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# EXPRESSION OF PINEAL ULTRAVIOLET- AND GREEN-LIKE OPSINS IN THE PINEAL ORGAN AND RETINA OF TELEOSTS

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## Summary

**In teleostean bony fishes, studies on the adults of various species have shown that pineal photoreceptors are maximally sensitive to short- and middle-wavelength light, possibly utilising both rod-like and pineal-specific opsins. Until recently, however, very little was known about the pineal opsins present in embryonic and larval teleosts and their relationships to opsins expressed by retinal photoreceptors. Our immunocytochemical studies have revealed that, in Atlantic halibut, herring and cod, pineal photoreceptors express principal phototransduction molecules during embryonic life before they appear in retinal photoreceptors. In cDNA from embryonic and adult halibut, we identified two partial opsin gene sequences, HPO1 and HPO4, with highest homology to teleost green and ultraviolet cone opsins (72–83 % and 71–83 % amino acid identity, respectively). In halibut, these opsins are expressed in the pineal organ of embryos and appear in the retina of larvae. Our recent *in situ* hybridisation studies with RNA probes for HPO1 and HPO4 demonstrate the presence of green-like opsin mRNAs in the pineal organ and the retina of herring, cod, turbot, haddock, Atlantic salmon, zebrafish and three species of cichlid, and of ultraviolet opsins in the retinas of zebrafish, Atlantic salmon, turbot and the three cichlid species. We conclude that the halibut pineal organ appears to have the potential for both ultraviolet and green photosensitivity from the embryonic stage and that the retina may acquire the same potential during the larval stages. In the other teleosts studied, although both pineal and retinal photoreceptors seem to utilise a green-like opsin from the larval stage, ultraviolet photoreception appears to be restricted to the retina.**

Key words: pineal organ, retina, fish, cloning, mRNA, *in situ* hybridisation.

## Introduction

The pineal organ is a component of the vertebrate circadian system whose primary activity regulator is light. In teleosts, pineal photoreceptors convey photic information to the central nervous system by modulating the synthesis and secretion of indoles (mainly melatonin) and by modulating neurotransmission to second-order neurons that project into various parts of the brain (see Ekström and Meissl, 1997).

Although photoreceptors in the pineal organ and the retina are employed in different photosensory functions, they display many structural, functional and biochemical similarities (see Meissl, 1997). Extraretinal photoreceptors typically exhibit maximal sensitivities in the short (blue) to middle (green) wavelengths (Shand and Foster, 1999), as do several cone types in the retina (Levine and MacNichol, 1979; Hárosi, 1994). The presence of retinal ultraviolet-sensitive cones is well established (see Tovée, 1995), whereas evidence for extraretinal ultraviolet photoreceptors is scarce (Uchida and Morita, 1990). Several studies suggest that the pineal organ of adult teleosts utilises two or more photopigments. Immunocytochemical detection of opsin proteins in the pineal organ of various species indicates that pineal photoreceptors contain retinal-like opsins, possibly structurally similar to both cone opsin(s) and rod opsin (Ekström and Meissl, 1997; Forsell et al., 1997; Meyer-Rochow et al., 1999). Intracellular recordings and microspectrophotometric studies of the pineal organ in rainbow trout *Oncorhynchus mykiss* have demonstrated photoreceptors with maximal spectral sensitivities in the short to middle wavelengths (Meissl and Ekström, 1988; Kusmic et al., 1993). In the pike *Esox lucius*, ultraviolet light causes a long-lasting inhibition of the action potential discharge of pineal chromaticity neurons (Falcon and Meissl, 1981), although the implications of this finding for the number of pineal photoreceptor types in this animal are unknown.

Recent molecular studies in teleosts have identified gene sequences encoding a rod-like opsin expressed in the brain and the pineal organ (Mano et al., 1999; Philp et al., 2000a) and a vertebrate ancient (VA) opsin expressed in various extraretinal tissues including the pineal organ (Soni and Foster, 1997; Kojima et al., 2000; Moutsaki et al., 2000; Philp et al., 2000b). Other 'novel' opsin molecules (e.g. pinopsins) have been identified in the pineal organ/complex of birds, lampreys and lizards (Okano et al., 1994; Kawamura and Yokoyama, 1997; Yokoyama and Zhang, 1997). Both pinopsin (in chicken) and VA opsin (in salmon) are short-wavelength-sensitive when reconstituted with vitamin $A_1$ (the wavelength of maximum sensitivity, $\lambda_{max}$, is 470 nm in the chicken and 451 nm in the salmon; Okano et al., 1994; Soni et al., 1998).

In many vertebrates, the retina contains cone photoreceptors with maximum absorption in the ultraviolet region of the spectrum ($\lambda_{max}$ in the range 355–400 nm; Hárosi, 1994; Tovée, 1995). Microspectrophotometric investigations have demonstrated the presence of ultraviolet-sensitive retinal photopigments in a large number of teleost species (Hárosi and Hashimoto, 1983; Bowmaker et al., 1991; Hárosi, 1994; McFarland and Loew, 1994; Carleton et al., 2000; Novales Flamarique and Hárosi, 2000). Gene sequences encoding different opsins, including opsins in the ultraviolet class (SWS-1, short-wavelength-sensitive class 1 opsins; see Yokoyama and Yokoyama, 1996), have been identified in goldfish *Carassius auratus*, zebrafish *Danio rerio*, killifish *Oryzias latipes* and Lake Malawi cichlids (Johnson et al., 1993; Hisatomi et al., 1996; Hisatomi et al., 1997; Vihtelic et al., 1999; Carleton et al., 2000). In the retina of adult goldfish and killifish, ultraviolet opsin mRNA is expressed in miniature single cones (Hisatomi et al., 1996) and in short single cones (Hisatomi et al., 1997), respectively. A corresponding distribution of ultraviolet opsin protein in the zebrafish retina has been demonstrated using immunocytochemistry (Vihtelic et al., 1999). All single cones are ultraviolet-sensitive in the Lake Malawi cichlid *Metriaclima zebra* (Carleton et al., 2000), whereas only the so-called corner cones appear to be ultraviolet-sensitive in salmonids (Bowmaker and Kunz, 1987; Beaudet et al., 1993; Hawryshyn and Hárosi, 1994).

Immunocytochemical studies of developing teleosts have demonstrated that opsin proteins and other phototransduction molecules are present in the pineal organ during embryonic development (Östholm et al., 1987; Forsell et al., 1997), a time when the retina is much less, or not at all, differentiated. The temporal pattern of opsin mRNA expression has been studied in the retina of goldfish and zebrafish. In these animals, rod opsin is expressed first, followed by green, red and ultraviolet opsins (Raymond et al., 1995; Stenkamp et al., 1996). Although the pineal organ is probably involved in early photosensory processes, only a small number of pineal opsin sequences have been identified and, consequently, little is known about the temporal correlation of opsin mRNA expression between the pineal organ and the retina.

Our work focuses on the development of the pineal organ and the retina of different teleosts, with special emphasis on the differentiation of photoreceptor cells and second-order neurons. This research combines molecular and histochemical methods including reverse transcriptase/polymerase chain reaction (RT-PCR) and gene cloning techniques, non-radioactive *in situ* hybridisation and immunocytochemistry. Here, we review and expand upon data from a marine flatfish, the Atlantic halibut (*Hippoglossus hippoglossus*), and two other marine species, the Atlantic herring (*Clupea harengus*) and the Atlantic cod (*Gadus morhua*), concerning the cellular and molecular differentiation of pineal and retinal photoreceptors (Holmqvist et al., 1996; Forsell et al., 1997; Forsell et al., 1998; J. Forsell, P. Ekström, W. J. DeGrip, J. V. Helvik and B. Holmqvist, in preparation). In addition, we present new data from recent *in situ* hybridisation studies on seven other teleost species.

## Early differentiation of the pineal organ

In halibut, herring and cod, the pineal organ differentiates during the embryonic period (Forsell et al., 1997; Forsell et al., 1998). In the halibut pineal organ, the first immunoreactive phototransduction molecules to appear are opsins; they are expressed at 70 % of the embryonic period (or 11 days post-fertilisation at a rearing temperature of 6 °C in the dark; Fig. 1A). Expression of opsins is followed by expression of α-transducin and arrestin at 90 % of the embryonic period, when putative melatonin-producing pineal photoreceptors also appear. The halibut retina does not show corresponding photoreceptor molecules until the larval stage (Forsell et al., 1998), and it only becomes morphologically differentiated several weeks after hatching (Kvenseth et al., 1996). Although the halibut brain is poorly differentiated compared with that of other teleosts at 70 % of the embryonic period (Fig. 1C; Holmqvist et al., 1996), the pineal organ possesses neuronal connections with the brain, suggesting the presence of neural signalling pathways between the two structures (Fig. 1D). In the Atlantic herring (Fig. 1B) and cod, photoreceptors in the

Fig. 1 (A) Immunoreactive opsin in the pineal organ (arrow) of the halibut embryo. Note the undifferentiated lateral eyes (e). (B) Herring embryo, with opsin-immunoreactive pineal cells (arrow), at an earlier developmental stage than the halibut in A. (C) Parasagittal section through the brain of a halibut embryo showing the generally low level of differentiation. The earliest differentiated neuronal cell bodies and axonal pathways are immunoreactive for acetylated α-tubulin (arrows). Note the absence of immunoreactivity in the optic tectum (ot). (D) Axonal connections (arrowheads) between the pineal organ (pin) and deep brain regions in the halibut embryo; pc, posterior commissure. (E) Expression of ultraviolet-opsin mRNA in the pineal organ of the halibut embryo. (F) Expression of ultraviolet-opsin mRNA in the pineal organ of the halibut larva. (G) The adult pineal organ of halibut (transverse section at the level of the end-vesicle) displays a large number of photoreceptors expressing green-opsin mRNA; bv, blood vessel; asterisk, lumen of the pineal organ. (H) Photoreceptors in the ventral retina of the halibut larva express ultraviolet-opsin mRNA (arrows). Scale bar: A, 50 μm; B, 140 μm; C, 80 μm; D 30 μm; E,F, 12 μm; G, 40 μm; H, 15 μm.

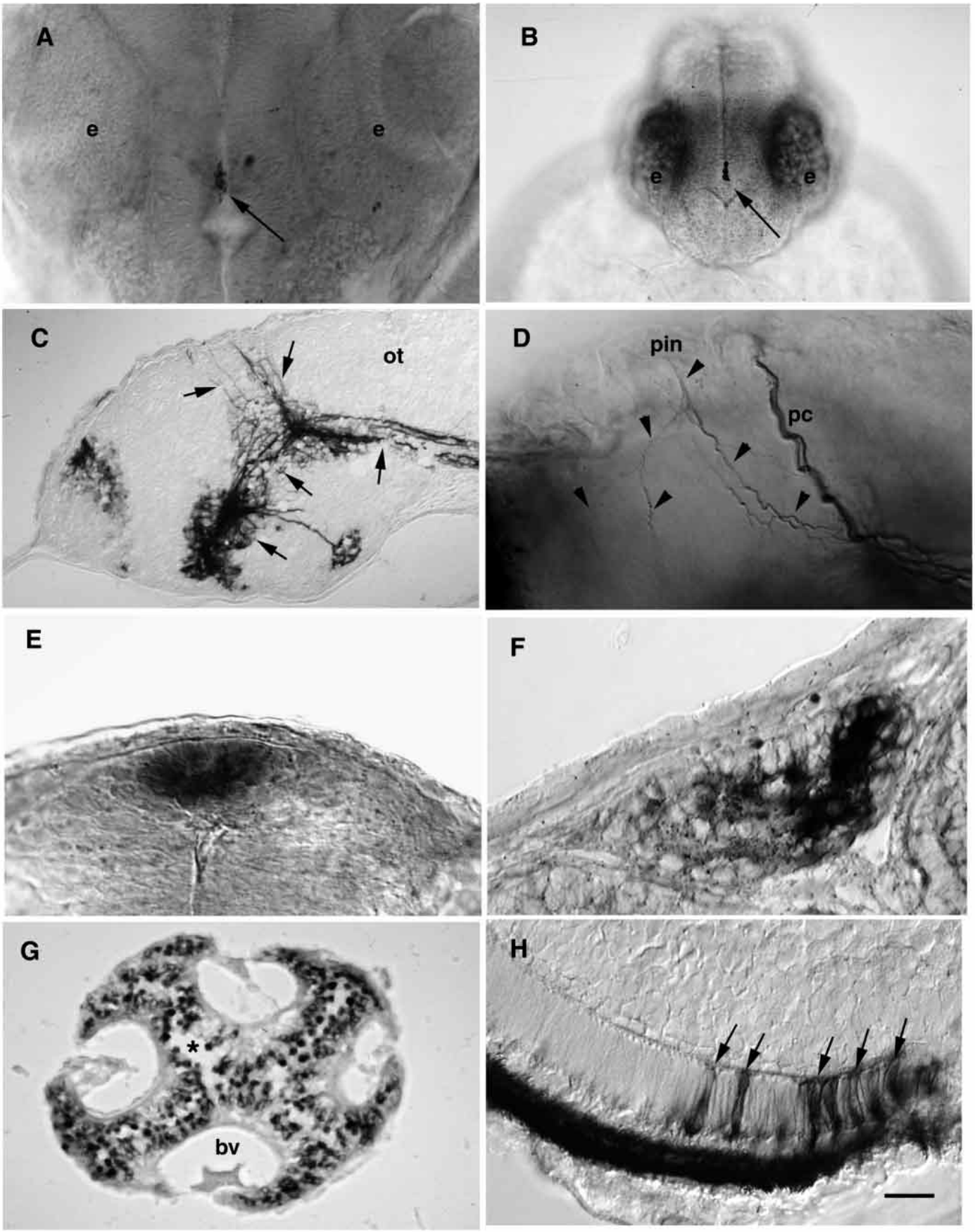


Fig. 1

pineal organ are opsin-immunoreactive from the embryonic stages (approximately 30 % and 70 % of the embryonic period, respectively. The temporal differences in the development of the brain and retina between halibut, herring and cod do not affect the general sequence of photoreceptor differentiation or the expression of phototransduction molecules, which remain similar for the three species. The early presence of short- and/or middle-wavelength-sensitive opsin proteins in pineal photoreceptors of halibut, herring and cod has been demonstrated by immunocytochemistry (Forsell et al., 1997; Forsell et al., 1998; J. Forsell, B. Holmqvist, W. J. DeGrip, J. V. Helvik and P. Ekström, in preparation).

The pineal organ differentiates earlier than the retina in other species, including sticklebacks *Gasterosteus aculeatus* (Ekström et al., 1983), Atlantic salmon *Salmo salar* (Östholm et al., 1987), rainbow trout (Omura and Oguri, 1993) and a cichlid *Aequidens pulcher* (Negishi and Wagner, 1995). In other teleosts species, there is a less obvious time lag between pineal and retinal differentiation (see Vigh et al., 1986; Ali et al., 1988). As noted above, the halibut probably exhibits the most pronounced differences observed to date. Since the retina is not functional until around 30 days after the pineal organ has differentiated, the pineal organ may act as a monitor of environmental light to control the timing of light-influenced hatching (Helvik and Walther, 1992; Forsell et al., 1997) and may possibly influence vertical migration during the embryonic stage (Mangor-Jensen and Waiwood, 1995). In other species, such as herring (J. Forsell, B. Holmqvist, W. J. DeGrip, J. V. Helvik and P. Ekström, in preparation), additional extraretinal structures (such as deep encephalic photoreceptors) as well as the retina may participate in early photoreception.

## Molecular identification of ultraviolet and green opsins in the halibut

Because, in the halibut, the pineal organ differentiates much earlier than the retina, and because behavioural evidence suggests early photoreception in this species, we carried out further investigations into the molecular identity of its pineal opsins (J. Forsell, P. Ekström and B. Holmqvist, in preparation). Using RT-PCR and related cloning techniques, two 681 base pair (bp; not counting primer regions) partial opsin gene sequences were identified from pineal cDNA, termed halibut pineal opsin 1 (HPO1; GenBank accession number AF1580979) and halibut pineal opsin 4 (HPO4; GenBank accession number AF158098). Identical fragments were identified in cDNA from microdissected adult pineal tissue and from whole embryos. The HPO1 and HPO4 fragments encode amino acid sequences that are within the region of vertebrate opsins spanning from transmembrane segment 2 to transmembrane segment 7 and show highest similarities to teleost retinal Rh2 (green cone) and SWS-1 (ultraviolet) opsins, respectively. HPO1 and HPO4 display high homology with teleost green and ultraviolet cone opsins (72–83 % and 71–83 %, respectively). The highest identity of HPO1 is to Lake Malawi cichlid (*Metriaclima zebra*, *Dimidiochromis compressiceps*, *Labeotropheus fuelleborni*) green cone opsin (83 %) and that of HPO4 is to ultraviolet cone opsin (83 %) (Carleton et al., 2000). HPO1 and HPO4 display low homology (39–49 % identity) with teleost extraretinal opsins such as salmon VA opsin (Soni and Foster, 1997) and catfish parapineal opsin (Blackshaw and Snyder, 1997). Several amino acid residues that are thought to displace the $\lambda_{max}$ of photopigments to shorter wavelengths (Chang et al., 1995; Bowmaker and Hunt, 1999) are present in the halibut pineal ultraviolet-like opsin HPO4.

*In situ* hybridisation was performed using digoxigenin-labelled RNA sense and anti-sense probes prepared from whole cloned cDNA fragments (see Holmqvist et al., 2000) of HPO1 and HPO4. Both anti-sense probes revealed expression in pineal photoreceptors of halibut embryos, larvae and adults (Fig. 1E,F), and our immunocytochemical data suggest corresponding distributions of opsin proteins (J. Forsell, P. Ekström and B. Holmqvist, in preparation). Furthermore, mRNAs for both ultraviolet- and green-like opsins were expressed in the retinal photoreceptors of halibut larvae (Fig. 1H). The sense probes did not produce any labelling. In halibut, pineal sensitivity to both short and middle wavelengths may thus be essential for light-influenced events during embryonic and initial larval development.

## Expression of ultraviolet- and green-like opsins in other teleosts

The results discussed so far suggest that green and ultraviolet opsins are expressed in photoreceptors of different teleost species from the early life stages. HPO1 and HPO4 show high identity to the green and ultraviolet cone opsins of several teleost species, and the immunocytochemical results suggest the presence of corresponding short-wavelength-sensitive pineal and retinal opsin proteins in the halibut, herring and cod (Forsell et al., 1997; J. Forsell, B. Holmqvist, W. J. DeGrip, J. V. Helvik and P. Ekström, in preparation). To investigate this further, we performed *in situ* hybridisation using the HPO1 and HPO4 (sense and anti-sense) RNA probes on tissue from the retina and the pineal organ of the following species: Atlantic herring (*Clupea harengus*), Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), turbot (*Scophthalmus maximus*), Atlantic salmon (*Salmo salar*), two Lake Malawi cichlids (*Labidochromis cearuleus* and *Pseodotropheus* sp.), a Central American cichlid (*Archocentrus nigrofasciatus*) and the zebrafish (*Danio rerio*). The endogenous opsin gene sequences in these species are not known or are presumed to be different from those in halibut. We therefore defined the specificity of the *in situ* hybridisation results (i.e. the hybridisation and visualisation of ultraviolet- and green-like opsin mRNAs) using the following criteria: (i) labelling had to be restricted to photoreceptor cells, (ii) no labelling was to occur using the corresponding sense probe, (iii) in herring and cod, there had to be a correlation with the distribution of immunoreactive opsin proteins (J. Forsell,

B. Holmqvist, W. J. DeGrip, J. V. Helvik and P. Ekström, in preparation) and (iv) in zebrafish and salmonids, the specificity of HPO4 labelling had to correspond to the morphologically and microspectrophotometrically defined putative ultraviolet-sensitive cones in the retina (Raymond et al., 1993; Robinson et al., 1993; Kunz et al., 1994). In all experiments, *in situ* hybridisation was performed simultaneously on several species under the same stringency conditions, using halibut retina and pineal organ as positive controls.

Labelling with the HPO1 probe met all the criteria detailed above. Labelling with the HPO4 probe also fulfilled all the criteria, but at a lower hybridisation temperature (57 °C instead of 65 °C for HPO1), indicating relatively lower sequence similarities of labelled ultraviolet-like mRNAs (when compared with green-like mRNAs). The endogenous mRNA transcripts that hybridised with the HPO1 and HPO4 probes were not identified in this study. However, the high sequence homologies between teleost opsins, the fulfilled stringency criteria of the *in situ* hybridisations and the good correlation between the distribution of *in situ* hybridisation labelling and the morphological and microspectrophotometric descriptions of photoreceptors and visual pigments together provide evidence that the observed hybridisations represent expression of putative ultraviolet- and green-like opsins in the pineal organ and retina of the teleost species examined. The results obtained for each species detailing the expression patterns in the pineal organ and/or the retina are summarised in Table 1.

The pineal organ displayed hybridisation with the HPO1 probe at high stringency conditions in all species tested (Table 1). In herring, cod, haddock and all the cichlid species, HPO1 produced strong labelling of pineal photoreceptors (Fig. 2A–D). The labelling was confined to cell bodies located mainly near the pineal lumen. In the pineal organ of Atlantic salmon and zebrafish (Fig. 2E), HPO1-labelled cells were

Table 1. *Hybridisation pattern with probes for HPO1 and HPO4 in the pineal organ and retina of teleosts at different developmental stages*

| Species | Life stage | Structure | HPO1 (green-like) anti-sense probe | HPO4 (UV-like) anti-sense probe |
|---|---|---|---|---|
| Atlantic halibut | Embryo | Pineal | + | + |
| *Hippoglossus hippoglossus* | | Retina | – | – |
| | Larva | Pineal | + | + |
| | | Retina | + | + |
| | Adult | Pineal | + | + |
| | | Retina | nt | nt |
| Zebrafish | Embryo | Pineal | + | – |
| *Danio rerio* | | Retina | + | – |
| | Adult | Pineal | + | – |
| | | Retina | + | + |
| Atlantic salmon | Alevin | Pineal | + | – |
| *Salmo salar* | | Retina | + | + |
| | Parr | Pineal | + | – |
| | | Retina | + | + |
| Atlantic cod | Larva | Pineal | + | – |
| *Gadus morhua* | | Retina | + | – |
| Haddock | Larva | Pineal | + | – |
| *Melanogrammus aeglefinus* | | Retina | + | – |
| Atlantic herring | Larva | Pineal | + | – |
| *Clupea harengus* | | Retina | + | – |
| Turbot | Larva | Pineal | + | – |
| *Scophthalmus maximus* | | Retina | + | + |
| Cichlids | Larva | Pineal | + | – |
| *Labidochromis cearuleus* | | Retina | + | + |
| *Pseodotropheus* spp. | Adult | Pineal | nt | nt |
| *Archocentrus nigrofasticus* | | Retina | + | + |

Specific labelling of photoreceptor cells with HPO1 and HPO4 anti-sense probes is shown with +, whereas the absence of hybridisation is shown with –.

Note that HPO1 and HPO4 sense probes did not produce labelling of any structure in any species tested.

nt, not tested.

relatively few, and the labelling was considerably weaker compared with that in the other species. Hybridisation with the HPO4 probe in the pineal organ was only observed in the halibut (Table 1).

In the zebrafish retina, HPO4 hybridisation was restricted to short single cones (Fig. 3A,C). This labelling is consistent with previous microspectrophotometric determinations of ultraviolet-sensitive visual pigment (Robinson et al., 1993) and with the immunocytochemical localisation of ultraviolet opsin (Vihtelic et al., 1999) in this cone type. Strong HPO1 labelling of double cones was obtained throughout the retina of the adult.

In the retina of Atlantic salmon alevins and parr, HPO4 hybridisation was restricted to the small single corner cones (Fig. 3E,F; Kunz et al., 1994). These cones are known to be ultraviolet-sensitive in all salmonid species examined to date, from microspectrophotometric and combined morphological/electrophysiological determinations (Bowmaker and Kunz, 1987; Beaudet et al., 1993; Kusmic et al., 1993; Hawryshyn and Hárosi, 1994; Novales Flamarique, 2000). HPO1 hybridisation was present in double cones and in some rods throughout the retina.

In the Central American cichlid *Archocentarus*

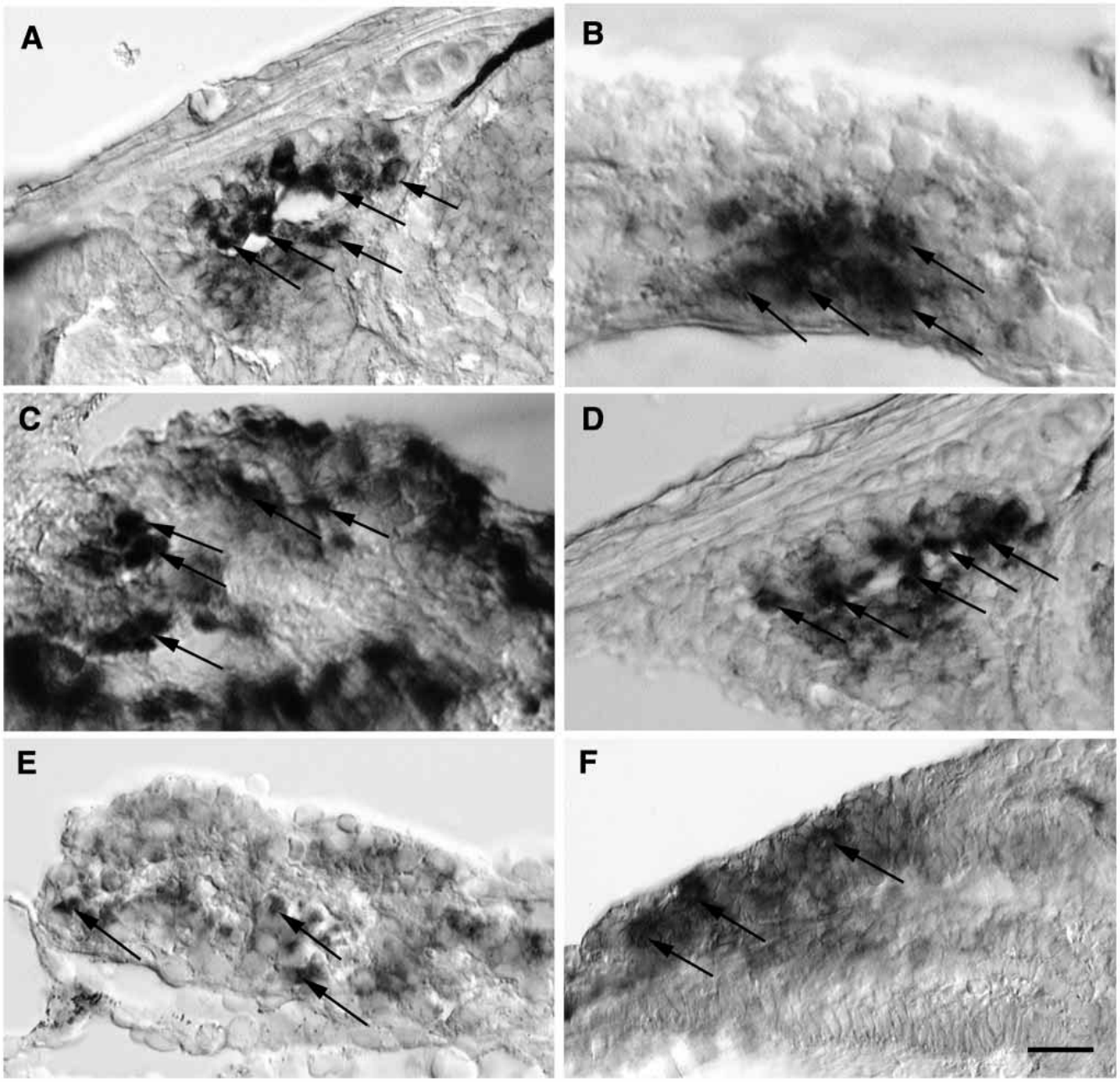


Fig. 2. Pineal photoreceptors displaying hybridisation with the HPO1 (green-opsin) probe (arrows). Sagittal sections of the pineal organ of (A) cod larva, (B) early herring larva, (C) adult cichlid (*Labidochromis cearuleus*), (D) haddock larva, (E) adult zebrafish and (F) Atlantic salmon parr. Scale bar: A,C–F, 15 μm; B, 9 μm.

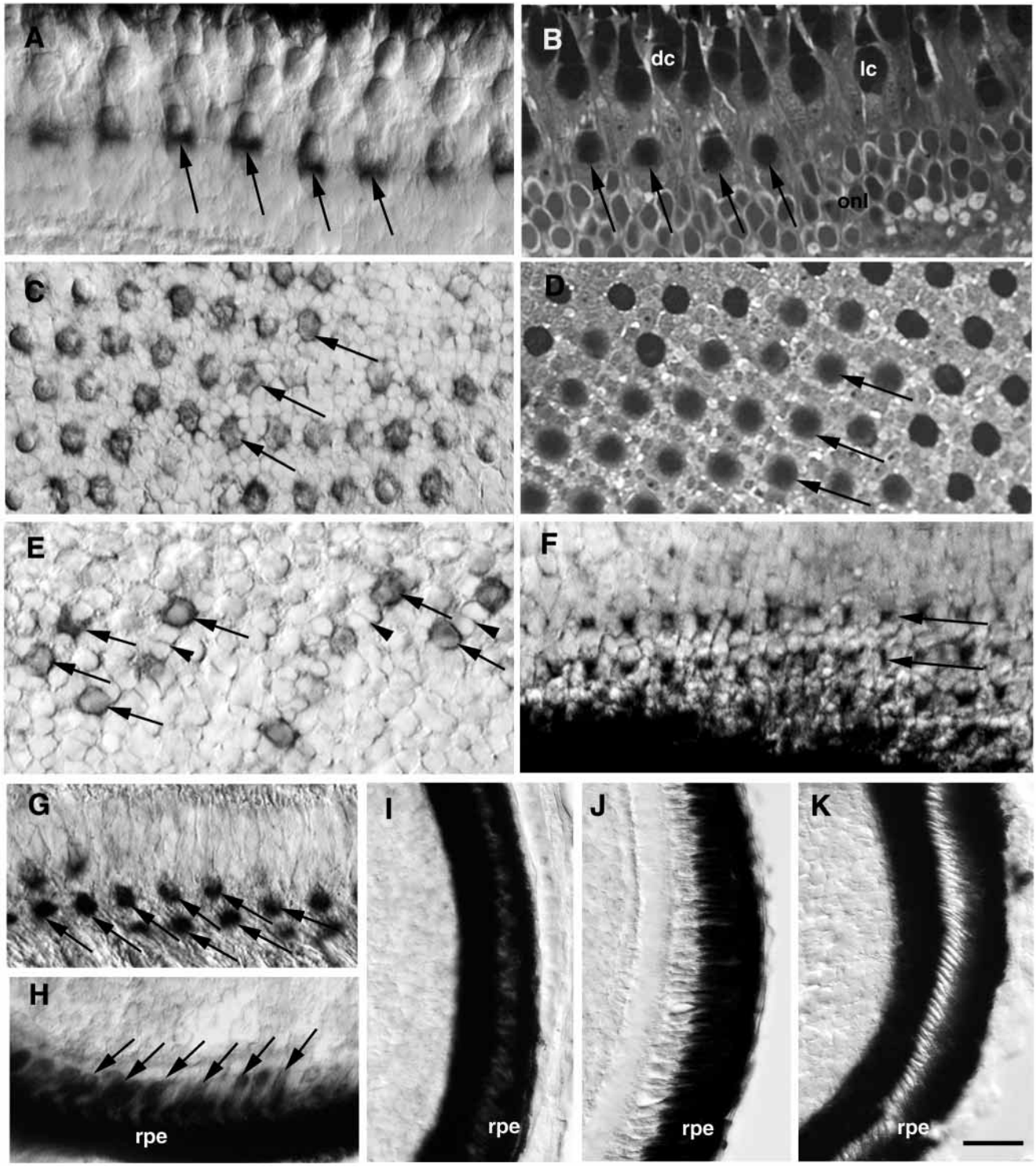


Fig. 3. Hybridisation of retinal photoreceptors with the HPO4 (ultraviolet-opsin) probe (A,C,E–H) and with the HPO1 (green-opsin) probe (I–K). (A,C) In zebrafish, HPO4 hybridisation is restricted to short single cones (arrows); these cones, which form rows with long single cones, are the most vitreally located cone types, as observed histologically (B,D; arrows). (E) In Atlantic salmon parr, HPO4 hybridisation is restricted to single corner cones (arrows); note that these cones face the partitioning membranes of adjacent double cones (arrowheads). (F) The same corner cones are labelled by HPO4 in the salmon alevin. (G) In the cichlid *Labidochromis cearuleus*, HPO4 hybridises to single cones (arrows), as is the case in the other cichlid species investigated *Pseodotropheus* spp. and *Archocentrus nigrofasciatus*. (H) In turbot larva, as in halibut larva (see Fig. 1H), HPO4 hybridises to single photoreceptors in the ventral portion of the retina (arrows). (I) Intense HPO1 hybridisation in the retinal photoreceptor layer of cod larva. (J) In the same cod retina, no hybridisation is observed with the HPO1 sense probe applied to a section adjacent to that shown in I. (K) HPO1 hybridisation in the retinal photoreceptor layer of the herring larva. dc, double cone; lc, long single cone; onl, outer nuclear layer; rpe, retinal pigment epithelium. Scale bar: A,B,E,F,H, 12 μm; C,D, 18 μm; G, 15 μm; I–K, 21 μm.

*nigrofasciatus* and the two Lake Malawi cichlid species, HPO4 hybridisation in the retina was restricted to single cones (Fig. 3G). These findings are consistent with microspectrophotometric-derived absorbance spectra from the Lake Malawi cichlid *Metriaclima zebra*; in this species, all single cones are ultraviolet-sensitive, with $\lambda_{max}$ at 368 nm (Carleton et al., 2000). The specificity of the HPO4 labelling in cichlids is further supported by its high structural identity (83 %) with the ultraviolet opsins of different Lake Malawi cichlids (Carleton et al., 2000). In all the cichlid species, HPO1 hybridisation was localised in double cones and some rods.

Both HPO1 and HPO4 hybridisation was detected in the retina of larval turbot. The HPO4-positive photoreceptors were restricted to the ventral retina (Fig. 3H); this localisation and the morphological appearance of photoreceptors were similar to those observed for HPO4-labelled photoreceptors in the retina of larval halibut (Fig. 1H). The presence of putative green-like opsins in this animal is in accordance with behavioural responses to middle-wavelength light observed at the larval stage (H. I. Browman, personal communication). In the herring and in the gadid species (cod and haddock), the expression of green opsin mRNAs and the absence of expression of ultraviolet opsin mRNAs (Fig. 3I–K) are consistent with results from spectral sensitivity studies of larval herring (Blaxter, 1968) and with preliminary microspectrophotometric observations of retinal visual pigments in cod and haddock larvae (F. I. Hárosi, personal communication), respectively.

Together, our results suggest that various teleost species express both green- and ultraviolet-like opsins in retinal photoreceptors and green-like opsins in pineal photoreceptors. In the species studied here, the expression of these opsins is similar in the larval and adult stages. Only the halibut appears to express a pineal ultraviolet opsin similar to HPO4. It is possible that the halibut compensates for what appears to be delayed retinal differentiation with a functional pineal organ possessing green and ultraviolet opsins from the early stages of development. Further studies with different fish species at various life stages are required to assess the extent of ultraviolet opsin expression among teleost phyla. Such investigations may, in turn, provide valuable clues to the origin and evolution of ultraviolet photoreceptors in teleost fishes and to the various functions of ultraviolet sensitivity.

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