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TOPOGRAPHY OF DIFFERENT PHOTORECEPTOR CELL TYPES IN THE LARVAL RETINA OF ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*)

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Summary

The identities of single cone cells in the retina of Atlantic halibut (*Hippoglossus hippoglossus*) larvae were studied by *in situ* hybridisation using RNA probes for the five different halibut opsins. Four different cone opsins (ultraviolet-, blue-, green- and red-sensitive) are expressed in Atlantic halibut at the end of the yolk-sac period, whereas rod opsin is expressed later in development. Photoreceptor cells expressing ultraviolet-sensitive opsin are found only in the ventral retina, presumably to optimise detection of the downwelling ultraviolet light. The majority of the photoreceptors (approximately 90 %) in the retina express green-sensitive opsin and its distribution shows no regional differences. In contrast, blue- and red-sensitive opsins are expressed much less frequently (in approximately 10 % of photoreceptors), although these two opsins are also found over the entire retina. The expression patterns of the different visual pigments indicate some form of mosaic expression in the single-coned larval retina, and this is reminiscent of the square mosaic expression found in post-metamorphic Atlantic halibut. These findings suggest plasticity in green-opsin-expressing cells during development, resulting in a square mosaic expression pattern.

Key words: opsin, cone, rod, mosaic, photoreceptor, flatfish, *Hippoglossus hippoglossus.*

Introduction

During their life cycle, fishes may change habitat and go through different developmental phases that have different light environments. Such changes in habitat are often related to remodelling processes within the organism known as metamorphosis. In salmonid species, the migration to the marine environment takes place after the embryonic, larval and early juvenile phases in fresh water. Concomitant with this change of habitat, an ontogenetic remodelling known as smoltification occurs. This involves changes in the visual pigments from being porphyropsin-dominated to rhodopsin-dominated. In addition, there is a loss of ultraviolet-sensitive photoreceptors (Browman and Hawryshyn, 1992; Alexander et al., 1994). In contrast to salmonids, which spend their early life stages in fresh water, the European eel (*Anguilla anguilla*) spends its embryonic and larval phases in the marine environment, while the juvenile and young adult phases occur in fresh water. Maturation and return to the sea induce changes in opsin expression, with the opsin type in the rod photoreceptor cells changing from green- to blue-sensitive (Archer et al., 1995).

In the marine environment, there are examples of vertical migration during ontogeny that are associated with changes in the photic environment. Flatfish provide a very good example of such visual adaptation (Beaudet and Hawryshyn, 1999). The early stages of flatfish are consistent with a pelagic, surface-dwelling life style. The bilaterally symmetrical larvae have one eye on each side of the body and have typical retinas adapted to a bright light environment; the retina consists of single-cone cells only (Blaxter, 1968; Evans and Fernald, 1993; Pankhurst and Butler, 1996)

The larvae of many fish hatch with, or rapidly develop, a retina that consists entirely of single cones. This includes fishes such as herring *Clupea harengus* (Blaxter and Jones, 1967), perch *Perca fluviatilis* (Ahlbert, 1973), greenback flounder *Rhombosolea tapirina* (Pankhurst and Butler, 1996), black bream *Acanthopagrus butcheri* (Shand et al., 1999), red sea bream *Pagrus major* (Kawamura et al., 1984), New Zealand snapper *Pagrus auratus* (Pankhurst and Eagar, 1996) and Atlantic halibut *Hippoglossus hippoglossus* (Kvenseth et al., 1996). These fish are examples of species showing indirect development with a larval stage in which the retina consists of only cone cells; the rod cells appear during metamorphosis (Evans and Fernald, 1990).

The larval period in flatfish ends with metamorphosis, during which the pelagic larva is remodelled to a juvenile form adapted to a benthic life style. This remodelling is associated

with changes in the general body plan: one eye migrates to the contralateral side of the head adjacent to the other eye, such that both eyes can scan the surroundings above the body plane. Microspectrophotometric analysis of the single cone cells in winter flounder (*Pseudopleuronectes americanus*) revealed only one kind of opsin (wavelength of maximum sensitivity, λ_{max}=519 nm; Evans et al., 1993), suggesting that these larvae are not capable of wavelength discrimination and that prey detection is probably based on differences in brightness contrast. They further showed that the visual system in post-metamorphic winter flounder gains three new types of cone opsins (with λ_{max} values of 457 nm, 532 nm and 547 nm) and one rod opsin (λ_{max}=506 nm), whereas the larval visual pigment disappears. These results indicate that, in addition to the induction of rod opsin expression, there is a major change in cone opsin expression between the pre- and post-metamorphic phases, suggesting a regulation of opsin expression within existing cone photoreceptor cells.

Two species of fish, zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*), are commonly used as models for retinal development in vertebrates, and most of the work related to gene expression and mosaic formation has been performed in these two closely related freshwater fishes. Retinal development in zebrafish and goldfish, in which both cones and rods appear simultaneously (Raymond et al., 1995; Stenkamp et al., 1996), is more closely related to that in species showing direct development (Evans and Fernald, 1990). Recently, attention has been focused on elucidating whether the rod photoreceptors contribute to cone mosaic patterning in these species with direct retina development (Wan and Stenkamp, 2000). These studies have included manipulations to examine the development of cone mosaics in retinas that lack rod photoreceptors. An examination of these events in a naturally developing retina would be useful.

The halibut is a typical example of a marine species with a pelagic larval stage occurring in a bright light environment. Concomitant with ontogenetic transformation to a juvenile life form, the halibut performs a vertical migration to a benthic life style. It is not known how the retinas of marine fish larvae are adapted to the pelagic environment and how they change during development to adjust to the benthic life style with a reduced light intensity and spectrum. We have used in situ hybridisation to analyse the photoreceptor cells and visual pigments in the larval halibut retina adapted to the pelagic marine environment.

Materials and methods

Yolk-sac stages of Atlantic halibut (*Hippoglossus hippoglossus*) larvae were obtained from Austevoll Aquaculture Research Station, near Bergen, Norway. The eggs were stripped from one female and fertilised with sperm from two males (Norberg et al., 1991). The fertilised eggs were maintained in flow-through incubators (Mangor-Jensen et al., 1998). 1–2 days prior to hatching, the embryos were transferred to new incubators and the yolk-sac stage larvae were allowed to develop for 40 days at a temperature of approximately 6 °C (Harboe et al., 1994). Two different groups of halibut larvae were collected and fixed directly from incubators. At this developmental stage, the larvae were transferred to the feeding systems.

In situ *hybridisation*

Halibut larvae were fixed in 4 % paraformaldehyde-buffered phosphate-buffered saline (PBS), pH 7.2, for 48 h at 4 °C. After a brief wash in PBS, the specimens were kept overnight in a 25 % sucrose PBS solution with 30 % Tissue Tek (O.C.T. Sakura Finetek Europe, Netherlands). Several larval heads were placed in a mould containing 100 % Tissue Tek and orientated with the anterior–posterior axis perpendicular (transverse) or parallel (sagittal) to bottom of the mould before rapid freezing on an iron block pre-cooled in liquid nitrogen. Transverse or radial sections (10 µm) of the eye were cut in a cryostat (Leitz Cryostat 1720,Wetzlar, Germany), air-dried and stored at – 80 °C until use.

Digoxigenin (DIG)-labelled RNA probes were prepared from the five halibut opsins (J. V. Helvik, Ø. Drivenes, T. H. Næss, A. Fjose and H. C. Seo, unpublished results) following the manufacturer's instructions (Boehringer Mannheim, Germany), and the probe concentration was determined using spot test. *In situ* hybridisation was carried out according to the method of Barthel and Raymond (Barthel and Raymond, 1993) with some modifications. Briefly, the tissues were rehydrated, treated with proteinase K (10 mg ml^{-1}) for 5 min at 37 °C, followed by postfixation in 4 % paraformaldehyde in PBS, treated with acetic anhydride and dehydrated. Approximately 100 µl of hybridisation mixture containing 100 ng of DIG-labelled RNA probes was applied directly to each air-dried section, and the sections were incubated in a humidity chamber without coverslips for 15 h at 60 °C. The probes were detected using an anti-DIG antibody coupled to alkaline phosphatase. Labelling was visualized with chromogen substrates. For each of the labels, sense probes were applied as a negative control to the tissue to confirm the specificity of labelling.

Photography and computer graphics

A Leica DMLB microscope with a digital camera (Hitachi kP-160 CCD, Japan) was used to take images of serial sections. Adobe Photoshop (version 5.0) was used to process the images (the functions 'auto levels' and 'sharpen' were used) before printing on a Hewlett Packard laser jet printer. Regions of interest were copied from the printed images onto transparent paper. A Nikon microscope (Microphot-FXA, Tokyo, Japan) with Nomarski optics was used to take colour slides (Kodak Ektachrome 160 T), which were then digitised using a Nikon 35 mm film scanner (Nikon LS-2000, Tokyo, Japan).

Results

Cellular expression

During the larval stages, the retina appears to contain only morphologically identical single cone cells. We have

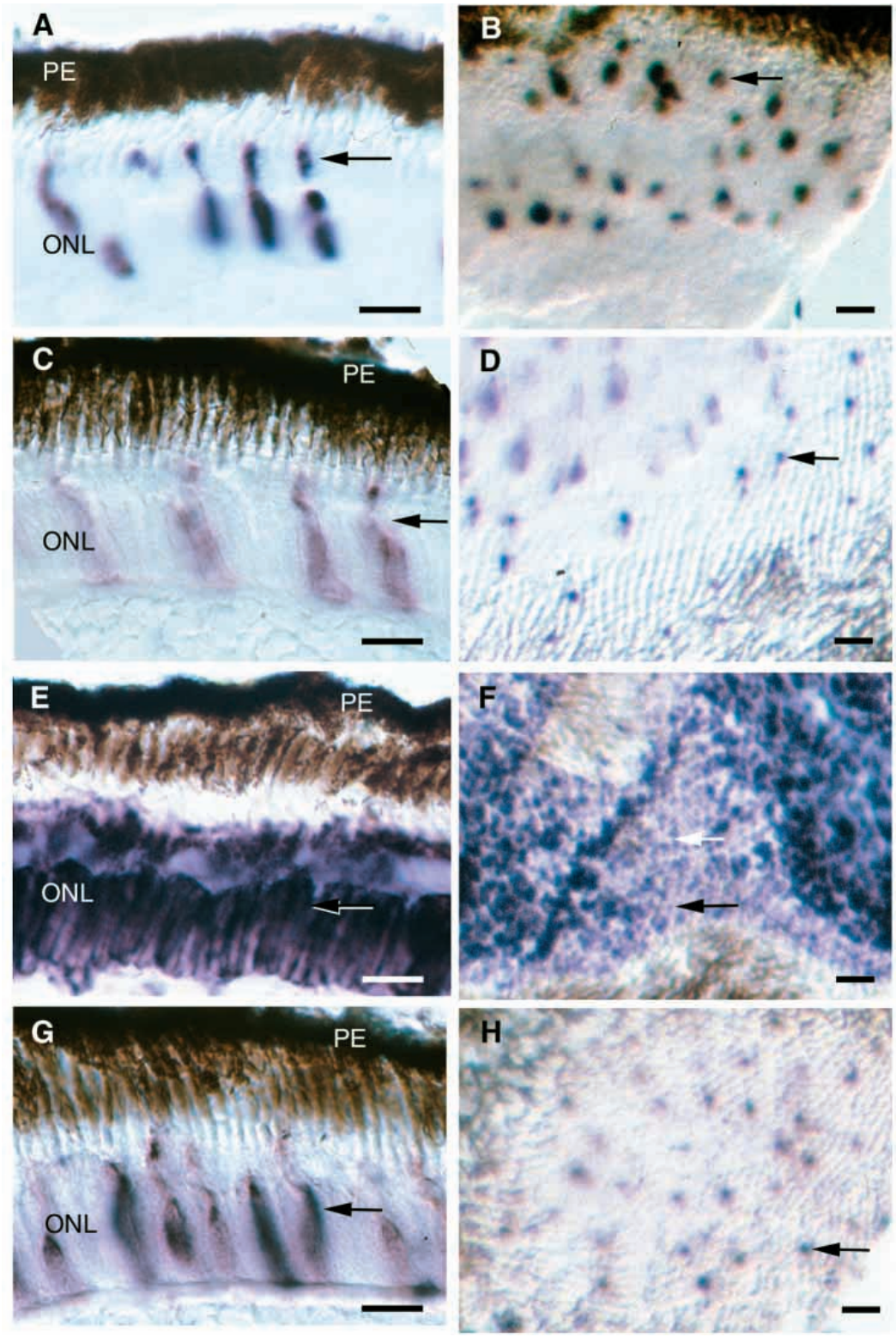

Fig. 1. Cellular expression of visual pigments and retinal mosaic expression in Atlantic halibut (*Hippoglossus hippoglossus*). The cellular identities of the different photoreceptors in the retina of Atlantic halibut larvae at the end of the yolk-sac period (40 days post-hatching) were investigated by *in situ* hybridisation analysis with RNA probes for the four different Atlantic halibut cone opsins: ultraviolet (A,B), blue (C,D), green (E,F) and red (G,H). A,C,E,G are radial sections, while B,D,F,H are tangential sections. Expression patterns in tangential sections from the central retina are with blue, green and red opsin probes, respectively, while tangential sections in the ventral retina are with ultraviolet opsin probe. Black arrows indicate photoreceptor cells expressing the respective opsins. The white arrow indicates photoreceptor cells that do not express green-sensitive opsin. ONL, outer nuclear layer; PE, pigment epithelium. Scale bars, 10 μm.

previously cloned five different opsins from halibut retina (J. V. Helvik, Ø. Drivenes, T. H. Næss, A. Fjose and H. C. Seo, unpublished results) that belong to the ultraviolet-, blue-, green- and red-sensitive and rod-type visual pigments. Expression studies using RNA probes from these opsins on juvenile retina reveal that ultraviolet and blue opsin probes detect single cone cells, that green and red opsin probes detects double cones, and that the rod opsin probe stains rod cells. These same RNA probes were used to identify photoreceptor cells in larval halibut retina (Fig. 1). The *in situ* staining shows that the larval retina consists of single cone photoreceptor cells packed in rows. The photoreceptor cell nuclei are large and cylindrical, and the outer nuclear layer contains a single nucleus. The entire nuclear layer and most of the inner segment of the photoreceptor cells are stained (Fig. 1A,C,E,G). The subcellular expression patterns appear to be similar for the four different cone-type opsins. The opsin mRNA seems to be more homogeneously distributed in the larval photoreceptors than in juvenile photoreceptors in which expression is more punctate in the myoid region (J. V. Helvik, Ø. Drivenes, T. H. Næss, A. Fjose and H. C. Seo, unpublished results). All four types of photoreceptor cell have an outer segment (Fig. 1A,C,E,G), indicating that all the cone classes are functional. We were not able to detect any rod-opsin-expressing cells in larval retina, suggesting that this type of opsin is not yet expressed at this stage of development. This is in contrast to retinas in post-metamorphic halibut, in which we detected rod-specific

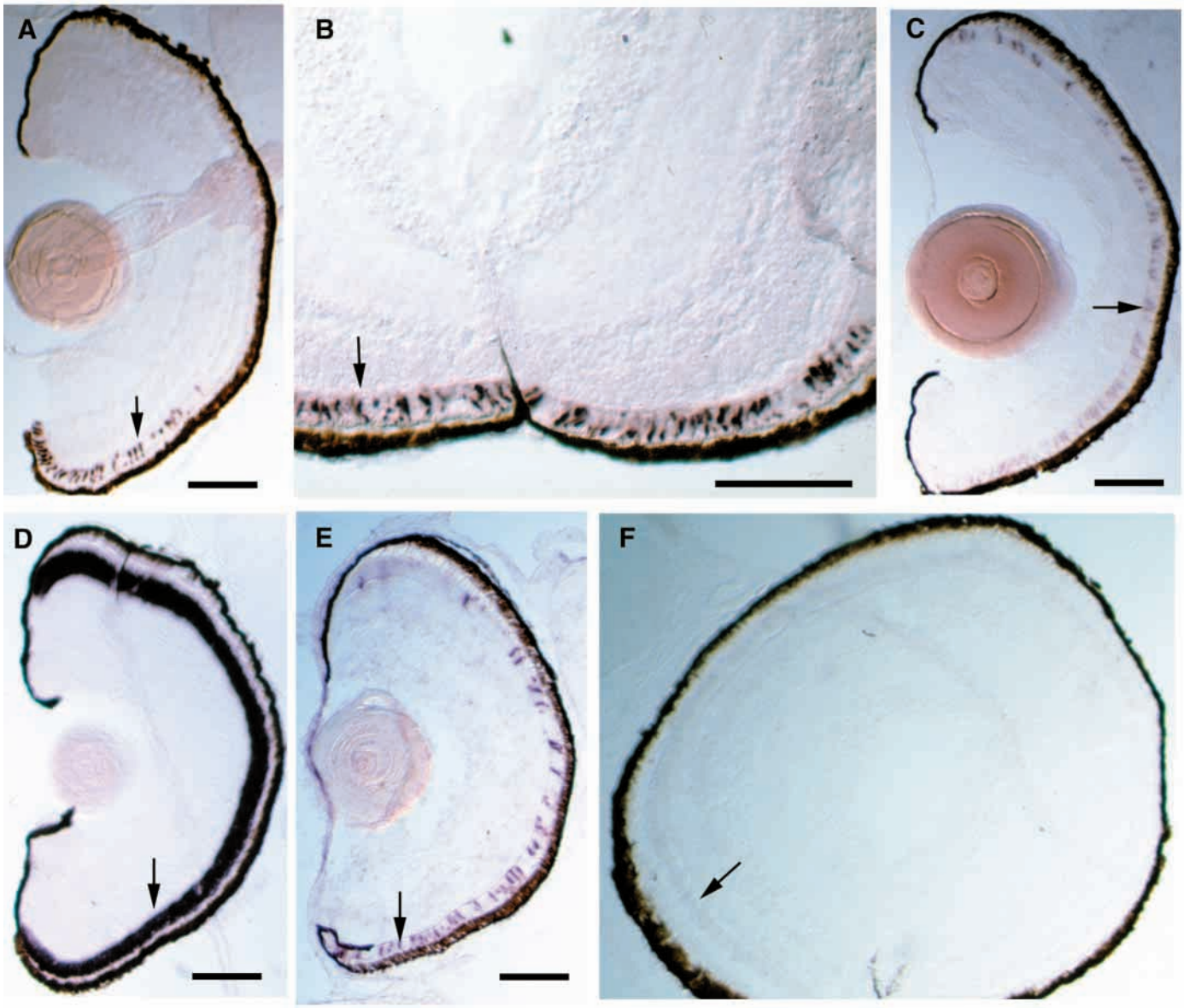

Fig. 2. Cellular distribution of various photoreceptor cells. Transverse sections (A,C,D,E) and sagittal sections (B,F) of Atlantic halibut retina (dorsal is up) at the end of the yolk sac stage (40 days post-hatching) are shown. The various photoreceptors were visualised by in situ hybridisation analysis with specific opsin probes: ultraviolet (A,B), blue (C), green (D), red (E) and rod (F). Arrows indicate the photoreceptor layer. Scale bars, 100 μm.

signals (J. V. Helvik, Ø. Drivenes, T. H. Næss, A. Fjose and H. C. Seo, unpublished results).

Mosaic distribution

The distribution patterns of the four different cone photoreceptor cells in the tangential plane were analysed by *in situ* hybridisation as shown in Fig. 1B,D,F,H. Tangential sections of the central retina at the level of the photoreceptor cells were used to demonstrate the distribution of the blue-, red- and green-sensitive opsin-expressing photoreceptor cells (Fig. 1D,F,H), while a tangential section of the ventral retina was used in the case of ultraviolet-sensitive opsin-expressing cells (Fig. 1B). The ultraviolet sensitive opsin-expressing cones in the ventral retina are surrounded by non-ultraviolet opsin-expressing cells (Figs 1A,B, 2A,B), which express other opsins such blue-, green- or red-sensitive opsin. A similar pattern of expression in single cone cells surrounded by non-opsin expressing cells is also observed for blue- and red-sensitive opsin-expressing cells (Fig. 1C,D and G,H, respectively). The green-sensitive opsin-expressing cones are different from the other opsin-expressing cones: a single green-sensitive opsin-expressing cone cell is surrounded by and in close contact with other green opsin expressing cells (Fig. 1E,F) and also with cones expressing different opsins.

Counting the number of blue- and red-sensitive opsin-expressing cells in an array covering 16×16 cells (Fig. 1D,H) shows that approximately 10 cells each express blue- or red-sensitive opsin. In Fig. 1F, the entire array is covered by green-sensitive opsin-expressing cells, and there are only a few white spots (see white arrow) representing non-green opsin-expressing cells. In total, there are approximately 250 cells in the counted array and since about 20 of them expressed either blue-sensitive or red-sensitive opsin and the rest green-sensitive opsin, an estimation indicates that the majority of the photoreceptor cells (90 %) seem to express green-sensitive opsin, while a minority (10 %) express either blue-sensitive or red-sensitive opsin. A similar correlation seems also to be the case for the ultraviolet-sensitive opsin-expressing cone in the ventral retina.

Topography of cone cells

Expression of ultraviolet-sensitive opsin is restricted to photoreceptor cells in the ventral part of the retina (Fig. 2A,B, Fig. 3A). Ultraviolet-sensitive opsin-expressing cells are located both nasal and temporal to the choroid fissure and cover approximately one-third of the retinal hemisphere. Within this region, the ultraviolet-sensitive opsin-expressing cells are intermingled with photoreceptor cells that do not express ultraviolet-sensitive opsin. The blue-sensitive opsin-expressing photoreceptor cells are evenly distributed over the entire retina with no apparent regional distribution (Fig. 2C, Fig. 3B). The distribution of green-sensitive opsin-expressing cells also covers the entire retina without any major regional specificity (Fig. 2D, Fig. 3C). The density of green-sensitive opsin expressing cells is too high to see the single cells in the 10 µm thick cryosection (Fig. 1E, Fig. 2D). The distribution

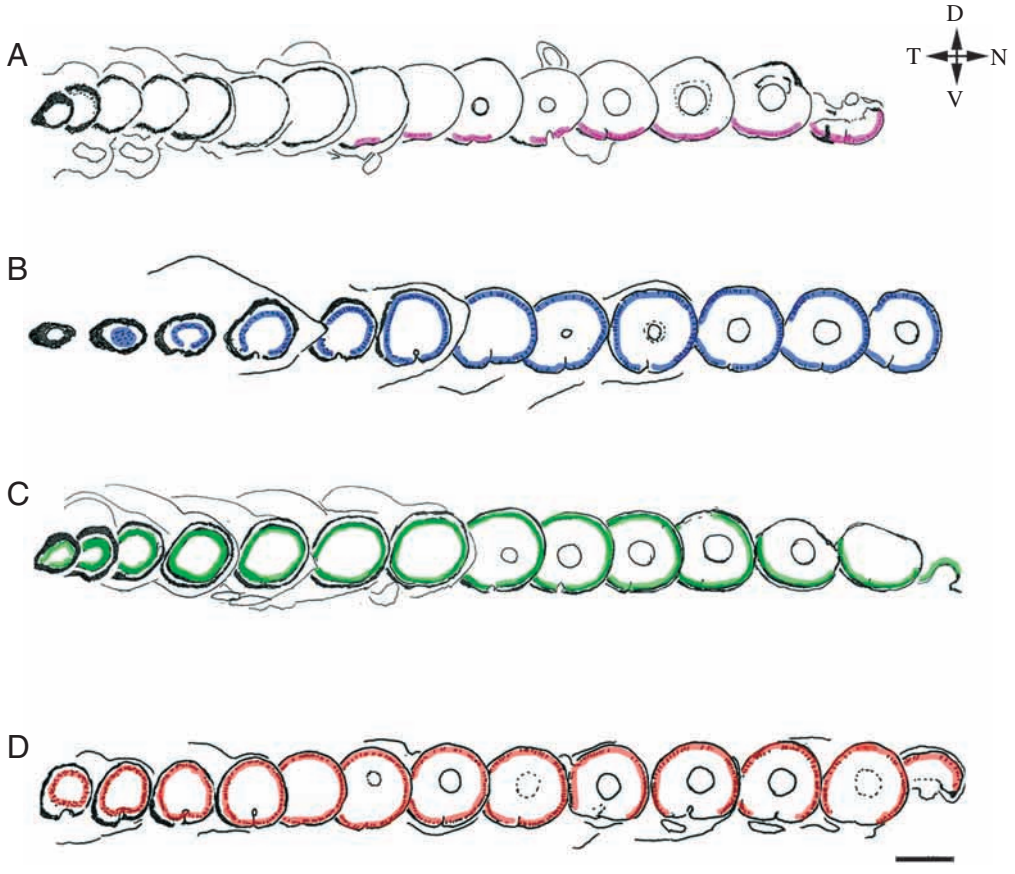

Fig. 3. Retinal location of the various cone opsins. Drawing of selected sections from a section series of Atlantic halibut larvae (40 days post-hatching). Four different halibut cone opsins probe are used. Cone distribution is indicated with colour code as follows: violet for ultraviolet- (A), blue for blue- (B), green for green- (C) and red for red sensitive-opsin. Scale bars, 0.5 mm. D, dorsal; N, nasal; T, temporal; V, ventral.

pattern of red-sensitive opsin-expressing cells is similar to that for blue-sensitive opsin, both with respect to distribution over the entire retina and also to the number of photoreceptor cells (Fig. 2E, Fig. 3D). Analysis of the retina at the first feeding and end of the yolk-sac stage revealed no photoreceptor cells with expressing rod-type opsin (Fig. 2F).

The overall topography of the various cone photoreceptor cells in the left eye of halibut larvae is presented in a set of two-dimensional illustrations that represent drawings of serial sections stained with the different opsin probes (Fig. 3). The area containing ultraviolet-sensitive opsin-expressing cells is shown with violet, with black dots indicating single ultraviolet cones. Ultraviolet-sensitive opsin-expressing cells are only observed in the most ventral retina at the larval stage (Fig. 3A). Blue-, green- and red-sensitive opsin-expressing cells are present in all the sectors of the retina (Fig. 3B–D). Single photoreceptors are illustrated by black dots in red- and blue-sensitive opsin expressing cells (Fig. 3B,D), while the density of green-sensitive opsin expressing cells was too high to distinguish individual cells.

Discussion

In this report, we show various opsins that are expressed in single cone photoreceptor cells using *in situ* hybridisation in the larval retina of Atlantic halibut. At the phase of first feeding, the retina of halibut larvae consists of morphologically identical photoreceptor cells that are probably single cone cells. Whereas conventional light microscopy failed to distinguish different types of larval photoreceptor cells (Kvenseth et al., 1996), we were able to distinguish them using specific markers for the various visual pigments. Atlantic halibut express ultraviolet-, blue-, green- and red-sensitive-type visual pigments at the end of the yolk-sac stage prior to first feeding.

The spectral characteristics of photoreceptors examined to date have been closely related to their morphological characteristics (Bowmaker, 1995). Thus, in fish larvae whose retinas contain only a single type of cone, one might assume that all photoreceptors contain the same visual pigment. In the winter flounder (*P. americanus*), which is closely related to the halibut, it has been shown that this single visual pigment absorbs in the green region of the spectrum (Evans et al., 1993). This result indicates that fish larvae have visual systems based on one cone type and that their visual system is transformed into one based on three or four cone types during metamorphosis. This study shows for the first time that photoreceptor cells that look identical at the morphological level may express different visual pigments. Furthermore, different cone opsins are present even though the classic morphological characteristics are not yet fully developed. Microspectrophotometry characterises photoreceptors according to their absorbance spectrum. However, the disadvantage with this method is that one can easily miss rare classes of photoreceptors or their regional distribution. This is especially problematic in larval retina, in which the absence of morphological characteristics in the single cones complicates the collection of cones for study.

The early life history of many marine teleosts includes a prolonged larval period, such that they follow an indirect developmental programme with delayed appearance of rod cells (Evans and Fernald, 1990; Balon, 1999). On the other hand, fishes with a more direct type developmental program (e.g. goldfish and zebrafish) develop a retina in which rod-type opsin is expressed first, followed by cone-type opsins red-, green-, blue- and lastly ultraviolet-sensitive opsin, during a restricted period in the late embryonic or early larval phase (Raymond et al., 1995; Stenkamp et al., 1996). Since our results also show that all different cone type opsins are expressed in the larval retina of fish with any indirect developmental program, the main difference between fish with these two different developmental programs seems to be related to the timing of expression of rod-type opsins, rather than the pattern of expression of cone-type opsin.

The retina of juvenile halibut is organised into a ‘square mosaic’, which first appears during metamorphosis (Kvenseth et al., 1996). *In situ* hybridisation staining of the photoreceptor cells in larval retina clearly shows an expression pattern that indicates some type of pattern of mosaic formation at this early stage of retinal development, when the retina consists only of single cones organised into a hexagonal row array. The identical morphology of the single cone cells makes it impossible to determine whether a green-sensitive opsin-expressing cell is a precursor for a double cone, although it is clear that the majority of the photoreceptor cells express green-sensitive opsin (see Fig. 1E,G).

In a typical square mosaic pattern, there are two double cones for each single cone. Since green- and red-sensitive opsins are expressed in equal numbers in double cones while blue-sensitive opsin is expressed in single cones, the ratio should be 2:2:1 for green:red:blue cones. Likewise, if all double cones express green-sensitive opsin, the ratio should be 4:1 (green:blue cones). In Atlantic halibut larvae, our results indicate a ratio of green:red:blue cones of 25:1:1 in the central retina. This result, a transformation from a green-sensitive opsin-dominated retina at the larval stage to a square mosaic expression pattern later in development, implies changes in opsin expression within individual cones. A similar result was obtained using microspectrophotometry data in winter flounder (Evans et al., 1993). Further studies such as calculations of the number of cells and their location in the retina during larval stages and metamorphosis are needed to elucidate the pattern of mosaic formation in a flatfish like halibut. Nonetheless it is very interesting that the early ‘mosaic pattern’ of opsin expression that we report here is present in a retina that lacks rod photoreceptors. These data, obtained from a naturally developing retina, support recent data suggesting that rod cells may not have an inductive role in the development of the cone mosaic (Wan and Stenkamp, 2000).

Ultraviolet-sensitive opsins are expressed in halibut larvae at the time of first feeding, implying that marine fish larvae living in the pelagic environment may be able to detect

ultraviolet light. The ventral distribution of the ultraviolet photoreceptor cells shows that it is the downwelling ultraviolet light that is important for the larvae. The implications for the visual system are not yet clear, but it has been suggested that detection of ultraviolet light could increase the contrast of a zooplankton prey against the water background (Loew et al., 1993; Browman et al., 1994). In the case of halibut, the location of ultraviolet opsin in the ventral retina suggests that halibut see the prey as dark objects against a bright ultraviolet background.

The present *in situ* hybridisation study shows that green cones dominate the retina of larval halibut. Photoreceptor cells expressing blue- and red-sensitive opsins are also distributed over the entire retina, but much less extensively. In the pelagic marine environment, the spectral surroundings are also dominated by green light, so the domination of green-sensitive opsin-expressing photoreceptor cells correlates with the spectral profile of the surroundings. Spectral absorption analysis (i.e. microspectrophotometry) of visual pigments is needed to confirm the significance of this finding. It is worth noting that the halibut retina probably has higher resolution in the green region of the spectrum as a result of the high numbers of green-sensitive opsin-expressing cells compared with red- and blue-sensitive opsin-expressing cells. The implication of these finding for visual function remains unclear at this time.

Further molecular identification of the visual pigments and *in situ* hybridisation studies on the photoreceptor cells in other marine species are needed to verify if marine pelagic fish larvae have a green-sensitive cone-dominated retina and ultraviolet-sensitive cones distributed only in the ventral part of the retina.

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