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Occlusable corneas in toadfishes: light transmission, movement and ultrastruture of pigment during light- and dark-adaptation

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Summary

The toadfishes *Tetractenos hamiltoni* and *Torquigener pleurogramma* (Tetraodontidae) possess occlusable yellow corneas. We examine the light transmission and location of the yellow/orange pigment throughout the cornea, the temporal properties of pigment migration and the ultrastructure of the pigmented processes during light- and dark-adaptation. Each species was dark-adapted during the day and light-adapted during the night and then exposed to either sun illumination or darkness for different lengths of time (0–70 min). Movement of corneal pigment could be induced in both species regardless of time of day or night. The pigment was able to migrate in a dorsal or ventral direction and changed from minimal to maximal pigmentation within 60 min. Three types of transmission curves were found with varying degrees of transmission in the 400–500 nm waveband, indicating that the pigment distribution is not uniform across the cornea; some areas of the cornea transmit near UV light, while others absorb blue light. The gradual change of the transmission characteristics in different areas of the cornea indicates the presence of different concentrations of a single type of pigment. Ultrastructural examination of the corneas showed that the layer containing the pigment is situated within the scleral cornea either surrounding (*T. pleurogramma*) or abutting (*T. hamiltoni*) an iridescent layer. Long sheet-like processes or chromatophores extending centrally from dorsal and ventral reservoirs are filled with pigment during the light-adapted state but empty in the dark-adapted state.

Key words: occlusable cornea, ocular media transmission, ultrastructure, pigment movement, chromatophores, toadfish, *Torquigener pleurogramma*, *Tetractenos hamiltoni*.

Introduction

The ability of some fish to regulate their corneal pigment cover was first described for *Hexagrammos octogrammus* (Orlov and Gamburtseva, 1976). Since then, occlusable corneas have been recorded for a range of freshwater and marine species belonging to the orders Channiformes, Perciformes, Scorpaeniformes and Tetraodontiformes (Orlov and Gamburtseva, 1976; Appleby and Muntz, 1979; Gamburtseva et al., 1980; Gnyubkina and Gamburtseva, 1981; Kondrashev et al., 1986; Gnyubkina and Levin, 1987; Gnyubkin, 1989) (see also Orlov's website: Fishes with changeable corneal coloration. http://www.iitp.ru/projects/posters/cornea/list.htm).

The cells containing the coloured pigment are specialised chromatophores. These chromatophores consist of a cell body situated in the periphery and a single long process that extends into the centre of the cornea (Gnyubkina and Levin, 1987). The width of these processes has not been investigated. In some fish, these corneal colouration cells (CCCs; Gamburtseva et al., 1980), or corneal staining cells (CSCs; Kondrashev and Khodtsev, 1984), contain two different pigment types of either yellow or orange appearance (Orlov and Gamburtseva, 1976). Both types of pigment are present in the cornea of *Hexagrammos octogrammus*, and the yellow pigment differs from the orange pigment in its response to illumination. When the dark-adapted fish is exposed to light, the yellow pigment expands first, and does so irrespective of light composition. The orange pigment, on the other hand, migrates more slowly and does not disperse when illuminated in the 420–560 nm waveband (Kondrashev and Khodtsev, 1984).

Three different pigmentation patterns have been described (Gamburtseva et al., 1980). In most fishes with occlusable corneas, the cell bodies of the pigment cells are found in two sickle-shaped aggregations located in the dorsal and ventral periphery (e.g. in *Hexagrammos octogrammus*; Orlov and Gamburtseva, 1976). During light-adaptation, the pigment is shifted from the cell bodies into the processes, which extend into the centre of the cornea, so that after 1–2 h the entire cornea is covered with pigment. In other species, the pigment cell bodies are found mainly dorsally, where they form one sickle-shaped reservoir (e.g. in *Chirolophis japonicus*; Gamburtseva et al., 1980). During light-adaptation, the pigment is shifted into the cell processes, which extend into

the centre of the cornea just covering the pupil zone. In *Bathymaster derjugini*, the pigment cell bodies lie in a ring around the perimeter of the cornea (Gamburtseva et al., 1980). During light-adaptation, the pigment is shifted into the cell processes progressively, covering the entire cornea within 1–2 h of light-adaptation. All of the experiments to date have been performed during the day.

The mechanisms underlying pigment expansion and retraction have not yet been resolved, but possibilities include nervous, humoral or autonomous control of the pigment migration. Central control (neural or humoral) seems unlikely, because unilateral illumination only induced pigment movement in the illuminated eye, and optic nerve sectioning (in one eye) has no effect, i.e. no response difference was found between two illuminated eyes (Appleby and Muntz, 1979). Kondrachev and Khodtsev (1984) found differential changes in corneal colouration when left and right eyes were exposed to different illumination conditions in the same fish, and therefore agree that central control is unlikely.

Local (retinal) humoral control has been proposed by Kondrachev and Khodtsev (1984), who suggest that the illumination of the photoreceptors in each eye induces release of a hormone that triggers pigment expansion. In darkness, the retina releases acetylcholine, which reaches the pigment cell bodies in the cornea *via* the choroid, where it stimulates pigment aggregation. The rate of pigmentation change is slow, which supports the hypothesis that pigment migration is under humoral rather than neural control (Appleby and Muntz, 1979; Kondrashev and Khodtsev, 1984).

Another possibility is that pigment migration is under autonomous control within the pigment cells. Kondrachev and Khodtsev (1984) believe this possibility is unlikely, because the response of the pigment is dependant on the stimulating wavelength, i.e. the red pigment only expanded when illuminated with wavelengths between 560 nm and 620 nm. Also, they found that pigment migration was disturbed in isolated corneas (Kondrachev and Khodtsev, 1984).

Recently, novel opsins localised in extraretinal tissues have been implicated in the control of skin pigmentation, pupillary aperture, and circadian and photoperiodic physiology (Provencio et al., 1998; Philp et al., 2000). Melanopsin, which possesses an invertebrate opsin character with a stable metastate that retains the chromophore, is converted from one of the two possible stable states into the other, independently of the supply of the 11-*cis* chromophore (Provencio et al., 1998). Provencio et al. conclude that this independence would permit melanopsin to act in a large variety of tissues. It is, therefore, possible that the corneal pigment cells contain such novel opsins, which may regulate the pigment response in a wavelength-dependent manner as proposed by Kondrashev and Khodtsev (1984).

In addition to CCCs, fish corneas contain other types of chromatophores, such as erythrophores, melanophores and xanthophores (Gamburtseva et al., 1980). None of these pigment cell types possess long processes or play a role in the changeable filter properties of fish corneas (Orlov and Gamburtseva, 1976; Gamburtseva et al., 1980).

The chemical composition of the yellow pigment is believed to be of carotenoid origin, based on the shape of its absorption curve (Moreland and Lythgoe, 1968; Bridges, 1969). The orange pigment is believed to be different from the yellow pigment as its absorption curve does not show the three intermediate peaks, but rather displays a single broad absorption band (half-band about 400–550 nm; Orlov and Gamburtseva, 1976).

Several functions have been proposed for yellow ocular filters, including protection from potentially deleterious UV wavelengths (Zigman, 1971) and the elimination of scattered light. The reduction of chromatic aberration has also been suggested for these yellow filters to improve visual acuity and contrast discrimination (Walls and Judd, 1933a,b). In contrast to these functional advantages yellow filters decrease sensitivity during low light conditions. However, fish with occlusable yellow corneas can take advantage of coloured filters during high intensity illumination conditions while avoiding the disadvantage of losing sensitivity during low light conditions by retracting their pigment (Gamburtseva et al., 1980; Heinermann, 1984).

In this study, we compare pigment movement induced by light- and dark-adaptation during the day with that caused by light- and dark-adaptation during the night to test the influence of a possible underlying diurnal pigment migration rhythm on the observed pigment migration pattern. We also determine the temporal properties of pigment retraction and expansion during the day and measure the changes in transmission properties across the cornea as the pigment moves. Ultrastructural examination of the corneas of the two toadfish species in the light- and dark-adapted states reveals that the pigment migration within large sheet-like processes causes concomitant changes in corneal thickness.

Materials and methods

Species collection

28 toadfish [*Torquigener pleurogramma* (Regan 1903) and *Tetractenos hamiltoni* (Richardson 1846) Tetraodontidae] were captured with a seine net in tide pools close to the Moreton Bay Research Station (University of Queensland) situated on North Stradbroke Island, Queensland, Australia. 14 similar-sized individuals (5–6 cm standard length) of each of the two species were collected and transferred into holding aquaria at the Station. Both species are schooling species that live in shallow water over a sandy bottom (Kuiter, 1993). At low tide, they are often found buried in sand in shallow water with only their iridescent eyes visible (Fig. 1A,B).

Dark- and light-adaptation

14 specimens of each species were used for experiments during the day, while the other 14 were used during the night. Six fish of each species were kept in a glass aquarium that was illuminated from above by sunlight on a bright day to ensure

that the pigment was maximally extended and the remaining two fish of each species were dark-adapted by placing them into an aquarium kept in a dark room for at least 60 min. The pigment distribution of one of the day-adapted fish was used as a control for the maximal pigment cover of the cornea. The five remaining light-adapted fish were dark-adapted for different lengths of time (10–70 min) by placing them into a second aquarium in the darkroom. In the second part of the

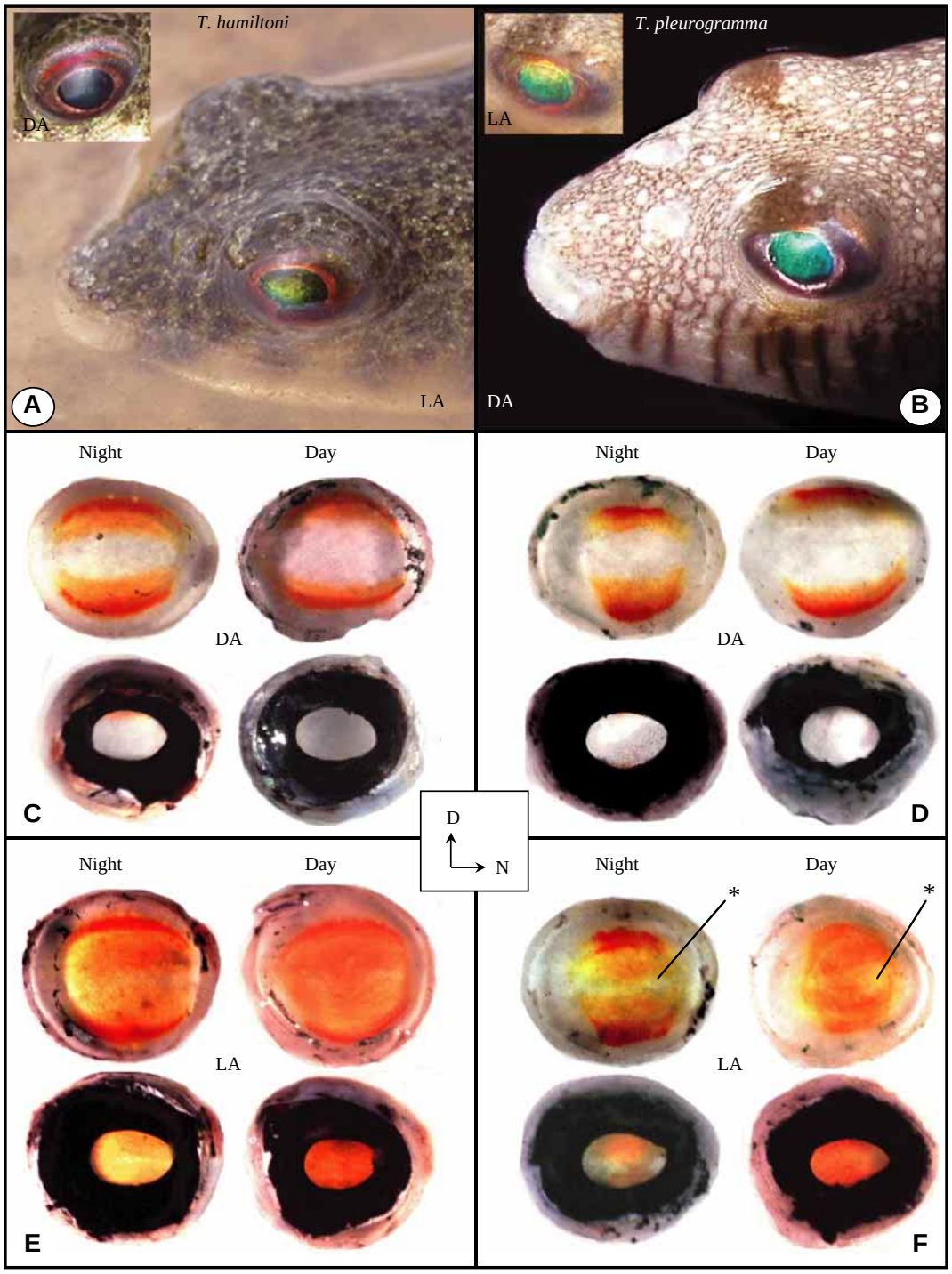

Fig. 1. (A) The common toadfish *Tetractenos hamiltoni*. (B) The weeping toado *Torquigener pleurogramma*. The colour of the iridescent cornea appears to be different in dark- (DA) and light-adapted (LA) conditions. (C–F) Pigment cover of the corneas of both toadfish during dark- (C,D) and light-adaptation (E,F) during the day and night. The light-adapted *T. pleurogramma* cornea (F) has a less densely pigmented temporal area, the 'pseudopupil' (asterisk). In each case (C–F), the isolated cornea (top) and the cornea with intact pupil (bottom) is shown. There are slight differences between the pigmentation during the night and the day, possibly indicating that there is an underlying diurnal rhythm in pigment migration. D, dorsal, N, nasal orientation.

daytime experiment one of the fish of each species that had been placed into the dark aquarium was used as a dark reference while the second fish of each species was light-adapted again (for 70 min).

The 14 night-adapted fish were kept in an aquarium outside the station in an area that was shaded from street illumination. One specimen of each species was used to determine the minimal pigment cover prior to the light-adaptation experiment. For 10–70 min, five fish of each species were exposed to a cold-light halogen lamp (KL1500, 150 W, Schott, Mainz, Germany) in combination with the normal room lighting (Fig. 2). The lamp was placed above the tank so that the entire area was illuminated uniformly and the walls of the tank were lined with Teflon to maximise brightness. The remaining two fish of each species were light-adapted for 60 min. One specimen of each was used as a light control while the other one was dark-adapted again.

For the analysis of pigment movement patterns, transmission properties and ultrastructure we present results for the dark-adaptation during the day and the light-adaptation during the night, simulating the natural direction of pigment movement at dusk and dawn. The results of the light-adaptation following dark-adaptation during the day and of the dark-adaptation following light-adaptation during the night were used to test whether pigment movement could be induced in both directions during the night and day.

Spectral transmission analysis

At the end of each experimental period, the fish were anaesthetised with methane tricaine sulfonate salt (MS222, 1:2000) and killed by decapitation in the dark (dark-adapted fish) or light (light-adapted fish). The eyes were enucleated and a window was cut into the back of the scleral eyecup so that the lens and vitreous could be removed without destroying the cornea. The eyes were then placed on white filter paper and photographed with a SLR camera (Canon EOS 1000). Remains of the retina and iris were then removed, and the cornea was rinsed in saltwater to remove traces of blood. Another photograph was taken of the isolated cornea before light transmission analysis. Spectral transmission spectra (350–800 nm) were obtained using ‘Sub-Spec’, a portable spectrophotometer (modified version of Oriel Instruments Intraspec IV system; described in Marshall, 1996). All samples were measured in air (Douglas and McGuigan, 1989). A ‘Spectralon’ white tablet was used as a 99% reflection standard. The instrument beam was aimed at the white tablet through different areas of the cornea under visual control. The cornea was mounted on a holder with which the position of the cornea could be controlled. Xenon illumination was provided with a camera flash with the front UV filter removed. Transmission spectra were normalised with respect to the transmission level at 700 nm (Douglas and McGuigan, 1989; Siebeck and Marshall, 2000, 2001).

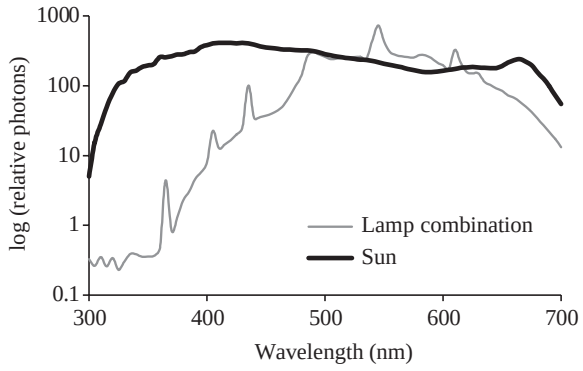

Fig. 2. Spectral composition of the two light regimes used in the experiment. The spectrum of the sun is much broader than that of the combination of the halogen lamp and the background room lighting. The illumination of the lamp combination is relatively poor in short wavelengths. The sharp spikes in the spectrum of the lamp combination are due to the overhead fluorescent room illumination.

During preliminary experiments, it was observed that the pigment of a dark-adapted isolated cornea, kept in a dish with salt water, dispersed under illumination. This pigment migration ceased if the isolated cornea was placed on filter paper that absorbed the excess water. All test corneas were therefore placed on filter paper as soon as the dissection was finished. Additionally, the overall illumination surrounding the experimental set up was matched to the adaptation condition to negate any post-enucleation pigment migration. We cannot completely rule out that anaesthesia and handling during the dissection and isolation of the corneas may also influence the pigment dispersal. We therefore took care that the preparations were made as quickly as possible. Approximately 5 min were needed for anaesthesia and preparation of the first cornea of each fish. We found that pigment migration from maximal to minimal (and *vice versa*) pigment cover takes about 60 min. It therefore seems that any changes caused by handling etc during the 5 min cannot be very large. Also, we compared the pigmentation pattern of the first cornea of each fish with that of the second cornea, which was left in the fish until the measurements of the first cornea were completed. A time difference of 5 min did not appear to affect our results. All corneas were treated exactly the same and any artefact due to handling should therefore affect all corneas in the same way. Differences between the different preparations must therefore be due to different stages of light- and dark-adaptation.

The locus of each transmission measurement was noted on both detailed drawings and photographs of the corneal pigmentation pattern. For each cornea, seven locations were scanned. Since the direction of the pigment movement was from the dorsal and ventral rims into the centre and back, five positions were selected along a vertical line through the centre of the cornea; dorsal rim, halfway point between the centre and the dorsal rim, centre, half-way point between the centre and the ventral rim, and the ventral rim. Also, two measurements were made along a horizontal line through the centre, one at the halfway point between the nasal rim and the centre and one at the halfway point between the temporal rim and the centre.

At each of the positions three measurements were made and averaged.

Characterisation of corneal pigment distribution

To characterise the pigment distribution after the different light- and dark-adapted treatments, corneal photographs were scanned using a Canon slide scanner (Nikon LS-1000, Tokyo, Japan). Images were then imported into Adobe Photoshop and a grid was superimposed over the cornea. The area of the isolated cornea, the pupil zone and the different regions of pigment cover were determined by counting the grid cells. The pupil zone was defined as the area of the cornea that was not covered by the immovable iris.

Temporal analysis of pigment migration

The pigment cover of the whole cornea, the pupil zone and the ventral and dorsal corneal hemifields of each species were determined during the day (light-adapted) and night (dark-adapted) as described above. Various measurements of pigment cover were performed after 10, 20, 30, 40, and 60 min of dark-adaptation of the light-adapted fish during the day and at the same time points of light-adaptation of the dark-adapted fish during the night. The two corneas of each fish were analysed and their results averaged. The two corneas of each fish were analysed as described above and their results were averaged (mean).

Ultrastructural analysis

For each species, both eyes of two specimens were fixed in 0.1 mol l^{-1} phosphate buffer containing 3.5% paraformaldehyde, 0.25% glutaraldehyde and 2% sucrose. Two eyes were fixed in the light-adapted condition and two in the dark-adapted condition. The corneas were postfixed in 2% osmium tetroxide with 1.5% potassium ferrocyanide in 0.1 mol l^{-1} sodium cacodylate buffer (as described in Collin and Collin, 1996). The tissue was then dehydrated in acetone and embedded in resin (polybed/812, Polysciences Inc.). For light microscopy thick sections (1 μm) were cut, stained with Toluidine Blue and examined with a Zeiss compound microscope (Axioskop, Jena, Germany) fitted with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, USA). Thin sections (60 nm) were prepared for transmission electron microscopy, stained with lead citrate and uranyl acetate and examined using a JEOL 1010 Transmission Electron Microscope (Peabody, USA). Negatives of all micrographs were digitised with a LeafScan 45 Negative Digitiser (Tel Aviv, Israel).

Results

Pigment distribution

During the day, the light-adapted cornea of *Tetractenos hamiltoni* is completely covered with orange pigment (Fig. 1). In contrast, only 83% of the light-adapted cornea of *Torquigener pleurogramma* is covered with yellow/orange pigment, leaving unpigmented areas in temporal and, to a lesser degree, nasal cornea (Fig. 1). The pattern is symmetrical, indicating that the dorsal and ventral parts of the cornea may contain the same amount of pigment. The central nasal cornea of *T. pleurogramma* contains an area (‘pseudopupil’) that appears less densely pigmented (visibly lighter) than the surrounding area in the light-adapted state (Fig. 1F, asterisk). In both species, the pupil zone is completely covered with pigment.

During the night, the dark-adapted corneas of *T. hamiltoni* and *T. pleurogramma* both show a 53% and 42% level of corneal pigmentation. In both cases, the pigment is retracted to the ventral and dorsal rims of the cornea and within these areas, two zones can be distinguished on the basis of a difference in colouration (Fig. 1C–F). The outermost zone appears orange, while the more central zone appears yellow. The centre of the corneas of both species is clear, i.e. the pupil zone contains no pigment.

After dark-adaptation during the day, the pigment is retracted so that the pupil zone is almost free of pigment (Fig. 1). Pigment retraction is not as strong as after dark-adaptation at night. Similarly, after light-adaptation during the night, the pigment expansion is not as strong as during light-adaptation during the day (Fig. 1).

Temporal changes in pigment migration

A change in the pigment cover of the cornea was induced by dark-adapting fish during the day, as well as by light-adapting fish during the night (Fig. 1). After 60–70 min illumination at night, the pigment of both species of toadfish appeared to be extended almost as far as it was during the day in bright sunshine. The pigment cover found in fish that were dark-adapted during the day was similar to that found in night-adapted fish (Fig. 1). However, the distribution pattern of the yellow and orange pigments appeared to be different in both species. During light-adaptation at night, the yellow pigment extended as far as it did during the day while the coverage of the orange pigment did not change (Fig. 1).

In 60 min of light-adaptation at night, pigmentation of the whole cornea changed from an average of 42% cover to 87% cover in *T. pleurogramma* and from 53% cover to 96% in *T. hamiltoni* (Fig. 3A). The time course in both species is very similar. After 10 min of exposure to light, pigment cover had already reached 79% and 80% of the entire cornea, respectively. In the first 10 min of illumination, the pigment cover within the pupil zone changed from 0% to over 30% in *T. hamiltoni* and from 0% to over 60% in *T. pleurogramma* (Fig. 3B). After 30 min, the pigment cover within the pupil zone had reached 84% and 91%, respectively. The ventral and dorsal hemifields showed a similar symmetrical pigmentation change in both species (Fig. 3C,D). The pigmentation change in *T. hamiltoni* was found to be slightly slower than that in *T. pleurogramma*.

In 60 min of dark-adaptation during the day, the pigmentation decreased from 83% to 37% cover in *T. pleurogramma* and from 100% to 42% in *T. hamiltoni* (Fig. 3A). However, the time course in the two species is quite

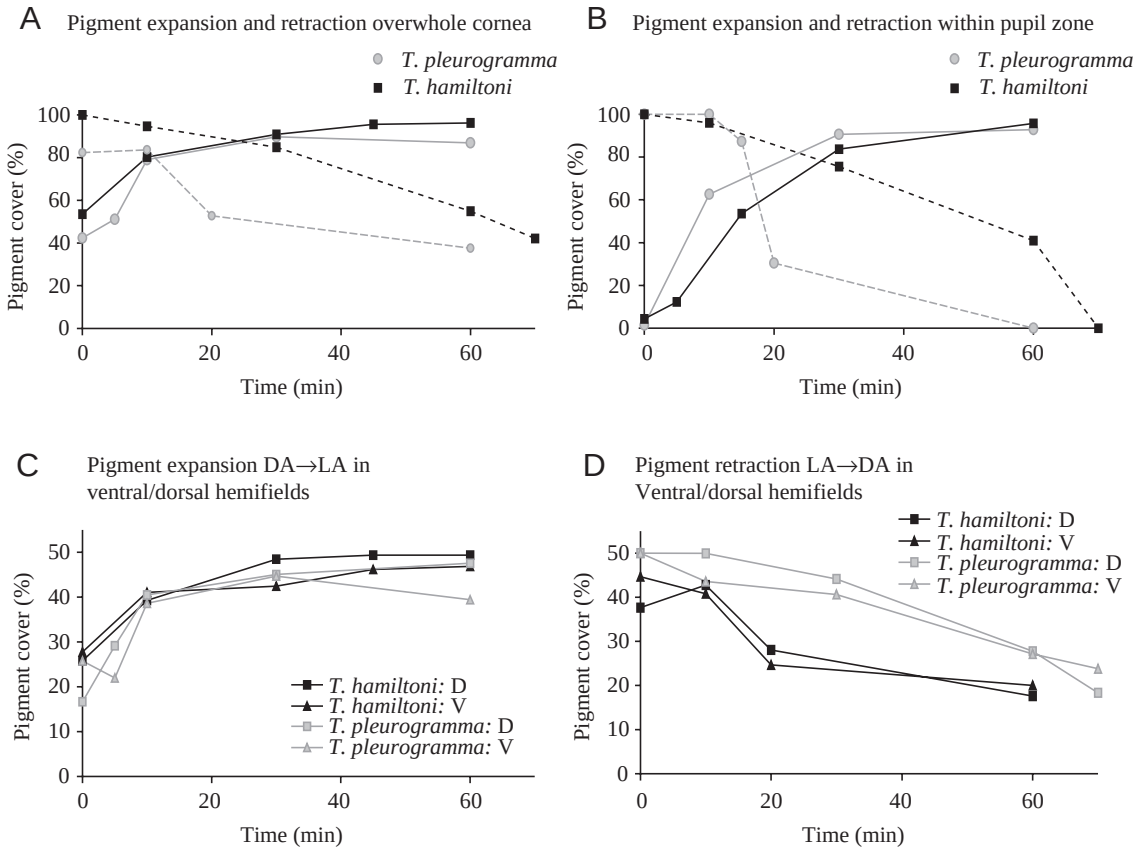

Fig. 3. Time course of pigment movement (expansion and retraction) during the day in both species of toadfish. (A) Pigment movement across the whole cornea and (B) within the pupil zone, and (C,D) a comparison of pigment movement in the ventral and dorsal hemifields of the whole cornea. (A) Corneal pigment cover varies from 53% during dark-adaptation (broken lines) to 100% during light-adaptation (solid lines) in *T. hamiltoni* and from 40% to 80%, respectively, in *T. pleurogramma*. (B) During light-adaptation (solid lines) the entire pupil zone is 100% pigmented, but is free of pigment after dark-adaptation (broken lines). (C,D) Pigment movement is symmetrical in the ventral (V) and dorsal (D) corneal hemifields of both species. The time course of the pigment movement is similar in both species during pigment expansion (C) while there are differences during pigment retraction (D). In *T. pleurogramma* the pigment retracts at a slow constant rate, whereas in *T. hamiltoni* it retracts in two phases, quickly between 10–20 min and slowly between 20–60 min.

different. *T. pleurogramma* shows an abrupt change from 83% to 53% pigment cover between 10 and 20 min of dark-adaptation, whereas *T. hamiltoni* reaches 54% pigment cover after only 60 min of darkness (Fig. 3A). The pigmentation of the pupil area also shows the difference in the speed of the pigmentation change. After 10 min dark-adaptation during the day, the pigmentation of *T. pleurogramma* does not change while *T. hamiltoni* reduces its pigment cover from 100% to 96% (Fig. 3B). The pigment in the pupil zone of *T. hamiltoni* retracts at a slow but relatively constant rate until it reaches 0% after 70 min. In *T. pleurogramma*, on the other hand, the pigment movement increases after a slow start so that after 20 min, only 30% of the pupil zone contains pigment, and after 60 min the pupil zone is completely clear. The overall change in dorsal and ventral hemifield pigmentation is symmetrical in both species (Fig. 3C,D).

Spectral changes in light transmission

Different classes of transmission curves can be distinguished by the slope and the shape of the function (Siebeck and Marshall, 2001). Class I consists of curves with a very steep slope (<30 nm between 0% and 100% transmission) and a sharp cut-off. Class II consists of curves with a less steep slope and a gradual onset of the cut-off and Class III is characterised by the three intermediate maxima between maximal and minimal transmission. Transmission curves of the corneas of the two toadfish investigated here showed class I, II or III characteristics. Class I and II curves found here could be divided into two sub-groups that contained curves with 50% transmission values below 400 nm and curves with 50% transmission values above 550 nm. Transmission curves in the latter group typically show less than 10% transmission at wavelengths below 530 nm (Fig. 4).

The transmission characteristics of the corneas of both species changed during light- and dark-adaptation. In each adaptation condition a continuous spectrum of transmission curves was found across the cornea. Individual transmission curves differed in the amount light transmitted in the

400–500 nm wavelength band (Fig. 4). Here are shown the transmission curves of five selected areas of each cornea: the rim of the cornea, the area halfway between rim and centre of the cornea, the centre of the cornea, and areas nasal and temporal of the central cornea (Fig. 4). The transmission values for dorsal and ventral hemifields of the cornea were averaged as the pigment change was found to be symmetrical in both hemifields.

The largest transmission change was observed in the central cornea (Fig. 4C, Area 3), where the dark-adapted cornea transmits wavelength above 400 nm to more than 80% while in the light-adapted cornea the transmission for wavelengths

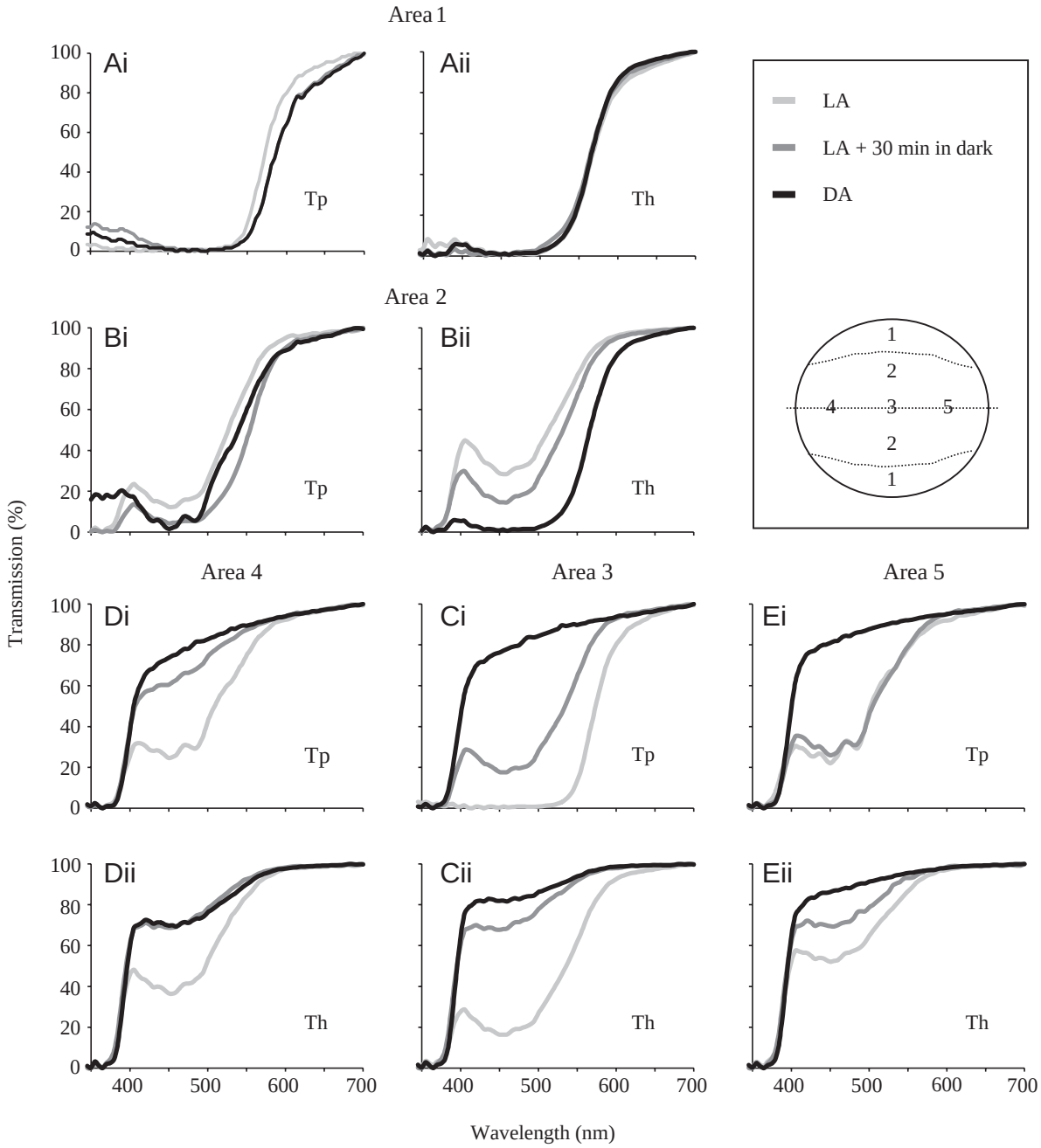

Fig. 4. Transmission change in different areas of the corneas of *T. hamiltoni* (Th; Aii–Eii) and *T. pleurogramma* (Tp; Ai–Ei) during three stages of light- and dark-adaptation (LA, light-adapted; LA+30 min dark, the halfway point of dark-adaptation; DA, dark-adapted). Mean transmission measurements are shown for five areas of each cornea (see inset). Due to the symmetrical distribution of the pigment in the ventral and the dorsal hemifields, the measurements for Areas 1 and 2 were averaged. The central cornea (Area 3; Ci,ii) shows the largest change in transmission properties during the adaptation change while the properties of the dorsal and ventral rims (Area 1; Ai,ii) remain constant. During dark-adaptation the central areas of the cornea (Areas 3–5; Ci,ii–E,i,ii) transmit increasing amounts of light in the 400–500 nm wavelength band while the reverse is true in Area 2 (Bi,ii), indicating that the pigment is shifted from the center towards the rim of the corneas.

between 400–500 nm is reduced to less than 40%. The nasal part of the central cornea (location of the pseudopupil in *T. pleurogramma*) shows the same general pattern of transmission change, with the difference that relatively more light of the 400–500 nm wavelength band is transmitted through the light-adapted cornea (Fig. 4D, Area 4). The temporal part of the central cornea also shows a similar trend (Fig. 4E, Area 5).

The dorsal and ventral rims of the cornea (Fig. 4A, Area 1) show no differences in transmission properties during light- and dark-adaptation. The 50% transmission cut-off of this area lies around 550 nm. The transmission properties of the area halfway between the centre and the rim of the cornea (Fig. 4B, Area 2) change depending on the adaptation condition; the transmission in the 400–500 nm wavelength band decreases with increasing dark-adaptation.

Corneal ultrastructure and changes in corneal pigmentation

The structure of the corneas of both species of toadfish is similar. In both species, the cornea comprises a dermal

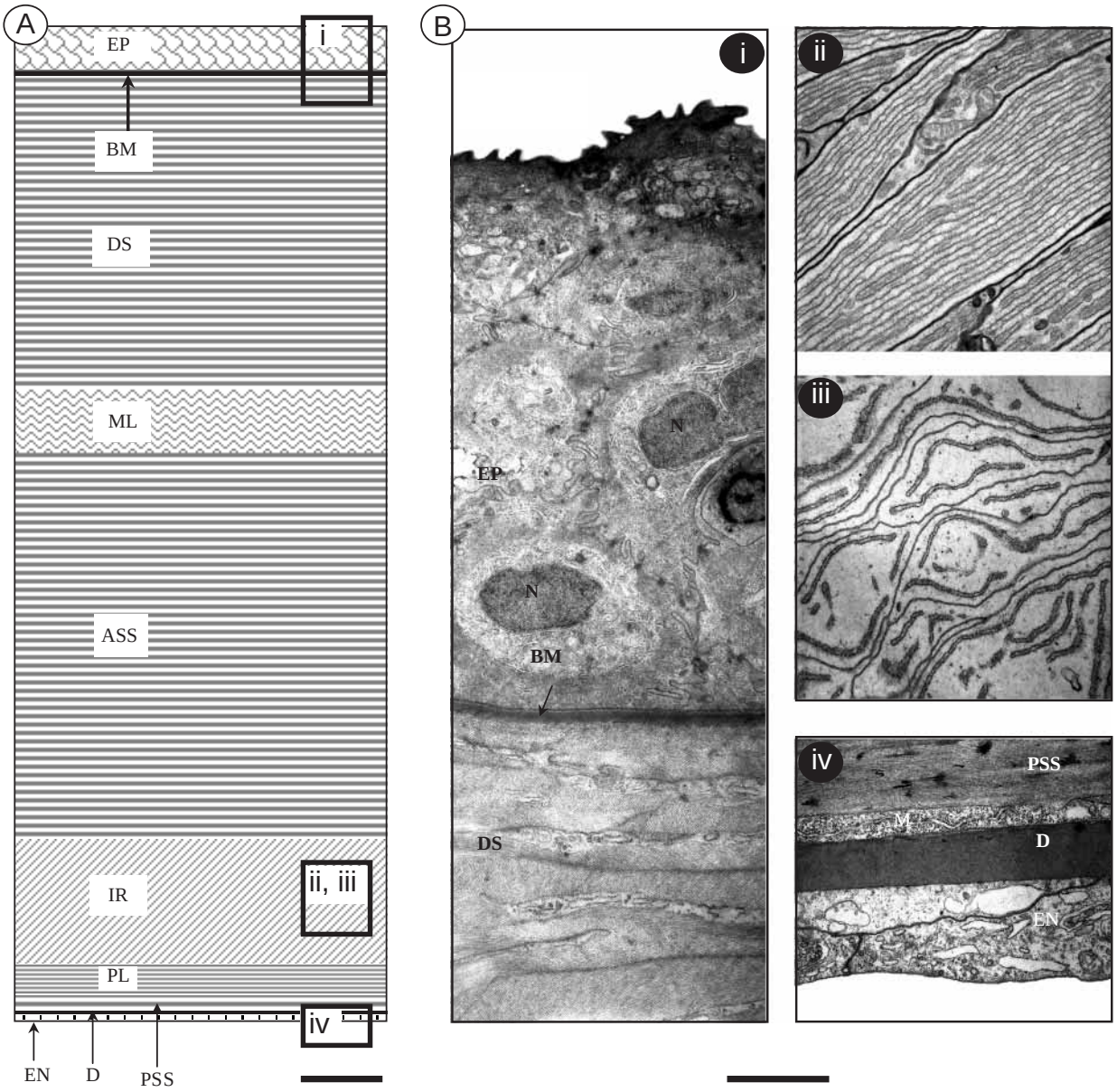

Fig. 5. Structure of the toadfish cornea. (A) Schematic diagram showing the thickness of the different layers in the central cornea. ASS, anterior scleral stroma; BM, base membrane; D, Desçemet's membrane; DS, dermal stroma; EN, endothelium; EP, epithelium; IR, iridescent layer; ML, mucoid layer; PL, pigment layer; PSS, posterior scleral stroma. Insets indicate which areas of the cornea are enlarged in B. Scale bar, 5 μm. (B) Electron micrographs showing a transverse section of (i) the epidermis (EP), base membrane (BM) and dermal stroma (DS); arrow, microprojections, N, nucleus; (ii,iii) the different structures of the iridescent layer (IR) comprising a series of aligned endoplasmic reticula in *T. hamiltoni* (ii) and *T. pleurogramma* (iii), and (iv) the posterior scleral stroma (PSS), a monolayer of cells (M), Desçemet's membrane (D) and endothelium (EN). Scale bar, 2 μm.

(secondary spectacle) and a scleral cornea loosely joined by a mucoid layer (Fig. 5A). The dermal cornea comprises an epithelium (2–3 cell layers thick), a basement membrane (0.4–0.5 μm thick) and a stroma consisting of many layers of collagen fibre bundles, which are arranged perpendicular to each other (Fig. 5Bi). The thickness of the dermal stroma of *T. hamiltoni* and of *T. pleurogramma* ranges between 96.5 μm and 151.9 μm (central to peripheral) and 58.7 μm and 55.5 μm (central to peripheral), respectively. A mucoid layer separates the dermal stroma from the anterior scleral stroma. Within the anterior scleral stroma of *T. hamiltoni*, two areas with a different affinity for osmium can be distinguished. The thickness of the scleral stroma ranges from 113.4 μm to 157.3 μm in *T. hamiltoni* and from 54.3 μm to 73.1 μm in *T. pleurogramma* (central to peripheral). An iridescent layer underlies the anterior scleral stroma (Fig. 5Bii,iii). The iridescent layer of both species consists of a series of aligned rough endoplasmic reticuli oriented approximately perpendicular to the incident light striking the cornea from above. The layer extends across the entire cornea and terminates just beyond the iris (Fig. 6). The iridescent layer ranges from 34.9 μm to 14 μm (central to peripheral) thickness in *T. hamiltoni* and from 29.3 μm to 23.6 μm in thickness in *T. pleurogramma*.

The pigment layer is located within the scleral cornea adjacent to the iridescent layer. In *T. pleurogramma*, the pigment layer is split and comprises two layers of processes situated on either side of the iridescent layer, while in *T. hamiltoni* a single

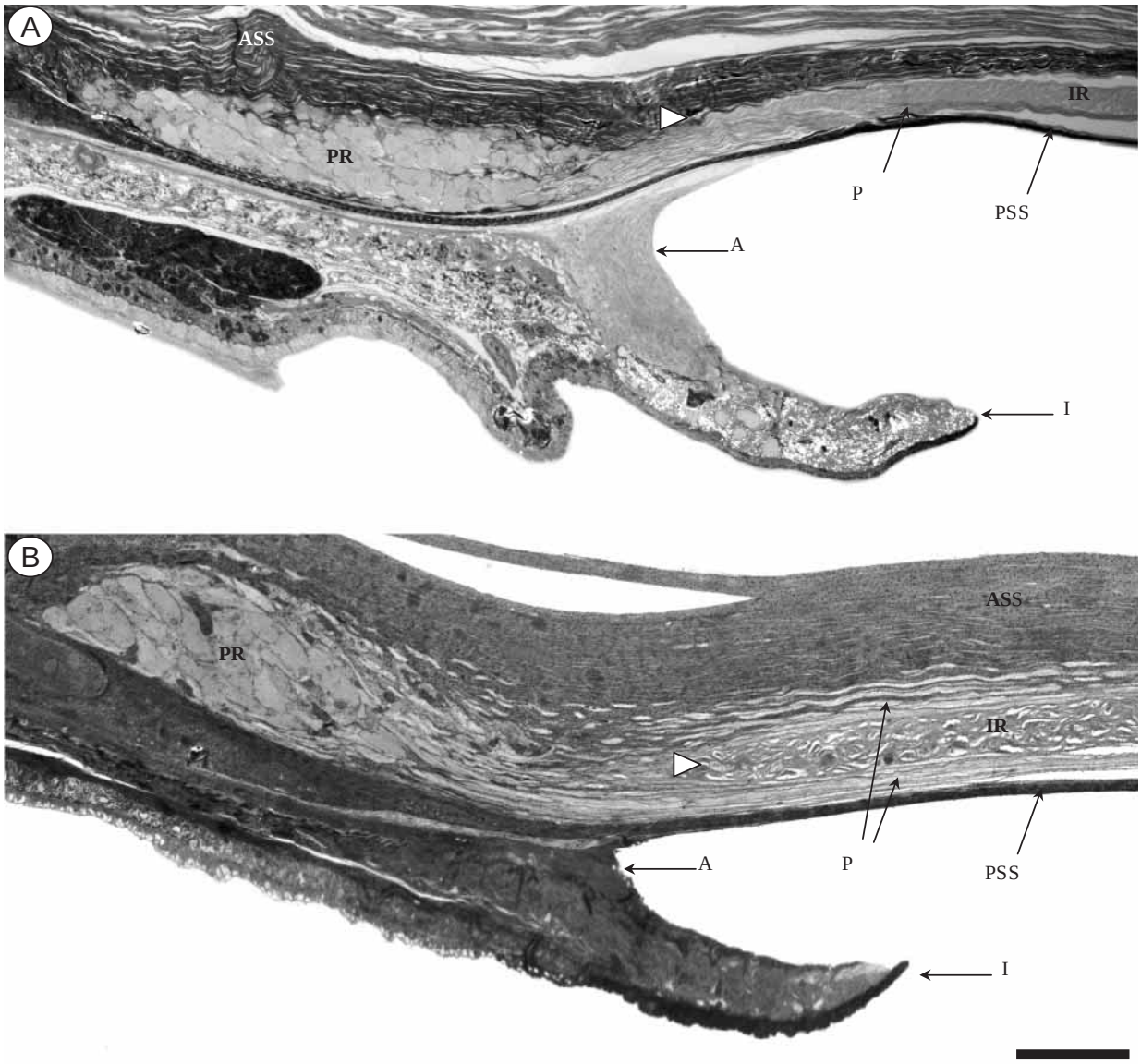

Fig. 6. Section through the scleral corneas of *T. hamiltoni* (A) and *T. pleurogramma* (B). The white arrowheads indicate the beginning of the iridescent layer (IR). In *T. pleurogramma*, the pigment processes can be found on either side of the iridescent layer, whereas in *T. hamiltoni* the processes are only found posterior to the iridescent layer. The anterior scleral stroma (ASS) has divided during histological processing. A, annular ligament; I, iris; P, pigment layer; PR, pigment reservoir with cell bodies; PSS, posterior scleral stroma. Scale bar, 500 μm.

pigment layer is situated beneath the iridescent layer (Fig. 6). The cell bodies of the pigment cells are found in the periphery of the cornea, outside the pupil zone (Fig. 6). The average diameter of the cell bodies in the pigment cell reservoir is 2.5 μm during light-adaptation and 13.8 μm during dark-adaptation (*T. pleurogramma*) and 8.7 μm during light-adaptation and 29 μm during dark-adaptation (*T. hamiltoni*). Long processes extend from each cell body into the center of the cornea. It appears that the processes are organised in thin sheets that terminate in the center of the cornea (Figs 7A, 8A). As the pigment processes originate in both the ventral and dorsal cornea, the processes extend throughout the entire cornea.

The chromatophores contain a variety of organelles and structures (Fig. 8B,C). The cytoplasm is granular and surrounds a large number of both lightly (<1 μm diameter) and darkly- (approximately 1 μm in diameter) stained vacuoles, which may contain lipid and contribute to at least part of the corneal pigmentation (Murphy and Tilney, 1974).

In the light-adapted state, the pigment cell processes are filled with granular cytoplasm and lipid-filled vacuoles (Fig. 7A,C). The width of the processes is 1.4±0.4 μm (mean ± S.D.) (*T. hamiltoni*) and 1.1±0.6 μm (*T. pleurogramma*). The cytoplasm appears to be closely associated with microtubules (0.2 nm in diameter), which lie parallel to the cell membrane (Fig. 8C). The pigment layer contains between 7–10 processes and, on average, reaches a width of 10.3±0.5 μm and 9.4±2.9 μm (*T. hamiltoni*, *T. pleurogramma*, respectively).

In the dark-adapted state, only the pigment reservoir contains the granular component of the cytoplasm and the lipid granules while the processes are empty, collapsing to less than half of their light-adapted diameter (*T. hamiltoni* 0.4±0.2 μm and *T. pleurogramma* 0.42±0.26 μm; Fig. 7B,C). As the pigment reservoir is situated outside the borders of the iris (pupil zone), the part of the cornea overlying the pupil is devoid of granules and lipid-filled vacuoles, providing at least circumstantial evidence in support of the structural

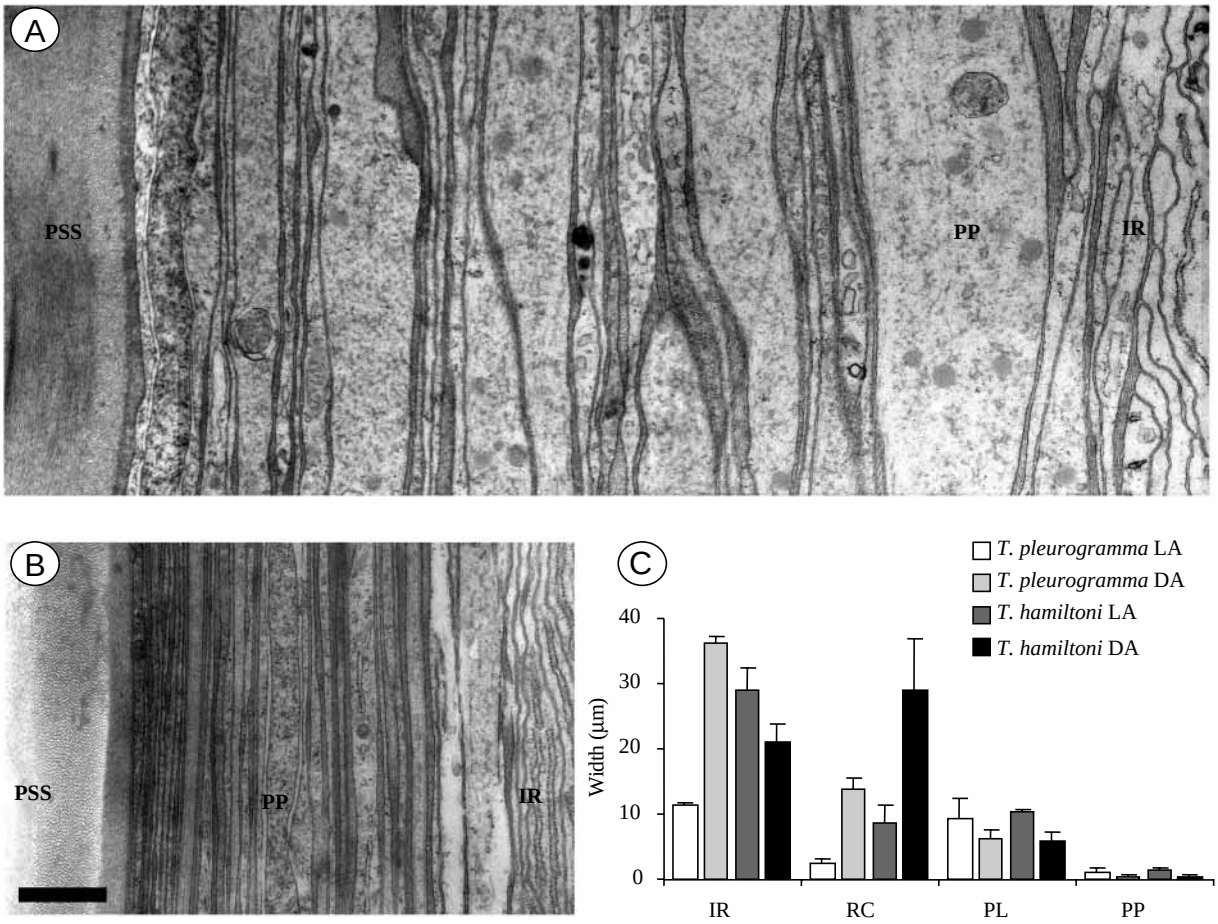

Fig. 7. Transverse sections through the central cornea showing the pigment layer during light- (A) and dark-adaptation (B). Scale bar, 1 μm. (C) Differences in width of the central iridescent layer (IR), the cells in the reservoir (RC), the central pigment layer (PL) and individual pigment processes (PP) in the central cornea in both species in the light- (LA) and dark-adapted (DA) condition. The difference between the widths of the iridescent layer is significant for both species (*T. pleurogramma*, F=2400, P<0.001; *T. hamiltoni*, F=33.3, P<0.001). Note that the IR width in *T. pleurogramma* is larger during dark-adaptation compared to light-adaptation, whereas the opposite is true for *T. hamiltoni*. During dark-adaptation, the cells in the reservoir of both species swell, the pigment processes in the central cornea empty leading to a decrease in width of the pigment layer. During light-adaptation, the central pigment processes swell, leading to an increased width of the pigment layer and a decreased size of the peripheral pigment reservoir cells.

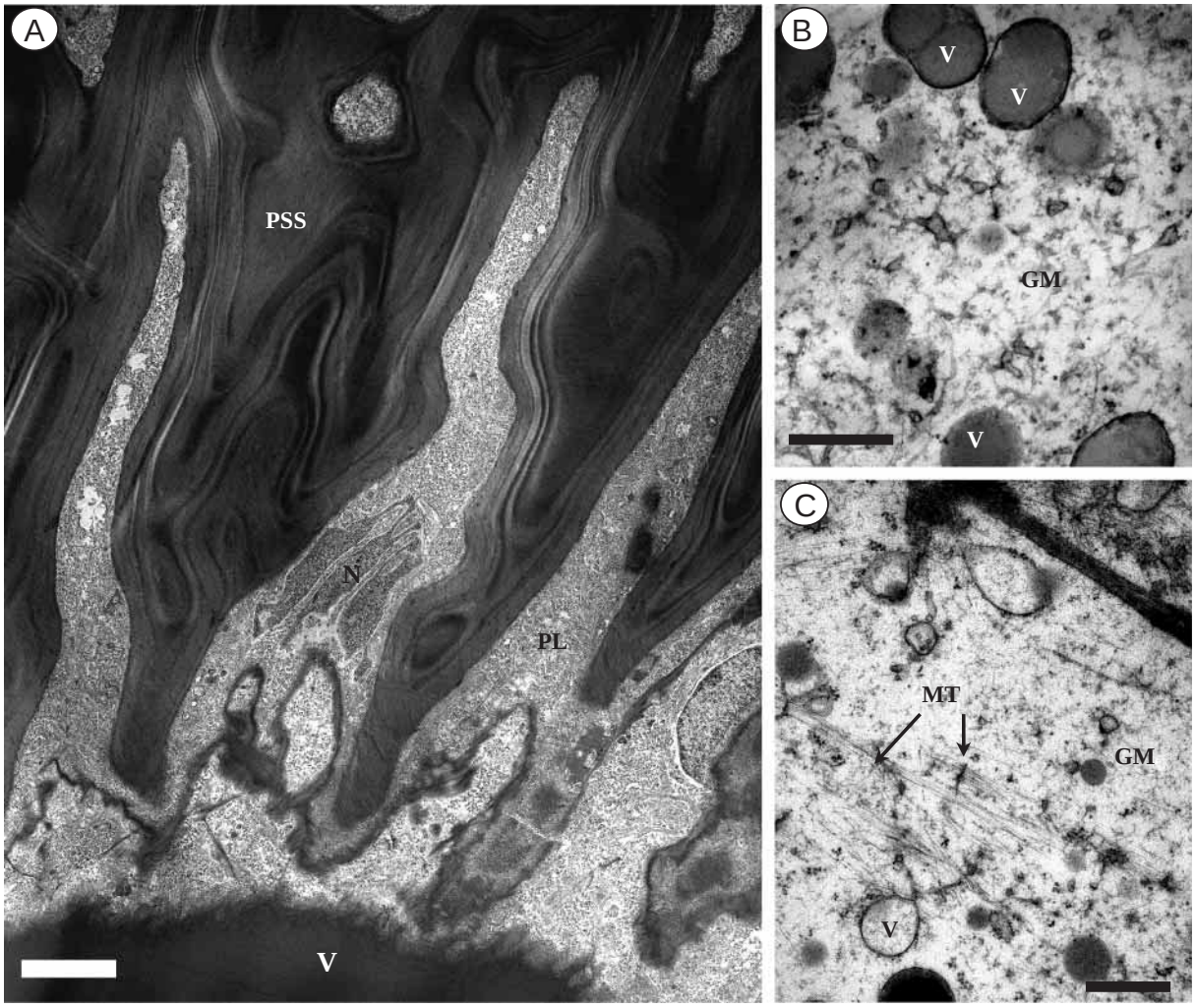

Fig. 8. (A) Tangential section through the pigment layer (PL) and posterior scleral stroma (PSS) in the light-adapted condition. The pigment cells have a sheet-like appearance. Scale bar, 5 μm. (B) Transverse section through a pigment reservoir cell in the dark-adapted condition. The cell contains granular material (GM) and vacuoles (V). Scale bar, 0.5 μm. (C) Transverse section through two pigment processes in the central cornea during light-adaptation. The cytoplasm contains granular material (GM) as well as vacuoles (V) of different sizes containing material with varying osmophilic affinity. Microtubules (MT) are arranged parallel to each other and the cell membrane. These may be involved in the transport of the pigment from the pigment reservoir into the pigment cell processes. Scale bar, 0.5 μm.

identification of the pigment when considered together with the transmission data.

The corneas of both species of toadfish contain a posterior scleral stroma ranging from 4.3 μm to 10 μm in *T. hamiltoni* and from 2.4 to 6.2 μm in *T. pleurogramma*, which lies anterior to a monolayer of cells, Desçemets membrane (0.89 μm in *T. hamiltoni* and 1 μm in *T. pleurogramma*) and an endothelium (0.8 μm in both *T. hamiltoni* and *T. pleurogramma*, Fig. 5Biv).

Discussion

Pigment migration characteristics

Both species of toadfish can change their corneal pigmentation. The colour of the cornea changes from clear to yellow/orange and back again in response to changes in the intensity of light. In order to test whether this mechanism is controlled exclusively by a diurnal rhythm, light-adapted fish were exposed to darkness during the day followed by light-adaptation, and dark-adapted fish to light during the night followed by dark-adaptation. Since no major differences in the response (pigment cover and time course) to light and dark-adaptation were observed and the reaction could be observed at any time, it is concluded that the underlying mechanism can be triggered by a variation in illumination intensity and is not exclusively controlled by a diurnal rhythm.

Slight differences were observed in the responses of the yellow and orange components of the pigmentation pattern during the night and day. One possibility is that these differences are due to an underlying diurnal rhythm. Alternatively, they could be caused by spectral differences between the illumination used for light-adaptation during the day and night. During the day, the broad spectrum of the sun

was used for light-adaptation, while the short-wavelength-deprived spectrum of the halogen lamp was used during the night. Kondrashev and Khodtsev (1984) report that the orange pigment in the corneas of two greenlings (*Hexagrammos octogrammus* and *H. stelleri*) did not disperse when illuminated with short wavelengths only, whereas the yellow pigment seemed equally sensitive to all wavelengths between 420–650 nm.

The pattern of pigmentation change found here is very similar to that described for other toadfish (Appleby and Muntz, 1979; Gamburtseva et al., 1980). However, in the light-adapted condition, the cornea of *Torquigener pleurogramma* is not uniformly coloured but shows less densely pigmented areas situated temporally and nasally, forming a pattern similar to that of species with constant pigment patterns (Kondrashev et al., 1986; Siebeck and Marshall, 2000). The function of these pseudopupils has not yet been resolved, but it is possible that they form a window through which the fish can look forward with maximal sensitivity, while still protecting the retina from downwelling (dorsal sunshield) and upwelling (ventral sunshield) light (Kondrashev et al., 1986).

The pigment of the toadfish *Torquigener pleurogramma* and *Tetractenos hamiltoni* takes approximately 60 min to expand fully when transferred from the dark into bright sunlight, which is similar to descriptions for the toadfish *Tetraodon steindachneri* (Appleby and Muntz, 1979). In all three species, pigment expansion follows a similar time course while pigment retraction seems more variable. All three species reach maximal pigment retraction at the same time; however, this is achieved by two different strategies. In *T. hamiltoni* the rate of pigment retraction remains constant, while in *T. pleurogramma* and *T. steindachneri* the pigment initially retracts slowly, followed by a rapid and a final slow phase.

During dark-adaptation, the minimal pigment cover (37–42%) in both species of toadfish investigated here differs from that in *T. steindachneri* (15%). Despite these differences in the degree of coverage, the pupil zone is still pigment-free in the species investigated here and presumably also in *T. steindachneri*. Therefore, there should be no functional differences between the various amounts of corneal pigment cover during dark-adaptation. The situation changes, however, when the fish are exposed to bright light, where there may be some advantage in screening their corneas as quickly as possible. Within 10 min, both *T. pleurogramma* and *T. hamiltoni* achieve a much larger corneal coverage than *T. steindachneri* (Appleby and Muntz, 1979), due to their larger initial coverage. It appears that the two species studied here have a time advantage over *T. steindachneri* as a result of the pigment 'standing by' just outside the pupil zone.

The relatively slow change of pigmentation cover found here is similar to what occurs in many species within at least nine teleost families, described by Gamburtseva et al. (1980). This slow response has been used as evidence that the response mechanism is unlikely to be under central control (Appleby and Muntz, 1979; Kondrachev and Khodtsev, 1984).

General structure of the cornea

The structure of the corneas of both toadfish species is very similar and shows the features typical of the corneas of some other teleosts (Collin and Collin, 2001). The division of the cornea into dermal (continuous with the underlying epithelium of the conjunctiva and skin) and scleral (continuous with the sclera surrounding the globe) components has previously been described for a range of shallow-water species by Walls (1942). However, a 'secondary spectacle' has recently been found in the pipefish, *Corythoichthyes paxtoni* (Collin and Collin, 1995), a range of gadiform deep-sea fish (Collin, 1997) and the salamanderfish *Lepidogalaxias salamandroides* (Collin and Collin, 1996), where the separation of the cornea by a mucoid layer has been attributed to the need to rotate the eye while maintaining a protective goggle, to reduce abrasion or to reduce the friction associated with eyes that project beyond the contour of the head (Collin, 1997).

The subdivision of the scleral stroma into an anterior and a posterior stroma was previously thought to be unique to deep-sea gadiforms (Collin and Collin, 1998). It is not clear why the shallow-water toadfish have three stromas, which, in the deep-sea teleosts examined, are thought to be an adaptation for strengthening the cornea and maintaining a robust intraocular pressure when subjected to the increased pressures at depth (Collin and Collin, 1998).

Iridescent layers have been found in many shallow-water marine teleosts (Lythgoe, 1974; Collin, 1997). Various types exist and can be situated in different layers of the cornea. The iridescent layer found in the toadfish as described here is situated between the scleral anterior stroma and the cellular processes containing pigment. The same position has been described for another toadfish, *Tetraodon samphongsi* (Lythgoe, 1974). In all three cases, the multi-layered stacks are comprised of rough endoplasmic reticulum. The orientation of the stacks is perpendicular to the incident light striking the cornea from above and therefore changes in relation to the orientation of the other layers in the cornea from dorsal to ventral cornea.

The iridescent layers of the two toadfish species investigated here do not seem to act as colour filters, as the transmission of the cornea in unpigmented areas is between 70% and 90%, which is similar to the findings of Lythgoe (1974). It is possible, however, that the transmission properties change when the cornea is separated from the rest of the eye in preparation for the transmission measurements. By cutting into the eye, the natural tension on the cornea caused by the intraocular pressure is destroyed and the cornea flattens. The iridescence that was clearly visible in the intact eye becomes invisible in the isolated cornea.

Transmission properties and pigment location

The cornea of *Hexagrammos octogrammus*, the first fish for which changeable corneal colouration was described, contains two types of pigment of yellow and orange appearance (Orlov and Gamburtseva, 1976). The authors conclude, from differences in their absorption spectra, that these colours are

not a function of differences in pigment density and propose that there are two different kinds of corneal chromatophores, one containing the yellow pigment and the other containing the orange pigment. The corneas of the two species of toadfish described here also have areas with orange and yellow pigmentation, and the transmission spectra of these areas resemble those of *H. octogrammus*. However, measurements taken in different areas of the cornea reveal that there is a gradual change in the transmission spectrum from the more typical spectrum (with three intermediate maxima) to a spectrum with a cut-off at wavelengths above 570 nm. It is, therefore, possible that the yellow and orange appearance of some areas of the corneas of *T. hamiltoni* and *T. pleurogramma* is due to different concentrations of the same pigment.

The transmission change in the different areas of the cornea can be explained with the pigment migration during the different adaptation conditions. During dark-adaptation, the long sheet-like processes extending from the pigment reservoir into the center of the cornea are empty and the transmission in the center of the cornea is maximal. The structures containing the pigment (presumably the granular cytoplasm and/or the lipid containing vacuoles) are concentrated inside the pigment reservoir, which is situated outside the pupil zone along the dorsal and ventral rim of the cornea. The transmission in that area is minimal and stays relatively constant during all adaptation conditions, indicating a continuously high concentration of pigment in the reservoir. In the process of light-adaptation, pigment is shifted from the reservoir along the sheet-like processes towards the center of the cornea, resulting in a swelling of the processes and in less shortwave light being transmitted through the central cornea. In the process of dark-adaptation, the pigment is moved back towards the pigment reservoir until the processes are empty and the transmission in the central cornea reaches maximal values. This pigment migration may be aided by the microtubules identified within the corneal chromatophores in both species of toadfish (Murphy and Tilney, 1974; Murphy, 1975).

Function of occlusable corneas

Several advantages of yellow corneas have been discussed in the literature. As early as 1933, Walls and Judd state that yellow filters increase visual acuity by '*reducing chromatic aberration, promote comfort by reducing glare and dazzle, enhance detail by the absorption of blue haze [scattered light] and also enhance contrast*' (Walls and Judd, 1933a,b). These are all theoretical benefits of short-wavelength filters that have not yet been confirmed with behavioural experiments. It is obvious, however, that the disadvantage of having short-wavelength filters is the loss of sensitivity during periods of low light intensity such as dusk and dawn and also at depth. This disadvantage no longer applies if the filter can be removed. Appleby and Muntz (1979) demonstrated this by calculating the effect of such filters on the sensitivity of *T. steindachneri* in different water types and at different depths (Jerlov, 1976). In clear water (Jerlov type 1A), they found a large sensitivity loss that increased with depth (i.e. a 0.45 log unit loss at 20 m for a corneal pigment density of 0.73). In order to absorb the same number of quanta, a fish with this corneal density would have to be 30 m shallower than a fish without a yellow filter (Appleby and Muntz, 1979).

Appleby and Muntz (1979) also evaluated the impact of occlusable *versus* permanent yellow corneas on vision at dusk and dawn, demonstrating that, while there is no advantage during sunset as the pigment moves as fast as the light diminishes, there might be a considerable advantage during sunrise. They concluded that *T. steindachneri* would possess a 3–5 min advantage over fish with permanent yellow corneas, which is considerable in the short tropical twilight when predation is highest. Since the two toadfish species studied here had slightly faster pigment movement rates, a greater advantage may be expected.

In all stages of pigment retraction or expansion, the ventral and dorsal hemifields of the cornea were equally covered with pigment. The area of highest pigment density in the corneas of fish with fixed colouration patterns was found in the dorsal hemifield or overlying the pupil zone, which is where it is also found in other species (Kondrashev et al., 1986). One of the reasons suggested for this pattern is that the yellow pigment acts as a protective sunshield from the intense downwelling light (Heinermann, 1984). The two toadfish species described here possess changeable 'sunglasses' that have their fastest and highest degree of protection in the dorsal and ventral corneal hemifields. Both species live in shallow water over a sandy bottom, which strongly reflects the downwelling light. With a symmetrical dorsal and ventral pigmentation, they achieve protection from downwelling and upwelling light simultaneously.

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References

Appleby, S. J. and Muntz, W. R. A. (1979). Occlusable yellow corneas in Tetraodontidae. *J. Exp. Biol.* **83**, 249-259.

Bridges, C. D. B. (1969). Yellow corneas in fishes. *Vision Res.* **9**, 435-436.

Collin, H. B. and Collin, S. P. (1995). Ultrastructure and organisation of the cornea, lens and iris in the pipefish, *Corithoichthyes paxtoni* (Syngnathidae, Teleostei). *Histol. Histopathol.* **10**, 313-323.

Collin, H. B. and Collin, S. P. (1996). The fine structure of the cornea of the salamanderfish, *Lepidogalaxias salamandroides* (Lepidogalaxiidae, Teleostei). *Cornea* **15**, 414-426.

Collin, S. P. (1997). Specialisation of the teleost visual system: adaptive diversity from shallow-water to deep-sea. *Acta Physiol. Scand.* **161**, 5-24.

Collin, S. P. and Collin, H. B. (1998). The deep-sea teleost cornea: a comparative study of gadiform fishes. *Histol. Histopathol.* **13**, 325-336.

Collin, S. P. and Collin, H. B. (2001). The fish cornea: adaptations for different aquatic environments. In *Sensory Biology of Jawed Fishes – New*

Insights (ed. B. G. Kapoor and T. J. Hara), pp. 57-96. Plymouth, UK: Science Publishers, Inc.

Douglas, R. H. and McGuigan, C. M. (1989). The spectral transmission of freshwater teleost ocular media – an interspecific comparison and a guide to potential ultraviolet sensitivity. *Vision Res.* **29**, 871-879.

Gamburtseva, A. G., Gnyubkina, V. P., Kondrashev, S. L. and Orlov, O. Y. (1980). Chromatophores and coloration of cornea of fishes. *Ecol. Physiol.* **591**, 495-503.

Gnyubkin, V. F. (1989). Response of pigmented corneas of whitespotted greenling to changes in light. *Sov. J. Mar. Biol.* **15**, 21-28.

Gnyubkina, V. P. and Gamburtseva, A. G. (1981). Structural pecularities of the corneal light filters. *Sov. J. Mar. Biol.* **7**, 175-178.

Gnyubkina, V. P. and Levin, A. V. (1987). Changeable corneal colouration in some Baikalian and River Sculpins (Pisces: Cottoidei). *Copeia* **3**, 758-762.

Heinermann, P. H. (1984). Yellow intraocular filters in fishes. *J. Exp. Biol.* **43**, 127-147.

Jerlov, N. G. (1976). *Marine Optics*. Amsterdam, New York: Elsevier Scientific.

Kondrashev, S. L., Gamburtzeva, A. G., Gnjubkina, V. P., Orlov, O. J. and Pham, T. M. (1986). Coloration of corneas in fish. A list of species. *Vision Res.* **26**, 287-290.

Kondrashev, S. L. and Khodtsev, A. S. (1984). Light-dependent and humoral control of pigment transport in corneal chromatophores in marine fishes. *Zool. Jb. Physiol.* **88**, 317-325.

Kuiter, H. R. (1993). *Coastal Fishes of South-Eastern Australia*. Bathurst: Crawford House Press.

Lythgoe, J. N. (1974). The ecology function and phylogeny of iridescent multilayers in fish corneas. In *Light as an Ecological Factor: II* (ed. G. C. Evans, R. Bainbridge and O. Rackham), pp. 211-247. Oxford: Blackwell Scientific Publications.

Marshall, N. J. (1996). Measuring colours around a coral reef. In *Biophotonics International*, vol. July/August 1996, pp. 52-56.

Moreland, J. D. and Lythgoe, J. N. (1968). Yellow corneas in fishes. *Vision Res.* **8**, 1377-1380.

Murphy, D. B. (1975). The mechanism of microtubule-dependent movement of pigment granules in teleost chromatophores. *Ann. NY Acad. Sci*. **253**, 692-701.

Murphy, D. B. and Tilney, L. G. (1974). The role of microtubules in the movement of pigment granules in teleost melanophores. *J. Cell Biol.* **61**, 757-779.

Orlov, O. Y. and Gamburtseva, A. G. (1976). Changeable colouration of cornea in the fish *Hexagrammos octogrammus*. *Nature* **263**, 405-407.

Philp, A. R., Bellingham, J., Garcia-Fernandez, J.-M. and Foster, R. G. (2000). A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish. *FEBS Lett.* **468**, 181-188.

Provencio, I., Jiang, G., De Grip, W. J., Hayes, W. P. and Rollag, M. D. (1998). Melanopsin: an opsin in melanophores, brain, and eye. *Proc. Natl. Acad. Sci. USA* **95**, 340-345.

Siebeck, U. E. and Marshall, N. J. (2000). Transmission of ocular media in labrid fishes. *Phil. Trans. R. Soc. Lond. B* **355**, 1257-1261.

Siebeck, U. E. and Marshall, N. J. (2001). Ocular media transmission of coral reef fish – can coral reef fish see ultraviolet light? *Vision Res.* **41**, 133-149.

Walls, G. L. (1942). *The Vertebrate Eye and its Adaptive Radiation*. Michigan: The Cranbrook Institute of Science.

Walls, G. L. and Judd, H. D. (1933a). The intra-ocular colour-filters of vertebrates. *Br. J. Ophthalmol.* **17**, 641-654

Walls, G. L. and Judd, H. D. (1933b). The intra-ocular colour-filters of vertebrates. *Br. J. Ophthalmol.* **17**, 705-720.

Zigman, S. (1971). Eye lens colour: Formation and function. *Science* **171**, 807-809.