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*Original Article*

# Role of the pineal organ in the photoregulated hatching of the Atlantic halibut

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**ABSTRACT The timing of hatching in the Atlantic halibut *(Hippoglossus hippoglossus)* has been suggested to be regulated by environmental light conditions. However, the photosensory organ that perceives the triggering light has not been identified. In the present study, we investigated the morphogenesis of the pineal organ and the neurochemical differentiation of photoreceptors in the pineal organ and the retina of the Atlantic halibut during embryonic development. Immunocytochemical techniques were used for detection of integral protein components of the phototransduction process: opsins, arrestin (S-antigen) and α-transducin. We also studied the expression of serotonin (5-HT), a precursor of the neurohormone melatonin known to be synthesized by pineal photoreceptors. In the pineal anlage, opsin immunoreactive (ir) cells appear at 11 days post-fertilization (pf), arrestin, α-transducin and serotonin ir cells appear at 14 days pf; hatching took place 15 days pf. The retina contained no immunoreactive cells in embryos or in newly hatched larva. During this period, the pineal anlage is morphologically discernible only as a wedge-shaped region in the diencephalic roof, where elongated cells are aligned with their long axes converging toward a centrally located presumptive pineal lumen. The results show that the pineal photoreceptors contain serotonin and molecules involved in the phototransduction cascade before hatching. We suggest that the pineal organ has the capacity to perceive and mediate photic information before hatching in halibut embryos, and may thereby influence the timing of hatching.**

KEY WORDS: *teleost, development, pineal organ, photoreceptors, phototransduction proteins, melatonin, hatching*

## Introduction

The Atlantic halibut is among the largest teleost species living in the North Atlantic. It spawns at 300-700 m depth during January to March (Haug, 1990). After spawning, the eggs, which are among the largest of pelagic teleost eggs, gradually move upwards in the water column to a depth of 100-250 m. Thus, deep in the ocean, halibut embryos experience only low light intensities, especially during the dark winter season.

In Atlantic halibut the environmental light condition is suggested to be the natural cue that times hatching via a "dark-signal" that mediates the initiation of the hatching process (Helvik and Walther, 1992). This is based on the observation that incubation of halibut eggs in continuous light results in sustained inhibition of hatching and that transfer of these light arrested eggs to darkness results in synchronous hatching within 90-120 min. Light intensities down to 0.1 lux seem to delay hatching.

The neurohormone melatonin is considered to serve as "the chemical expression of darkness" in vertebrates (Reiter, 1991). In the species studied, melatonin synthesis is high during the dark period and low during the light period. In fish, melatonin is synthesized by photoreceptors in both the pineal organ and the retina. However, at the time of hatching, the halibut retina consists only of neuroblastic cells and the first morphologically differentiated photoreceptors appear about 25 days (ca 150 day degrees) after hatching (Kvenseth *et al.*, 1996).

In fishes, as in other non-mammalian vertebrates, the pineal organ is suggested to be the most influential extraretinal light receptor. In adult fish, the pineal organ transmits information about the ambient photoperiod by melatonin, and by neural input to the brain. Melatonin, as well as neural signals, are believed to be involved in the regulation of several physiological and behavioral events (for review see Ekström and Meissl, 1997). Pineal photoreception has been suggested to be involved in mediating phototactic behavior in larval species such as the stickleback (Östholm *et al.*, 1987) and the Arctic charr (Vigh-Teichmann *et al.*, 1991) whereas this does not seem to be the case in larval zebrafish (Easter and Nicola, 1996). However, there are no data directly showing a

*Abbreviations used in this paper:* 5HT, 5-Hidroxytryptamine; ir, immunoreactive; pf, post-fertilization; v, ventricle.

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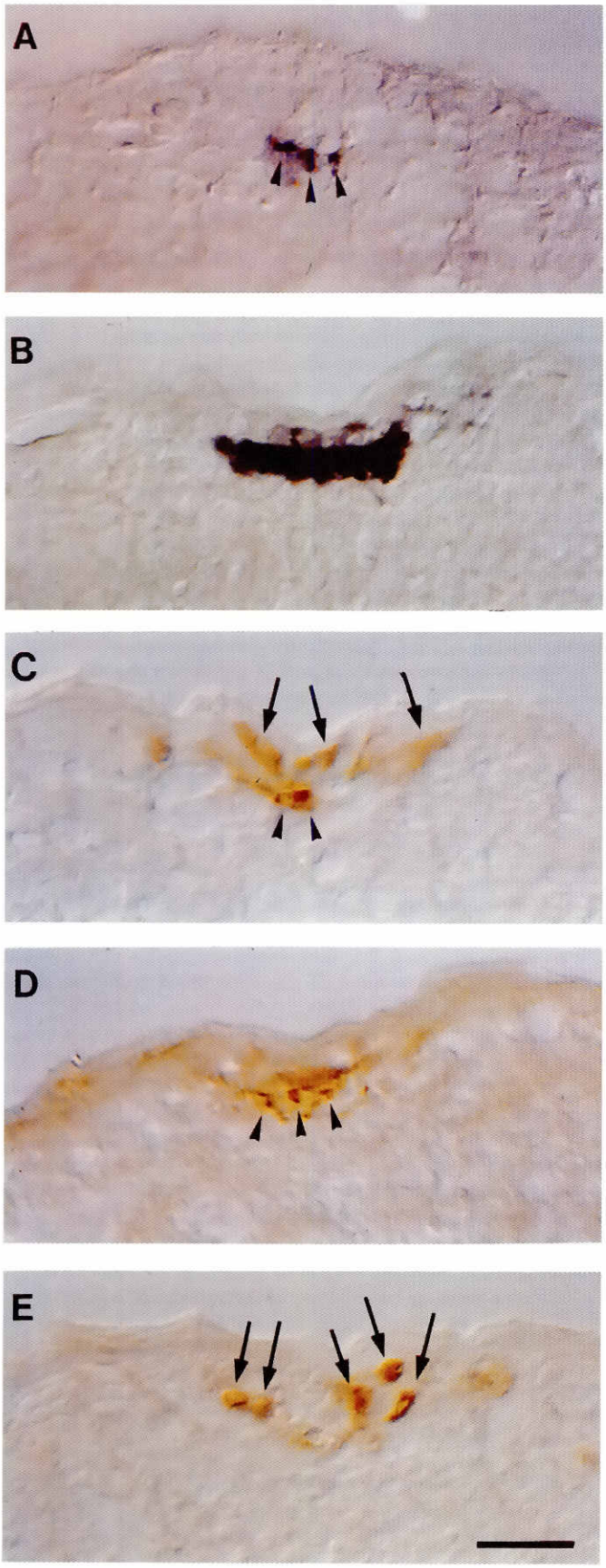


physiological role of pineal photosensitivity in embryonic stages of fish.

Pineal photoreceptors have been shown, using immunocytochemistry, to express phototransduction proteins similar to those in retinal photoreceptors: opsin (Vigh-Teichmann *et al.*, 1983; Östholm *et al.*, 1987), arrestin (Ekström *et al.*, 1987) and α-transducin (van Veen *et al.*, 1986). Antibodies against melatonin (Falcón *et al.*, 1981) and HIOMT (Falcón *et al.*, 1994) have been used to probe the site of melatonin synthesis. However, since serotonin is a precursor of melatonin, antibodies against serotonin have often been used as markers for presumed melatonin producing pineal cells (van Veen *et al.*, 1984; Ekström and Meissl, 1990).

In the present study, we have investigated the morphological development of the pineal organ and the expression of immunoreactivities for phototransduction molecules and serotonin in photoreceptors in the pineal organ and the retina during embryonic development of the halibut.

## Results

At 11 days pf the first opsin immunoreactive (ir) cells were detected by the OS-2 antibody in a circumscribed region of the diencephalic roof which corresponds to the pineal organ, i.e. the pineal anlage (Fig. 1A). At this stage the pineal anlage could not be morphologically distinguished from the surrounding cells in the diencephalic roof. The opsin immunoreaction was restricted to the outer portion of photoreceptor cells, i.e. outer segments. The opsin ir outer segments occupied the central portion of the pineal anlage. This central portion is elongated in the rostro-caudal direction.

During the subsequent development, the OS-2 opsin ir outer segments increased in both number and size. At one day before hatching (14 days pf) a more prominent and elongate central structure of densely packed opsin ir segments was present. Moreover, it appears that also the photoreceptor cell bodies are opsin ir at this stage (Fig. 1B). At 14 days pf the first cells immunoreactive for arrestin, α-transducin and serotonin were observed in the pineal anlage. The arrestin immunoreaction was confined to dorsally located cell bodies and also to a small number of outer segments (Fig. 1C). The α-transducin immunoreactivity was relatively weak and restricted to a small number of outer segments (Fig. 1D). The serotonin immunoreaction was present in photoreceptor cell bodies (Fig. 1E). The photoreceptors in the pineal anlage were immunonegative with the COS-1 antibody during the period 7 days pf to hatching.

At 14 days pf, when the photoreceptor markers give cellular labeling, the pineal anlage was still weakly differentiated with respect to the surrounding diencephalic roof. However, a slight

**Fig. 1. Immunoreactions in the halibut pineal anlage during embryonic development. (A)** *The first opsin immunoreaction (OS-2, detecting blue and green visual pigments) in photoreceptor outer segments (arrowheads) at 11 days postfertilization (pf).* **(B)** *Increased number of densely packed opsin immunoreactive (OS-2) photoreceptor cells at 14 days pf (one day prior to hatching).* **(C)** *Arrestin immunoreactive photoreceptors with dorsally located cell bodies (arrows) and centrally protruding outer segments (arrowheads) at 14 days pf.* **(D)** *Weak α-transducin immunoreaction in a small number of photoreceptor outer segments (arrowheads) at 14 days pf.* **(E)** *Serotonin immunoreaction confined to photoreceptor cell bodies (arrows) at 14 days pf. (A,C) Transversal sections (10 μm). (B,D,E) Sagittal sections (10 μm), rostral to the left. Bar, 20 μm.*

thickening of the roof was seen in the region containing the immunoreactive elements (Fig. 2A). This region could be distinguished as a wedge-shaped area containing elongated cells with their axes pointing towards a (somewhat ventrally displaced) central region (Fig. 2B). This central region appeared to be equivalent to the region displaying opsin ir, arrestin ir and α-transducin ir photoreceptor outer segments.

In the retina, immunoreaction against opsins (OS-2 and COS-1), arrestin, α-transducin and serotonin could not be detected during the period between 7 days pf to hatching.

## Discussion

In the present investigation of the Atlantic halibut we demonstrate that the pineal anlage is morphologically weakly differentiated before hatching but contains photoreceptors that are immunoreactive to opsin, arrestin, α-transducin and serotonin. In contrast, the retina does not show any immunoreaction, and it has been shown that the halibut retina contains only neuroblastic cells at the time of hatching (Kvenseth *et al.*, 1996). This suggests that the pineal anlage is endowed with the first functional photoreceptors in the halibut embryo. Since there are no indications of other photoreceptors, the pineal anlage is suggested to be the only mediator of environmental light conditions during the embryonic life stage.

### *Pineal morphology*

In contrast to the early appearance of phototransduction molecules and serotonin in pineal photoreceptors, the morphological differentiation of the pineal organ is late in the halibut. The late morphological differentiation may differ from that of other fish species. During the embryonic development of the rainbow trout (Omura and Oguri, 1993), the stickleback (Ekström *et al.*, 1983) and the Arctic charr (Vigh-Teichmann *et al.*, 1991), the pineal organ differentiates into a distinct pineal end-vesicle which protrudes over the habenular commissure. The late morphological differentiation of the pineal organ is similar to that observed in the retina (Kvenseth *et al.*, 1996) and in the central visual system in the halibut (Holmqvist *et al.*, 1996). When directly compared with two other marine species, the Atlantic herring and Atlantic cod that hatch after a similar developing time (13-15 days at 6-8°C incubation temperature), it has been shown that the development of the retina and retinal projections to the brain is much slower in the halibut (Holmqvist *et al.*, 1996).

### *Expression of phototransduction proteins in pineal photoreceptors*

In the halibut, the expression of opsin, arrestin and α-transducin in the pineal photoreceptors precedes that in retinal photoreceptors. This corresponds with the observations in stickleback (van Veen *et al.*, 1984; Östholm *et al.*, 1988), Atlantic salmon (Östholm *et al.*, 1987) and blue acara (Negishi and Wagner, 1995). However, in the halibut pineal anlage we found a different sequence of expression of phototransduction proteins. Opsin ir outer segments appeared several days before arrestin and α-transducin ir cells in the halibut, whereas these proteins are expressed simultaneously in the stickleback and salmon.

One explanation to this disparity may be that the extended time course of development in halibut (Holmqvist *et al.*, 1996) contributes to a higher temporal resolution of sequential events. However, the disparity could also indicate an interspecific variation in the expression of the phototransduction proteins among fishes due to their vast variations in biology and life strategies.

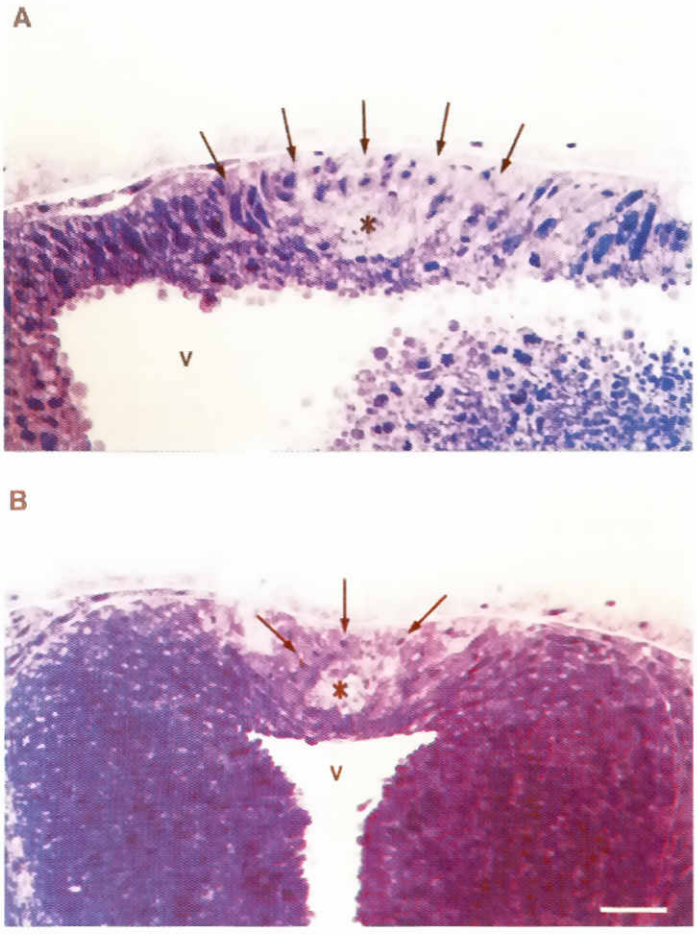


**Fig. 2. Morphological differentiation of the halibut pineal anlage.** *Toluidine blue stained sections of embryos at 14 days post fertilization (one day prior to hatching).* **(A)** *Sagittal section (rostral to the left) showing the weakly differentiated pineal anlage as a slim swelling in the dorsal roof (arrows).* **(B)** *Transversal section demonstrating the same area as in* **(A)**, *with a pineal anlage encompassing elongated cells (arrows) with their axes protruding towards a central region (asterisks). Note: immunoreactive photoreceptor outer segments (see Fig. 1) appear to be situated in a region equivalent to the region marked with asterisks in (A and B). V, ventricle. Sections, 5 μm. Bar, 20 μm.*

As shown in several investigations, opsin, arrestin and α-transducin play important roles in the phototransduction of retinal rods, and it has been suggested that they play similar roles in pineal photoreceptors (Korf *et al.*, 1989). Opsin is the peptide component of photopigments associated with vitamin-A aldehydes. Arrestin, also known as S-antigen, binds to photoexcited and phosphorylated rhodopsin upon illumination. α-transducin is a subunit of the heterotrimeric GTP-binding protein which acts as a mediator between photoactivated pigment and cGMP phosphodiesterase.

Pineal specific opsins found in chicken, "pinopsin" (Okano *et al.*, 1994) and "p-opsin" (Max *et al.*, 1995) have several unique

features when compared to retinal opsins, indicating different mechanisms in the pineal and retinal phototransduction process. Furthermore, a separation of the spectral sensitivity between pineal and retinal photoreceptors has been indicated in the channel catfish. In the catfish, pineal photoreceptors express a pineal specific blue-sensitive opsin, while the retinal counterparts express rhodopsin and a red-sensitive opsin (Grad *et al.*, 1996). In the present study of the halibut, two opsin antibodies were used, the OS-2 antibody and the COS-1 antibody demonstrating blue/green- and red/green-sensitive pigments, respectively, in the retina of lower and higher vertebrates (Szél *et al.*, 1988; Cserháti *et al.*, 1989; Vigh-Teichmann and Vigh, 1990). Since only the OS-2 antibody gives cellular staining in the pineal anlage, it appears that the halibut pineal opsin, at least during the early developmental stage around hatching, could be a blue-sensitive pigment. In the halibut, blue light seems to delay hatching more effectively than red or yellow light (Helvik and Walther, 1992). This wavelength-dependent hatching response in halibut supports the hypothesis that a blue-sensitive pineal opsin may be the specific opsin type involved in the regulation of hatching. However, to obtain more specific data about wavelength-dependent hatching, halibut eggs should be incubated in light with different wavelengths using narrower monochromatic filters together with isolation and characterization of halibut photopigments.

***Melatonin: a dark-generating hatching signal in the halibut?***

In some fish species, light-dark conditions are known to be the influential factor for hatching. Zebrafish and medaka (Schoots *et al.*, 1983), and Atlantic salmon (Brännäs, 1987), maintained in a light-dark cycle, hatch mainly during the light period. In contrast to these species, light arrests hatching in halibut (Helvik and Walther, 1992). Thus the embryo must attain a specific developmental stage, appropriate to receive the stimuli from triggering or suppressing factors such as light. In the halibut, a darkness-generated signal has been proposed to cause the release of the proteolytic hatching enzyme and initiate the hatching process (Helvik and Walther, 1992). The present study emphasizes the fact that pineal photoreceptors of the halibut express the melatonin precursor, serotonin, before hatching. Melatonin synthesis is regulated by the ambient light-dark cycle. Melatonin synthesis increases rapidly at the beginning of the dark period and remains high until the end of the period (Falcón *et al.*, 1987; Meissl and Brandstätter, 1992). Suppression of melatonin synthesis (Max and Menaker, 1992) as well as the delay of hatching in halibut (Helvik and Walther, 1992) are proportional to increasing light intensities, which suggests that melatonin may be the mediating signal of pineal origin that triggers darkness-induced hatching in halibut.

The pineal organ may mediate light information also via neuronal projections to central brain areas (Ekström and van Veen, 1984; Ekström and Korf, 1985). Pineal projections have been suggested to play a role in photo-neuroendocrine actions during parr-smolt transformation of the Atlantic salmon (Holmqvist *et al.*, 1994). Pinealofugal axons, coursing in the dorsoventral diencephalic tract, have been described in early life stages of zebrafish embryo (Wilson and Easter, 1991). In the halibut embryo, similar axons have been detected in close relation to the pineal organ (Forsell *et al.*, 1996). As the neural activity of the pineal organ is inversely proportional to the ambient irradiance (for review see Ekström and Meissl, 1997), it is also possible that the darkness-generated signal in the halibut embryo may be of neural nature.

In conclusion, pineal photoreceptors in halibut seem to have the capacity to receive and transmit ambient light information already before hatching and may thereby be responsible for photoinfluenced events such as hatching. It is suggested that pineal photoreceptors mediate the inhibitory effect of light on hatching, e.g. by suppression of a "dark signal" (possibly melatonin) that is required to induce hatching.

## Materials and Methods

Fertilized eggs of Atlantic halibut were obtained from Austevoll Marine Aquaculture station (Norway). Eggs were maintained in darkness in seawater at 6°C. A batch of 25-30 eggs or larvae were collected daily from day 7 pf to hatching (15 days pf). They were placed in cold 4% paraformaldehyde in 0.1 M Sörensen's phosphate buffer (pH 7.2) for 12-16 h. Egg shells and membranes were removed and the embryos and larvae were rinsed thoroughly in Sörensen's buffer. Specimens were left overnight in an infiltration solution with 25% sucrose and 20% Tissue Tek™ embedding matrix, in Sörensen's buffer. Whole embryos and larvae were embedded in Tissue Tek, frozen in liquid nitrogen and cryosectioned in sagittal or transversal sections (10 μm). Sections were mounted on chrome alum-gelatinized slides and processed for immunocytochemistry.

Eggs intended for histological examination were collected at 11 and 14 days pf. They were fixed by immersion in 2% paraformaldehyde and 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2). Egg shells and membranes were removed and the embryos were rinsed thoroughly in Sörensen's buffer, dehydrated in ethanol and embedded in Historesin™ (Reichert-Jung). Specimens were sectioned in sagittal and transversal sections (5 μm) on a microtome, and stained with toluidine blue.

Five different antibodies were used. Monoclonal mouse anti-chicken opsin antibody (OS-2) detecting blue- and green-sensitive pigments in the mammalian retina (Szél *et al.*, 1988) and the *Xenopus* retina (Röhlich *et al.*, 1989), diluted 1:15000. Monoclonal mouse anti-chicken opsin antibody (COS-1) detecting red- and green-sensitive pigments in the chicken and pigeon retina (Szél *et al.*, 1986; Cserháti *et al.*, 1989), and red-sensitive pigments in the green frog retina (Vigh-Teichmann and Vigh, 1990), diluted 1:5000. These two monoclonal opsin antibodies were kindly provided by Dr. A. Szél. Monoclonal mouse anti-bovine arrestin antibody (Donoso *et al.*, 1990; kindly provided by Dr. C.M. Kalsow), diluted 1:500. Polyclonal rabbit anti-α-transducin antibody (van Veen *et al.*, 1986; kindly provided by Dr. Th. van Veen) diluted 1:500. Polyclonal rabbit anti-serotonin antibody (Incstar) diluted 1:500.

All antisera were diluted in 0.1 M phosphate buffered saline containing 0.25% Triton-X (pH 7.2; PBS-TX) and 0.1% bovine serum albumin. The following immunocytochemical protocol was used: (1) Rinses in PBS-TX, 2x5 min. (2) Incubation in primary antibody, overnight at room temperature (RT). (3) Rinses in PBS-TX, 2x10 min. (4) Incubation in rabbit anti-mouse IgG serum (Dako, Copenhagen, Denmark) 1:50, or (for anti-α-transducin and anti-serotonin) swine anti-rabbit IgG serum (Dako, Copenhagen, Denmark) 1:50, in RT for 30 min. (5) Rinses in PBS-TX, 2x10 min. (6) Incubation in mouse peroxidase-antiperoxidase (PAP) complex (DAKO, Copenhagen, Denmark) or (for anti-α-transducin and anti-serotonin) in rabbit PAP complex (DAKO, Copenhagen, Denmark) 1:50. (7) Rinses in PBS-TX, 2x5 min. (8) Rinses in 0.05 M Tris-HCl buffer (pH 7.6; TRIS). (9) Incubation in 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.03% $H_2O_2$ or (for anti-opsins) 0.0125% DAB with 0.015% $H_2O_2$ and 0.025% nickel ammonium sulphate in TRIS, 10 min. (10) Buffer rinse, 10 min. (11) Dehydration, mounting with Permount.

Microphotographs were taken with a Zeiss Axiophote microscope, equipped with 10x Plan Neofluar, and 16x, 25x, 40x and 63x Plan Neofluar oil immersion objectives, and Nomarski differential interference contrast equipment.

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