: $12{\cdot}99 \pm 0{\cdot}87$ *v.* $11{\cdot}35 \pm 0{\cdot}64$ µm^2) and DS (SP *v.* NP: $18{\cdot}59 \pm 1{\cdot}03$ *v.* $20{\cdot}31 \pm 1{\cdot}67$ µm^2).

DISCUSSION

Descriptive studies of pineal anatomy, histology and function have been conducted in most fish orders (Ekström & Meissl, 1997). Unfortunately, little is known about

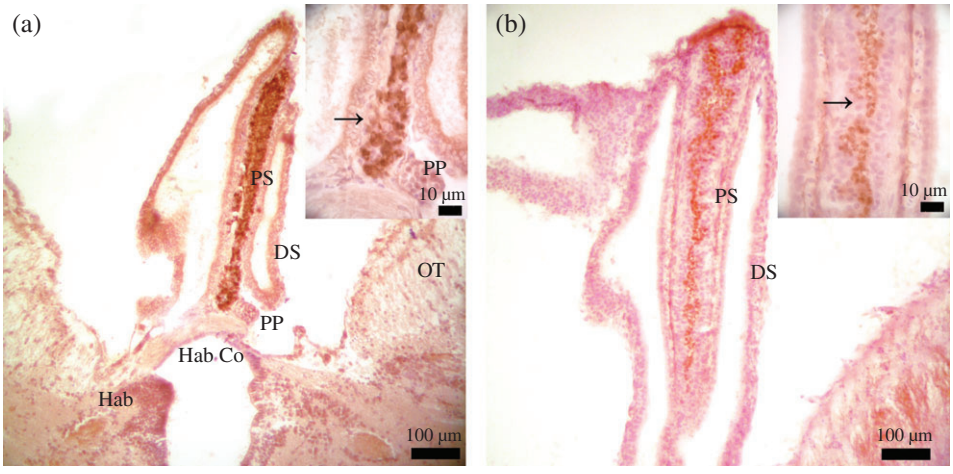

FIG. 6. Localization of immunoreactive cells for visual pigments in the pineal complex of adult *Cichlasoma dimerus*, employing anti-bovine rod opsin (1:250 dilution, CERN-922) and anti-human LW cone opsin (1:250 dilution, CERN-874) antisera. Immunostaining was observed for (a) rod opsins and (b) cone opsins in cells of the pineal organ. ⟶, immunoreactive cells [(a) and (b) insets]. Note the absence of immunolabel in the PP organ (a inset). PS, pineal stalk; PP, parapineal organ; DS, dorsal sac; Hab, habenula; Hab Co, habenular commissure; OT, optic tectum.

the characteristics of the pineal complex in cichlids and comparative studies between juvenile and adult stages are scarce.

In both juvenile and adult *C. dimerus*, the pineal complex presents three main structures: the pineal organ, the PP organ and the DS. In turn, the pineal organ is formed by two well defined and continuous structures: the PV and the PS. This organization of the pineal complex is similar to that observed in other teleosts (Omura & Oguri, 1969; Joy & Agha, 1993; Confente *et al.*, 2008; Herrera-Pérez *et al.*, 2011).

In *C. dimerus,* the pineal organ exhibits, histologically, at least two distinct cell types, both in the PV and PS. Type A cells, or putative photosensitive pinealocytes, have large, apical nuclei and short cytoplasmic projections towards the vesicle's lumen, indicating a secretory function. Smaller type B cells have irregular and dark-purple nuclei occupying a basal position, most feasible glia-like cells. These type of cells found in the pineal organ of *C. dimerus* present similar characteristics to those found in other fishes such as *Solea senegalensis* Kaup 1858 (Confente *et al.*, 2008), *Dicentrarchus labrax* (L. 1758) (Herrera-Pérez *et al.*, 2011) and *Catla catla* (Hamilton 1822) (Dey *et al.*, 2003).

The immunohistochemical study revealed divergent patterns of immunoreactivity for the opsins during the development of *C. dimerus*. At 59 dpf, cone and rod opsins-immunoreactive cells could be distinguished towards the central region of the PV, in cells immediately surrounding its lumen. In adult *C. dimerus*, the pineal organ presented positive staining for the two antisera, suggesting a late development of photoreceptive activity of the PS compared to that found in the PV. The presence of both opsins in the PV and the absence in the PS during the larval and juvenile stages could imply that the PV is the main photoreceptive structure at this point of the development. Also, other photoreceptors different from the ones tested could be present, *e.g.* exo-rhodopsin, UV opsin, parapinopsin, melanopsin and parietopsin

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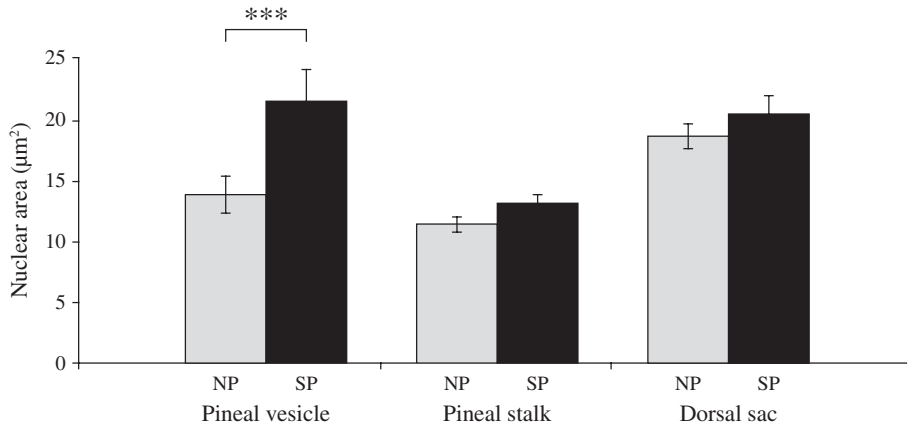

FIG. 7. Analysis of mean ± S.E. nuclear area for each of the components of the pineal organ and dorsal sac (DS). An effect of photoperiod was observed on the morphology of the cells of the pineal vesicle. Juveniles exposed to short photoperiod (SP) had a nuclear area (putative pinealocytes) significantly higher than those exposed to a natural photoperiod (NP) ($23{\cdot}45 + 2{\cdot}67$ *v.* $13{\cdot}82 + 1{\cdot}52$ μm^2; ***, $P < 0{\cdot}001$). There was no detectable effect of the photoperiod on the nuclear area of cells from the pineal stalk and the DS. ($P > 0{\cdot}05$).

(Blackshaw & Snyder, 1997; Mano *et al.*, 1999; Philp *et al.*, 2000*a*, *b*; Kojima *et al.*, 2000; Forsell *et al.*, 2001, 2002; Peirson *et al.*, 2009). Moreover, opsin levels could have fallen below the detection threshold in the PS during the juvenile stage, or could be affected by daily variations in the expression patterns of cone and rod opsin photopigments, as in the African cichlid *Astatotilapia burtoni* (Günther 1894) (Halstenberg *et al.*, 2005).

The DS of *C. dimerus* is a well-developed structure at 59 dpf. As was observed in other teleost species (Dey *et al.*, 2003; Confente *et al.*, 2008; Herrera-Pérez *et al.*, 2011), it is a saccular and highly folded structure which partially surrounds the PS, has a direct connection with the third ventricle and exhibits columnar and ciliated cells. Even though the function of the DS still remains unknown, it was considered to be part of the pineal complex, as it is in direct contact with the PS.

The PP organ in teleosts is a simple and small structure located dorsal to the habenular commissure (Guglielmotti & Cristino, 2006). It may present a small lumen, as in the case of *S. senegalensis* (Confente *et al.*, 2008), or non-lumen at all, as in *D. labrax* (Herrera-Pérez *et al.*, 2011). The PP organ of *C. dimerus* is an asymmetrical, small structure with a narrow lumen (as in the case of *S. senegalensis*), located dorsal to the habenular commissure and on the left side of the brain, thus presenting the characteristics previously described in other species (Borg *et al.*, 1983; Concha & Wilson, 2001). The role of the PP organ also remains unknown but a photoreceptive function has been suggested (Rüdeber, 1969; Vigh-Teichmann *et al.*, 1983; García-Fernández *et al.*, 1997). The absence of immunolabelling in the PP organ does not rule out its photosensitive potential, as other photoreceptors, different from the ones tested, could be present in this structure. Also, cone and rod opsin levels outside the PV could fall below the detection threshold.

Different photoperiods affected putative pinealocytes' morphology from the PV, but not from the PS or DS. Putative pinealocytes from juveniles exposed to short photoperiod (8L:16D) showed a greater nuclear area (55·3% larger) than those of fish exposed to natural photoperiod (14L:10D). Similar results were obtained by Dey *et al.* (2003) in adult females of *C. catla*, where exposure to continuous darkness for 30 days led to increased nuclear area of photoreceptive cells from the PV, but not from PS and DS. In teleosts with intra-pineal circadian control of melatonin secretion, the concentration of plasma melatonin varies according to the photoperiod, with values being in general higher all throughout the day under short photoperiod, compared to long photoperiod (Moniruzzaman & Maitra, 2012). This variation is in part consequence of the degradation of the arylalkyl amine N-acetyl transferase 2 enzyme (AANAT2, the key regulatory enzyme involved in the day and night rhythmic production of melatonin, by modification of serotonin) in the presence of light. In contrast, at night, *de novo* synthesis of AANAT2 protein occurs (Falcón *et al.*, 2001). At the same time, the *aanat2* messenger RNA has rhythmic variations, reaching the peak in the darkness (Bégay *et al.*, 1998; Coon *et al.*, 1999; Appelbaum *et al.*, 2006). The final result is a greater production of melatonin during the night (Falcón *et al.*, 2001). Furthermore, during the transition of light and dark, the pinealocytes become larger (Diehl *et al.*, 1984). Both events could be related, as a larger nuclear area often implicates increased transcriptional and translational activity, as nuclei of actively synthesizing cells are known to be larger than less synthetically active ones (Brown & Bertke, 1974). These studies, along with the results obtained in this work, show a stimulating action of a short photoperiod on the pineal organ, specifically in type A cells of the PV. A greater nuclear area of *C. dimerus* putative pinealocytes could implicate a greater synthesis of visual pigments, neurotransmitters, enzymatic regulatory elements or key enzymes such as AANAT2, therefore increasing overall pinealocyte activity and most likely melatonin synthesis. The fact that opsins immunolabel and responsiveness to photoperiod were only detected on pinealocytes from the PV suggests that in *C. dimerus* juveniles at 59 dpf the PV would be the main photosensitive structure within the pineal complex.

In summary, the pineal complex of adult and juvenile (59 dpf) *C. dimerus* was characterized at a histological, anatomical and morphological level. A difference in the expression pattern of opsins was described between juveniles and adults. A clear effect of the photoperiod on the activity of type A cells from the PV of juveniles was observed, indirectly measured by nuclear morphometry. Eventually, this technique could be used as an indicator of melatonin synthesis in those fishes where it is difficult to obtain plasma samples, *e.g.* due to its small size, in order to estimate pineal gland cell activity.

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References

Alonso, F., Cánepa, M. M., Moreira, R. G. & Pandolfi, M. (2011). Social and reproductive physiology and behavior of the Neotropical cichlid fish *Cichlasoma dimerus* under laboratory conditions. *Neotropical Ichthyology* **9,** 559–570. doi: 10.1590/S1679-62252011005000025

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Alonso, F., Honji, R., Moreira, R. G. & Pandolfi, M. (2012). Dominance hierarchies and social status ascent opportunity: anticipatory behavioral and physiological adjustments in a Neotropical cichlid fish. *Physiology and Behavior* **106,** 612–618. doi: 10.1016/j.physbeh.2012.04.003

Appelbaum, L., Vallone, D., Anzulovich, A., Ziv, L., Tom, M., Foulkes, N. S. & Gothilf, Y. (2006). Zebra fish arylalkylamine N-acetyltransferase genes - targets for regulation of the circadian clock. *Journal of Molecular Endocrinology* **36,** 337–347. doi: 10.1677/jme.1.01893

Ariëns Kappers, J. (1965). Survey of the innervations of the epiphysis cerebri and the accessory pineal organs of vertebrates. *Progress in Brain Research* **10,** 87–153. doi: 10.1016/S0079-6123(08)63448-2

Bégay, V., Falcón, J., Cahill, G. M., Klein, D. C. & Coon, S. L. (1998). Transcripts encoding two melatonin synthesis enzymes in the teleost pineal organ: circadian regulation in pike and zebra fish, but not in trout. *Endocrinology* **139,** 905–912. doi: 10.1210/en.139.3.905

Blackshaw, S. & Snyder, S. H. (1997). Parapinopsin, a novel catfish opsin localized to the parapineal organ, defines a new gene family. *Journal of Neuroscience* **17,** 8083–8092.

Borg, B., Ekström, P. & Van Veen, T. (1983). The parapineal organ of teleosts. *Acta Zoologica* **64,** 211–218. doi: 10.1111/j.1463-6395.1983.tb00802.x

Bowmaker, J. K. & Wagner, H.-J. (2004). Pineal organs of deep-sea fish: photopigments and structure. *Journal of Experimental Biology* **207,** 2379–2387. doi: 10.1242/jeb.01033

Brown, W. V. & Bertke, E. M. (1974). *Textbook of Cytology*. St Louis, MO: C. V. Mosby Co..

Concha, M. L. & Wilson, S. W. (2001). Asymmetry in the epithalamus of vertebrate. *Journal of Anatomy* **199,** 63–84. doi: 10.1046/j.1469-7580.2001.19910063.x

Confente, F., El M'Rabet, A., Ouarour, A., Voisin, P., De Grip, W. J., Rendón, M. C. & Muñoz-Cueto, J. A. (2008). The pineal complex of Senegalese sole (*Solea senegalensis*): anatomical, histological and immunohistochemical study. *Aquaculture* **285,** 207–215. doi: 10.1016/j.aquaculture.2008.08.040

Coon, S.L., Bégay V., Deurloo D., Falcón J. & Klalin D.C. (1999). Two arylalkylamine N-acetyltransferase genes mediate melatonin synthesis in fish. *Journal of Biological Chemistry* **274**, 9076–9082. doi: 0.1074/jbc.274.13.9076

Dey, R., Bhattacharya, S., Maitra, S. K. & Banerji, T. K. (2003). The morpho-anatomy and histology of the pineal complex in a major Indian carp, *Catla catla*: identification of the pineal photoreceptor cells and their responsiveness to constant light and constant darkness during different phases of the annual reproductive cycle. *Endocrine Research* **29,** 429–443. doi: 10.1081/ERC-120026949

Diehl, B. J., Heidbüchel, U., Welker, H. A. & Vollrath, L. (1984). Day/night changes of pineal gland volume and pinealocyte nuclear size assessed over 10 consecutive days. *Journal of Neural Transmission* **60,** 19–29. doi: 10.1007/BF01254762

Ekström, P. & Meissl, H. (1997). The pineal organ of teleost fishes. *Reviews in Fish Biology and Fisheries* **7,** 199–284. doi: 10.1023/A:1018483627058

Ekström, P. & Meissl, H. (2003). Evolution of photosensory pineal organs in new light: the fate of neuroendocrine photoreceptors. *Philosophical Transactions of the Royal Society B* **358,** 1679–1700.

Falcón, J., Galarneau, K. M., Weller, J. L., Ron, B., Chen, G., Coon, S. L. & Klein, D. C. (2001). Regulation of arylalkylamine *N*-acetyltransferase-2 (AANAT2, EC 2.3.1.87) in the fish pineal organ: evidence for a role of proteasomal proteolysis. *Endocrinology* **142,** 1804–1813. doi: 10.1210/en.142.5.1804

Falcón, J., Besseau, L., Sauzet, S. & Boeuf, G. (2007). Melatonin effects on the hypothalamo-pituitary axis in fish. *Trends in Endocrinology and Metabolism* **18,** 81–88. doi: 10.1016/j.tem.2007.01.00

Fiszbein, A., Cánepa, M. M., Rey Vázquez, G., Maggese, C. & Pandolfi, M. (2010). Photoperiodic modulation of reproductive physiology and behaviour in the cichlid fish *Cichlasoma dimerus*. *Physiology and Behavior* **99,** 425–432. doi: 10.1016/j.physbeh.2009.11.017

Forsell, J., Ekström, P., Flamarique, I. N. & Holmqvist, B. (2001). Expression of pineal ultraviolet- and green-like opsins in the pineal organ and retina of teleosts. *Journal of Experimental Biology* **204,** 2517–2525.

Forsell, J., Holmqvist, B. & Ekstroöm, P. (2002). Molecular identification and developmental expression of UV and green opsin mRNAs in the pineal organ of the Atlantic halibut. *Developmental Brain Research* **136,** 51–62.

García-Fernández, J. M., Jiménez, A. J., González, B., Pombal, M. A. & Foster, R. G. (1997). An immunocytochemical study of encephalic photoreceptors in three species of lamprey. *Cell and Tissue Research* **288,** 267–278. doi: 10.1007/s004410050812

Guglielmotti, V. & Cristino, L. (2006). The interplay between the pineal complex and the habenular nuclei in lower vertebrates in the context of the evolution of cerebral asymmetry. *Brain Research Bulletin* **69,** 475–488. doi: 10.1016/j.brainresbull.2006.03.010

Hafeez, M. A. (1971). Light microscopic studies on the pineal organ in teleost fishes with special regard to its function. *Journal of Morphology* **134,** 281–313. doi: 10.1002/jmor.1051340304

Halstenberg, S., Lindgren, K.M., Samagh, S.P., Nadal-Vicens, M., Balt, S., Fernald, R.D. (2005). Diurnal rhythm of cone opsin expression in the teleost fish *Haplochromis burtoni*. *Visual Neuroscience*. **22,** 135–141. doi: 10.1017/S0952523805222022

Herrera-Pérez, P., Servili, A., Rendón, M. C., Sánchez-Vázquez, F. J., Falcón, J. & Muñoz Cueto, J. A. (2011). The pineal complex of the European sea bass (*Dicentrarchus labrax*): I. Histological, immunohistochemical and qPCR study. *Journal of Chemical Neuroanatomy* **41,** 170–180. doi: 10.1016/j.jchemneu.2011.01.006

Jiménez, A. J., Fernández-Llébrez, P. & Pérez-Fígares, J. M. (1995). Central projections from the goldfish pineal organ traced by HRP-immunocytochemistry. *Histology and Histopathology* **10,** 847–852.

Joy, K. P. & Agha, A. K. (1993). A light-microscopic study on pineal organ structure and innervation in the catfish, *Heteropneustes fossilis*. *Journal für Hirnforschung* **34,** 545–553.

Kojima, D., Mano, H. & Fukada, Y. (2000). Vertebrate ancient-long opsin: a green sensitive photoreceptive molecule present in zebrafish deep brain and retinal horizontal cells. *Journal of Neuroscience* **20,** 2845–2851.

Laurá, R., Magnoli, D., Zichichi, R., Guerrera, M. C., De Carlos, F., Suárez, A. Á., Abbate, F., Ciriaco, E., Vega, J. A. & Germanà, A. (2012). The photoreceptive cells of the pineal gland in adult zebrafish (*Danio rerio*). *Microscopy Research and Technique* **75,** 359–366. doi: 10.1002/jemt.21064

Magnoli, D., Zichichi, R., Laurà, R., Guerrera, M. C., Campo, S., de Carlos, F., Suárez, A. Á., Abbate, F., Ciriaco, E., Vega, J. A. & Germanà, A. (2012). Rhodopsin expression in the zebrafish pineal gland from larval to adult stage. *Brain Research* **1442,** 9–14. doi: 10.1016/j.brainres.2012.01.021

Mano, H., Kojima, D. & Fukada, Y. (1999). Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. *Molecular Brain Research* **73,** 110–118.

Meijide, F. J. & Guerrero, G. A. (2000). Embryonic and larval development of a substrate-brooding cichlid, *Cichlasoma dimerus* (Heckel, 1840), under laboratory conditions. *Journal of Zoology* **252,** 481–493.

Meléndez-Ferro, M., Villar-Cheda, B., Manoel Abalo, X., Pérez-Costas, E., Rodríguez-Muñoz, R., Degrip, W. J., Yánez, J., Rodicio, M. C. & Anadón, R. (2002). Early development of the retina and pineal complex in the sea lamprey: comparative immunocytochemical study. *Journal of Comparative Neurology* **442,** 250–265. doi: 10.1002/cne.10090

Moniruzzaman, M. & Maitra, S. K. (2012). Influence of altered photoperiods on serum melatonin and its receptors (MT1 and MT2) in the brain, retina, and ovary in carp *Catla catla*. *Chronobiology International* **29,** 175–188. doi: 10.3109/07420528.2011.645753

Morandini, L., Honji, R. M., Ramallo, M. R., Moreira, R. G. & Pandolfi, M. (2014). The interrenal gland in males of the cichlid fish *Cichlasoma dimerus*: relationship with stress and the establishment of social hierarchies. *General and Comparative Endocrinology* **195,** 88–98. doi: 10.1016/j.ygcen.2013.10.009

NRC (2011). *Guide for the Care and Use of Laboratory Animals*, 8th edn. Washington, DC: National Academies Press.

Omura, Y. & Oguri, M. (1969). Histological studies on the pineal organ of 15 species of teleosts. *Bulletin of the Japanese Society for the Science of Fish* **35,** 991–1000. doi: 10.2331/suisan.35.991

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Pandolfi M., Cánepa M.M., Meijide F.J., Alonso F., Rey Vázquez G., Maggese M.C. & Vissio, P.G .(2009). Studies on the reproductive and developmental biology of *Cichlasoma dimerus* (Percifomes, Cichlidae). *Biocell* **33,** 1–18.

Peirson, S. N., Halford, S. & Foster, R. G. (2009). The evolution of irradiance detection: melanopsin and the non-visual opsins. *Philosophical Transactions of the Royal Society B* **364,** 2849–2865.

Philp, A. R., Bellingham, J., Garcia-Ferna´ndez, J. & Foster, R. G. (2000*a*). A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish. *FEBS Letters* **468,** 181–188.

Philp, A. R., Garcý´a-Ferna´ndez, J. M., Soni, B. G., Lucas, R. J., Bellingham, J. & Foster, R. G. (2000*b*). Vertebrate ancient (VA) opsin and extraretinal photoreception in the Atlantic salmon (*Salmo salar*). *Journal of Experimental Biology* **203,** 1925–1936.

Pombal, M. A., Yánez, J., Marín, O., González, A. & Anadón, R. (1999). Cholinergic and GABAergic neuronal elements in the pineal organ of lampreys, and tract-tracing observations of differential connections of pinealofugal neurons. *Cell and Tissue Research* **295,** 215–223. doi: 10.1007/s004410051227

Porter, M. J. R., Duncan, N., Handeland, S. O., Stafansson, S. O. & Bromage, N. R. (2001). Temperature, light intensity and plasma melatonin levels in juvenile Atlantic salmon. *Journal of Fish Biology* **58,** 431–438. doi: 10.1111/j.1095-8649.2001.tb02262.x

Ramallo, M. R., Grober, M. S., Cánepa, M. M., Morandini, L. & Pandolfi, M. (2012). Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish *Cichlasoma dimerus*. *General and Comparative Endocrinology* **179,** 221–231. doi: 10.1016/j.ygcen.2012.08.015

Rüdeber, C. (1969). Light and electron microscopic studies on the pineal organ of the dogfish, *Scyliorhinus canicula L.*. *Cell and Tissue Research* **96,** 548–581. doi: 10.1007/BF00973334

Takahashi, H. (1969). Light and electron microscopic studies on the pineal organ of the goldfish, *Carassius auratus*. *Bulletin of the Faculty of Fisheries, Hokkaido University* **20,** 143–157.

Tubert, C., Lo Nostro, F. L., Villafañe, V. & Pandolfi, M. (2012). Aggressive behavior and reproductive physiology in females of the social cichlid fish *Cichlasoma dimerus*. *Physiology and Behavior* **106,** 193–200. doi: 10.1016/j.physbeh.2012.02.002

Vigh-Teichmann, I., Korf, H. W., Nürnberger, F., Oksche, A., Vigh, B. & Olsson, R. (1983). Opsin-immunoreactive outer segments in the pineal and parapineal organs of the lamprey (*Lampetra fluviatilis*), the eel (*Anguilla anguilla*), and the rainbow trout (*Salmo gairdneri*). *Cell and Tissue Research* **230,** 289–307. doi: 10.1007/BF00213806

Vigh-Teichmann, I., Ali, M. A., Szél, A. & Vigh, B. (1991). Ultrastructure and opsin immunocytochemistry of the pineal complex of the larval Arctic charr *Salvelinus alpinus*: a comparison with the retina. *Journal of Pineal Research* **10,** 196–209. doi: 10.1111/j.1600-079X.1991.tb00816.x

Villamizar, N., Blanco-Vives, B., Migaud, H., Davie, A., Carboni, S. & Sánchez-Vázquez, F. J. (2011). Effects of light during early larval development of some aquacultured teleosts: a review. *Aquaculture* **315,** 86–94. doi: 10.1016/j.aquaculture.2010.10.036

Wagner, H. J. & Mattheus, U. (2002). Pineal organs in deep demersal fish. *Cell and Tissue Research* **307,** 115–127. doi: 10.1007/s00441-001-0482-y

Yáñez, J. & Anadón, R. (1998). Neural connections of the pineal organ in the primitive bony fish *Acipenser baeri*: a carbocyanine dye tract-tracing study. *Journal of Comparative Neurology* **398,** 151–161. doi: 10.1002/(SICI)1096-9861(19980824)398:2<151::AID-CNE1>3.0.CO;2-#