Multiple cone visual pigments and the potential for trichromatic colour vision in two species of elasmobranch

Nathan S. Hart[1],*, Thomas J. Lisney[2], N. Justin Marshall[1] and Shaun P. Collin[2]

[1]*Vision, Touch and Hearing Research Centre (Queensland Brain Institute)* and [2]*Department of Anatomy and Developmental Biology School of Biomedical Sciences, University of Queensland, Brisbane, Queensland 4072, Australia*

*Author for correspondence (e-mail: n.hart@uq.edu.au)

Summary

Elasmobranchs (sharks, skates and rays) are the modern descendents of the first jawed vertebrates and, as apex predators, often occupy the highest trophic levels of aquatic (predominantly marine) ecosystems. However, despite their crucial role in the structure of marine communities, their importance both to commercial and to recreational fisheries, and the inherent interest in their role in vertebrate evolution, very little is known about their visual capabilities, especially with regard to whether or not they have the potential for colour vision. Using microspectrophotometry, we show that the retinae of the giant shovelnose ray (*Rhinobatos typus*) and the eastern shovelnose ray (*Aptychotrema rostrata*) contain three spectrally distinct cone visual pigments with wavelengths of maximum absorbance (λ_{max}) at 477, 502 and 561 nm and at 459, 492 and 553 nm, respectively. The retinae of *R. typus* and *A. rostrata* also contain a single type of rod visual pigment with λ_{max} at 504 and 498 nm, respectively. *R. typus*, living in the same estuarine waters as *A. rostrata*, were found to have identical visual pigments to *R. typus* inhabiting coral reef flats, despite a considerable difference in habitat spectral radiance. This is the first time that multiple cone visual pigments have been measured directly in an elasmobranch. The finding raises the possibility that some species are able to discriminate colour – a visual ability traditionally thought to be lacking in this vertebrate class – and it is evident that the visual ecology of elasmobranchs is far more complex than once thought.

Key words: elasmobranch, microspectrophotometry, shovelnose ray, visual pigment, *Rhinobatos typus*, *Aptychotrema rostrata*, shark, vision.

Introduction

The eyes of elasmobranchs (sharks, skates and rays) were traditionally assumed to be specialized for dim light (scotopic) vision, having all-rod retinae, reflective tapeta and high photoreceptor to ganglion cell summation ratios that provide enhanced sensitivity at the expense of spatial acuity (Walls, 1942). Nevertheless, cone photoreceptors were described in the retinae of cat sharks (*Scyliorhinus* spp.; Neumayer, 1897) and dogfish (*Mustelus canis*; Schaper, 1899) at the end of the 19th century, although this was largely overlooked until Gruber et al. (1963) described cones in the retina of the lemon shark (*Negaprion brevirostris*). In fact, with the possible exception of some species of skate [*Raja* (*Leucoraja*) *ocellata* and *L. erinacea*] that are reported to possess only rods (Ripps and Dowling, 1991), almost all elasmobranch species studied to date have duplex retinae, although the density of cones varies markedly between species (Gruber, 1975; Gruber and Cohen, 1978). For example, peak rod to cone ratios range from approximately 3:1 in the Atlantic stingray *Dasyatis sabina* (Logiudice and Laird, 1994) to 40:1 in the southern fiddler ray

Trygonorhina fasciata (Braekevelt, 1992) and >100:1 in the smooth dogfish *Mustelus canis* (Stell and Witkovsky, 1973).

Cone photoreceptors mediate vision under bright light (photopic) conditions when rod photoreceptor responses are saturated; the primary benefit of a duplex retina is, therefore, to extend the range of light intensities over which the visual system can operate (Schultze, 1866). Since light intensity varies by approximately 10 $\log_{10}$ units during the day, from starlight to bright sunlight, it is not surprising that most vertebrates possess duplex retinae (Walls, 1942). However, in many vertebrates, cones also have a secondary function, that is colour discrimination. If two or more cone types are present, each containing a visual pigment with different spectral sensitivity, the animal may be able to compare the outputs from these distinct cell types and extract chromatic information from the retinal image.

At present, it is not known whether elasmobranchs have colour vision. Physically restrained lemon sharks (*Negaprion brevirostris*) have been conditioned to respond (signified by

extension of their nictitating membrane) when a coloured adapting light was silently substituted with another colour (Gruber, 1975). However, the results of this study were inconclusive as a significant difference in the conditioned responses was only obtained when the change in stimulus colour was accompanied by a change in stimulus brightness. Subsequent behavioural studies employing a two choice discrimination paradigm also failed to demonstrate the presence of colour vision in *N. brevirostris* (Gruber cited in Cohen, 1980). Furthermore, there is no evidence for multiple cone types in lemon sharks based on the available electrophysiological evidence (Cohen and Gruber, 1977; Cohen, 1980; Cohen and Gruber, 1985).

Nevertheless, the recent discovery in a jawless vertebrate, the lamprey *Geotria australis*, of multiple cone types and visual pigment opsin genes that are orthologous to the major classes of opsin genes found in jawed vertebrates (Collin et al., 2003a; Collin et al., 2003b), suggests that multiple cone visual pigments existed prior to the divergence of the jawed and jawless vertebrate lineages and would have been present in the ancestors of all elasmobranchs. Moreover, there are over 1100 extant species of elasmobranch and they occupy a diverse range of habitats, from freshwater to marine, coastal to pelagic and shallow to deep water. They share these niches with teleost fish, turtles and invertebrates that are known to employ colour vision and it would be surprising if at least some elasmobranch species did not share this visual ability. To this end, we used microspectrophotometry to measure the spectral absorption of rod and cone visual pigments in two species of predominantly shallow-dwelling elasmobranch, the giant shovelnose ray *Rhinobatos typus* and the eastern shovelnose ray *Aptychotrema rostrata*, and show for the first time that some elasmobranchs do, in fact, have multiple cone types and, therefore, the potential for colour vision.

Materials and methods

Animal capture and handling

All procedures were approved by the University of Queensland Animal Ethics Committee, and conducted under the ethical guidelines of the National Health and Medical Research Council of Australia. Animals were collected using seine nets under permits granted by the Great Barrier Reef Marine Parks Authority (Permit #G03/11864.1) and Queensland Fisheries (Permit #PRM0210F).

Light and electron microscopy

Following an overdose of tricaine methane sulfonate salt (MS222; 1:2000), four specimens of the giant shovelnose ray *Rhinobatos typus* Bennett 1830 (25–68 cm total length) were sacrificed for light microscopical and ultrastructural examination of the rod and cone photoreceptors. For light microscopy, enucleated eyes were immersion fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer for 1 h before being dehydrated and embedded in LR white resin (Sigma; Castle Hill, NSW, Australia). Sections (1 μm) were
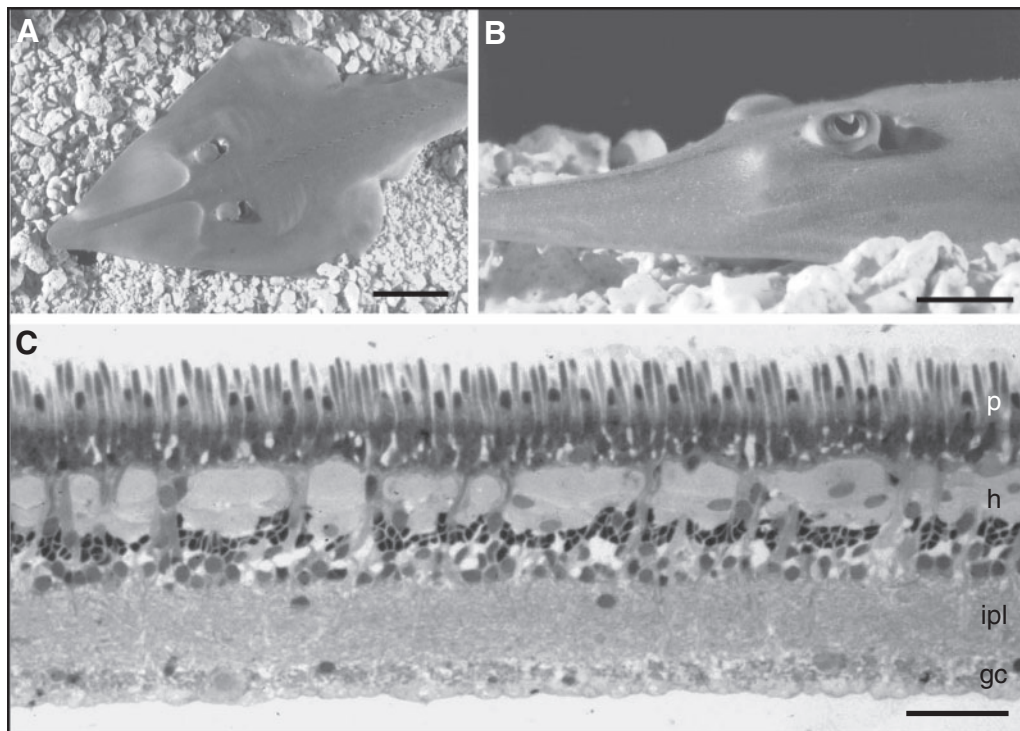

Fig. 1. Dorsal (A) and lateral (B) views of the giant shovelnose ray, *Rhinobatos typus*, showing the raised eyes and crescent-shaped pupil in the light-adapted state. (C) Light micrograph of the retina of *R. typus* showing the duplex nature of the photoreceptor layer (smaller receptors are cones). gc, ganglion cell layer; h, horizontal cell layer; ipl, inner plexiform layer; p, photoreceptor layer. Scale bars: (A) 50 mm; (B) 80 mm; and (C) 100 μm.

cut on an LKB ultramicrotome and stained with toluidine blue. For electron microscopy, retinal tissue was fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer (pH 7.4) and embedded in araldite before being sectioned on an LKB rotary ultramicrotome (Collin et al., 1999). Ultra-thin sections were stained with lead citrate and uranyl acetate, and examined on either a Phillips 410 or a Phillips CM10 transmission electron microscope set at 80 kV (Phillips Inc., Eindhoven, The Netherlands).

Microspectrophotometry

Two specimens of *R. typus* were caught in the shallows off Heron Island, Great Barrier Reef, Queensland (23°26′S 151°55′E) in May 2003. A third specimen was taken from shallow coastal waters off North Stradbroke Island, Moreton Bay, Queensland (27°30′S 153°25′E) in February 2004. All specimens were newborn or young-of-the-year females with a total length and disc width in the range 39–47 cm and 13–15 cm, respectively. Three adult specimens (total length and disc width range 42–76 cm and 15–26 cm, respectively) of the eastern shovelnose ray *Aptychotrema rostrata* Shaw and Nodder 1794 were also caught off North Stradbroke Island between October 2003 and April 2004. Animals were kept in darkness overnight and killed with an overdose of MS222 followed by spinal section and pithing.

Eye removal from dark-adapted animals and retinal dissection was conducted under the illumination provided by a bank of 24 infra-red (IR) light emitting diodes and visualized using an IR image converter (FJW Optical Systems Inc., Palatine, IL, USA) attached to one ocular of a stereo dissecting microscope. Following enucleation, eyes were hemisected and immersed in an elasmobranch Ringer solution (330 mmol l^{-1} urea, 350 mmol l^{-1} NaCl, 4 mmol l^{-1} KCl, 5 mmol l^{-1} CaCl$_2$, 2 mmol l^{-1} MgCl$_2$; approximate osmolality 1050 mOsm kg^{-1}). Small pieces (~1 mm^2) of retinal tissue were dissected away from the vitreous and choroids, and transferred to a drop of Ringer solution containing 10% dextran (MW 282,000; Sigma
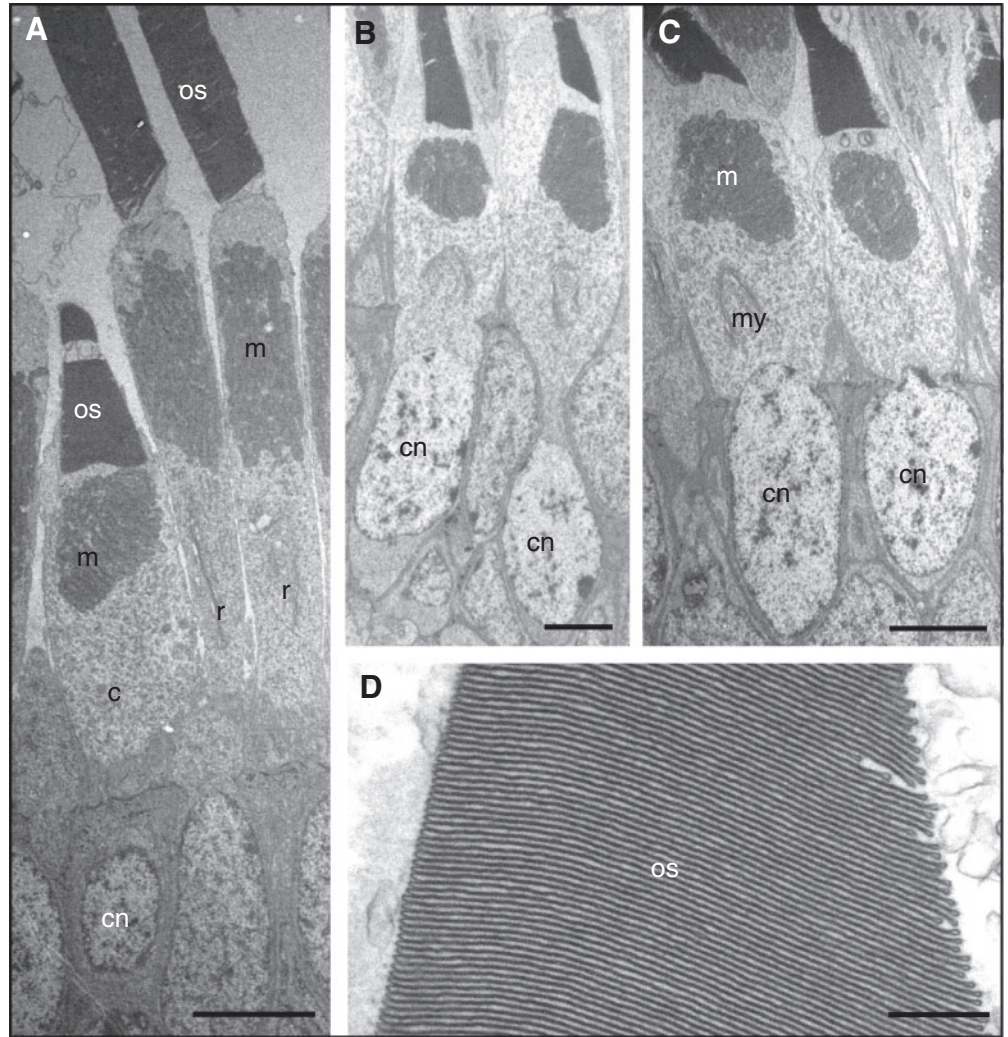

Fig. 2. Ultrastructure of rods and cones in *Rhinobatos typus*. (A) Typical rod (r) and cone (c) morphology. (B,C) Different types of cone photoreceptors can be distinguished based on minor differences in receptor size, mitochondrial alignment and the location of the nucleus in the outer nuclear layer. (D) High-power micrograph of the outer segment (os) of a cone photoreceptor showing the lack of a plasma membrane surrounding the discs. c, cone; cn, cone nucleus; m, mitochondria; my, myoid; r, rod. Scale bars: (A–C) 5 μm; and (D) 0.5 μm.

D-7265) placed in the middle of a 24×60 mm No. 1 glass coverslip. The retina was gently teased apart using mounted needles, covered with an 18 mm diameter No. 0 coverslip and the edges of the top cover slip sealed with nail varnish to prevent dehydration.

Transverse absorbance spectra (380–800 nm) of cone and rod outer segments were made using a computer-controlled, single-beam, wavelength-scanning microspectrophotometer (MSP) described in detail elsewhere (Hart, 2004). A sample scan was made by aligning the measuring beam (typical dimensions 1×3 μm) within a single outer segment and recording the amount of light transmitted at each wavelength. A baseline scan was made in an identical fashion from a cell-free area of the preparation adjacent to the measured cell. Baseline transmittance was subtracted from that of the sample at each corresponding wavelength to create a 'pre-bleach'

Table 1. *Spectral characteristics of rod and cone visual pigments in the retina of two species of shovelnose ray measured using microspectrophotometry*

| | Giant shovelnose ray *Rhinobatos typus* | | | | Eastern shovelnose ray *Aptychotrema rostrata* | | | |
	Rod	SWS cone	MWS cone	LWS cone	Rod	SWS cone	MWS cone	LWS cone
Mean prebleach λ_{max} (nm)	504.2±1.9	476.6±4.2	502.0±2.8	561.1±4.8	498.1±1.9	458.7±3.1	491.8±3.3	553.2±4.4
λ_{max} of mean prebleach spectrum (nm)	504.1	476.5	502.8	563.2	498.2	458.2	493.5	553.0
Mean difference spectrum λ_{max} (nm)	506.0±3.2	476.4±3.4	501.5±7.3	559.4±7.8	501.1±2.3	457.7±6.4	490.0±6.0	554.8±6.5
λ_{max} of mean difference spectrum (nm)	505.4	476.7	504.3	561.3	501.3	457.2	493.4	553.5
Mean transverse absorbance (decadic) at λ_{max} of difference spectrum	0.028±0.006	0.020±0.005	0.013±0.003	0.016±0.008	0.035±0.007	0.013±0.004	0.011±0.003	0.013±0.005
Number of cells averaged	14	10	5	10	15	10	5	11

Values are ±1 standard deviation. λ_{max}, wavelength of maximum absorbance.

spectrum that was subsequently converted to absorbance. Outer segments were then bleached with full spectrum 'white' light from the monochromator at its blaze angle for 3 min, and new sample and baseline scans made to create a 'post-bleach' spectrum. The post-bleach spectrum was deducted from the pre-bleach spectrum to create a bleaching difference spectrum for each outer segment. Visual pigment pre-bleach and difference spectra were then analyzed as described elsewhere (MacNichol, 1986; Govardovskii et al., 2000; Hart, 2002) to obtain an estimate of the wavelength of maximum absorbance (λ_{max}).

Spectroradiometry

Spectral radiance measurements were made using a calibrated, computer-controlled Ocean Optics S2000 spectroradiometer (Ocean Optics, Florida, USA) fitted with a 12 m ultraviolet-transmitting fibre-optic light guide and a radiometric (32° acceptance angle) head. Measurements of *R. typus* reef habitat were made on Heron Island reef flats and in the Wistari channel, Great Barrier Reef, Queensland (23°27′S 151°54′E). Measurements of *R. typus* and *A. rostrata* coastal/estuarine habitat were made off Amity Point, North Stradbroke Island, Queensland (27°25′S 153°27′E). All spectra were quantified in terms of photons rather than energy, as is appropriate for the consideration of visual systems.

Results

As with other batoid elasmobranchs, the eyes of both species are positioned on the dorsal surface and face laterally (Fig. 1A,B). Each has a reflective tapetum at the back of the eye, the reflectance of which is strongest along a horizontal band slightly dorsal to the optic nerve head, and a mobile *operculum pupillare* that partially occludes the pupil in the light adapted state (Fig. 1B).

Photoreceptor morphology

The retinae of *Rhinobatos typus* and *Aptychotrema rostrata* possess both rod and cone photoreceptors (Figs 1C and 2A–D). Rods were characterized by their relatively longer, cylindrical outer segments (typically 2.0–3.5 μm in diameter and at least 20 μm in length), narrower inner segments and smaller nuclei (Fig. 2A). Cones were easily distinguished from rods on the basis of their shorter, conical outer segments and wider, tapering inner segments, which at the level of the myoid were almost twice the width (6 μm) of the rods (Fig. 2A–C). Cone outer segments, which were located at the level of the rod ellipsoid, were typically 4–8 μm in length and tapered from a width of 1.5–3.5 μm nearest the inner segment to 1–2 μm at the tip. Unlike the rods, cone outer segment discs were not surrounded by a plasma membrane (Fig. 2D). Differences were noted in the position of cone nuclei in the outer nuclear layer and the alignment of their densely packed ellipsoidal mitochondria (Fig. 2B,C), suggesting that there are different morphological subtypes of cone.
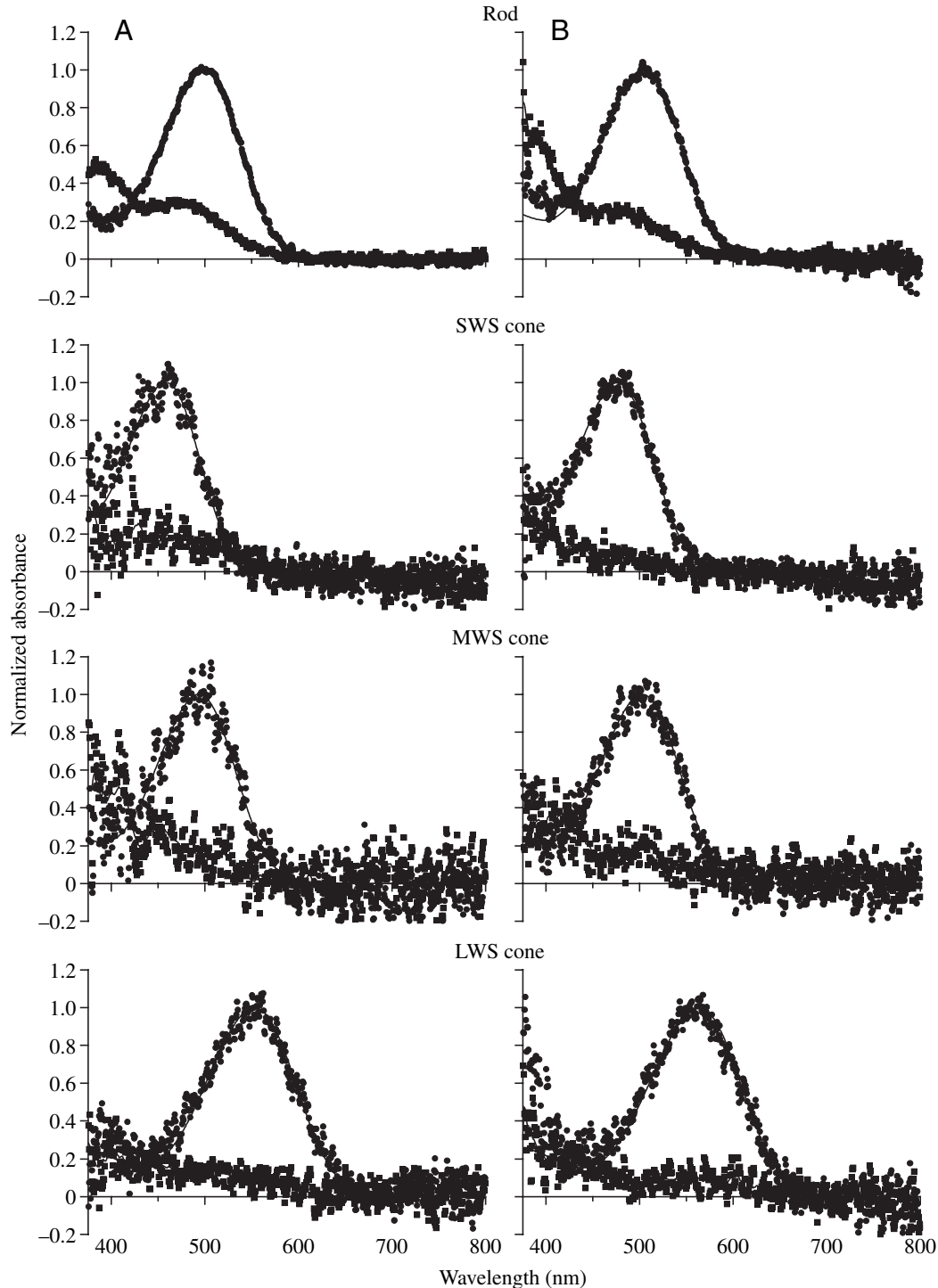
Fig. 3. Normalized absorbance spectra of rod and cone visual pigments in (A) the giant shovelnose ray *Rhinobatos typus*, and (B) the eastern shovelnose ray *Aptychotrema rostrata*. Prebleach spectra (circles) are best-fitted with a rhodospin visual pigment template of the appropriate λ_{max}; postbleach spectra (squares) are fitted with an unweighted running average. SWS, MWS and LWS refer to short-, medium- and long-wavelength-sensitive visual pigments, respectively.

Microspectrophotometry

Microspectrophotometric data for both species of elasmobranch studied are summarized in Table 1 and Fig. 3. On the basis of goodness-of-fit to mathematical visual pigment templates (Govardovskii et al., 2000), all absorbance spectra were considered to represent vitamin A_1-based visual pigments (rhodopsins). The retina of adult *R. typus* contained a single type of rod, the outer segments of which contained a medium-wavelength ('green') sensitive visual pigment with a mean wavelength of maximum absorbance (λ_{max}) at 504 nm. The

retina of *R. typus* also contained a large number of cone photoreceptors. Cone outer segments contained either a short-wavelength ('blue') sensitive pigment with a mean λ_{max} at 477 nm, a medium-wavelength ('green') sensitive pigment with a mean λ_{max} at 502 nm or a long-wavelength ('red') sensitive pigment with a mean λ_{max} at 561 nm. The visual pigment λ_{max} values of a single juvenile *R. typus* caught from estuarine waters off North Stradbroke Island (where the adult *A. rostrata* were also caught) did not differ from those of juvenile *R. typus* caught on the reef flats off Heron Island (Student's *t*-test; rods: $t=1.636$, d.f.=14, $P=0.124$; short wavelength-sensitive: $t=-0.674$, d.f.=11, $P=0.515$; medium wavelength-sensitive: insufficient data; long wavelength-sensitive: $t=1.023$, d.f.=17, $P=0.321$) and so the data were pooled. The retina of *A. rostrata* had morphologically similar rod and cone photoreceptors to *R. typus*. However, the λ_{max} values of the visual pigments in the rods (498 nm λ_{max}) and the three cone types (459, 492 and 553 nm λ_{max}) in *A. rostrata* were all at shorter wavelengths than the corresponding visual pigment types in *R. typus*.

Spectroradiometry

Spectral radiance along different lines of sight at different depths in typical reef (*R. typus*) and coastal (both *R. typus* and *A. rostrata*) waters are shown in Fig. 4A–D. At relatively shallow depths in both habitats, there is a relatively broad range of wavelengths available for vision (Fig. 4A,B; down-welling light), although wavelengths below 450 nm are clearly more rapidly attenuated in coastal waters than on the reef. At 5 m in the reef habitat, the down-welling light is still spectrally broad, although the horizontal and up-welling radiances are significantly richer in short wavelength light compared with shallow reef and deeper coastal waters (Fig. 4C). By comparison, at 3–5 m in the greener coastal waters the spectral distribution of light was quite uniform regardless of direction (Fig. 4D).

Discussion

The giant shovelnose ray *Rhinobatos typus* is widely distributed throughout the warm-temperate to tropical waters of the Indo-West Pacific, occurring off Malaysia, Indonesia and the entire north coast of Australia from Shark Bay (Western Australia; 25°30′S) to Forster (New South Wales; 32°12′S) (Compagno and Last, 1999). The eastern shovelnose ray *Aptychotrema rostrata* is restricted to the east coast of Australia from Halifax Bay (Queensland; 18°43′S) to Jervis Bay (New South Wales; 35°08′S) (Compagno and Last, 1999; Kyne and Bennett, 2002). Both species are active-foraging bottom dwellers and feed predominantly on benthic crustaceans, molluscs and fishes.
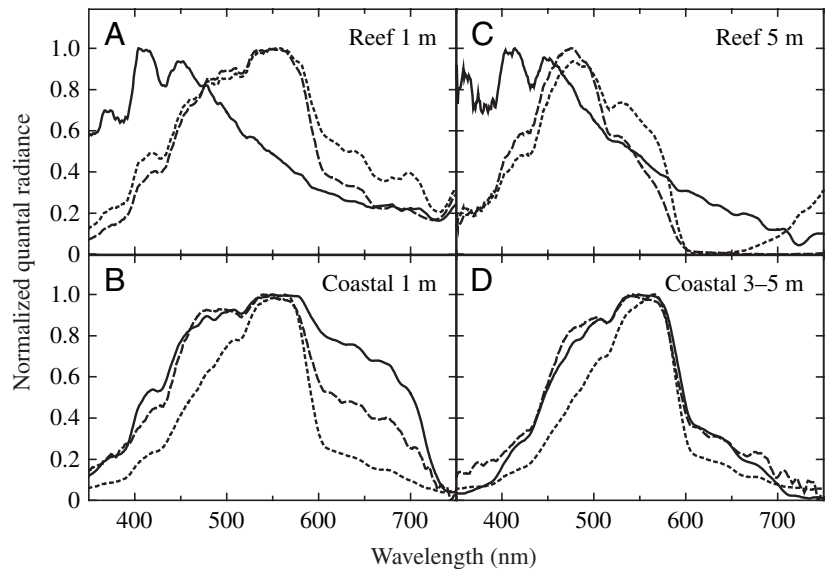

Fig. 4. Spectral radiance along different lines of sight at different depths in Australian reef and coastal waters. (A) Reef water 1 m depth; (B) coastal water 1 m depth; (C) reef water 5 m depth; (D) coastal water 3–5 m depth. Solid line, down-welling radiance; broken line, horizontal radiance; dotted line, up-welling radiance.

Multiple cone types and the potential for colour vision

The majority of elasmobranchs studied have duplex retinae, although the proportion of cones varies greatly between species. The presence of cones in the retina of *R. typus* was shown previously by Collin (1988), who reported a relatively high peak rod to cone ratio of 4:1. Although no quantitative analysis was performed in the present study, the retina of *A. rostrata* also contained a high proportion of cones. The relative abundance of cone photoreceptors and the presence of a highly mobile pupil suggest that both species are well-adapted to brightly lit environments. The morphology of the photoreceptors closely resembles that of other elasmobranch species studied (Braekevelt, 1992, 1994). Cones were easily distinguished from rods on the basis of their shorter, conical outer segments and wider, tapering inner segments. Differences in the relative location of cone nuclei in the outer nuclear layer and the alignment of their ellipsoidal mitochondria suggests that there are several morphological subtypes of cone in the shovelnose ray retina. In teleost fish (Downing et al., 1986) and birds (Morris and Shorey, 1967), different spectral cone types can be distinguished on the basis of nuclear and ellipsoidal location. However, as we did not attempt to correlate cone morphology with spectral type, further investigation is required to confirm if this holds true for shovelnose rays and other elasmobranchs.

Microspectrophotometric measurements revealed the presence of three spectrally distinct cone visual pigments in both species of ray. The presence of multiple cone types raises the possibility that these species have the potential for trichromatic colour vision, a visual ability traditionally thought to be lacking from elasmobranchs. A previous microspectrophotometric and extraction spectrophotometric

study on a closely related species, the Atlantic guitarfish *Rhinobatos lentiginosus*, found only one rod (λ_{max} 496 nm) and one almost spectrally identical cone visual pigment (λ_{max} 499 nm; Gruber et al., 1991). However, it is quite possible that other cone types in *R. lentiginosus* were missed: cones in both species of ray investigated in the present study were often obscured by the surrounding rods and cone outer segments were easily detached during preparation of the tissue. The only other microspectrophotometric study performed on the elasmobranch retina failed to obtain useful absorbance spectra from the cones of the two species investigated, the brown smooth-hound (*Mustelus henlei*) and the leopard shark (*Triakis semifasciata*), most probably due to the paucity of cones in these bottom-dwelling species (Sillman et al., 1996).

Two other lines of evidence suggest that multiple cone types and colour vision may be common in elasmobranchs, at least among the rays. Firstly, electroretinographic recordings of the early receptor potential (ERP) in the light-adapted retina of the common stingray (*Dasyatis pastinaca*) revealed three peaks in spectral sensitivity at 476, 502 and 540 nm (Govardovskii and Lychakov, 1977). As the ERP represents signals from the photoreceptors alone (Brindley and Gardner-Medwin, 1966), the three peaks in sensitivity can be correlated with the presence of three spectrally distinct visual pigments. Secondly, horizontal cells in the retina of the red stingray (*Dasyatis akajei*) have been shown to possess colour-coded responses (C-type S-potentials) to chromatic stimuli, hyperpolarizing when illuminated with short wavelength light and depolarizing at longer wavelengths (Toyoda et al., 1978). Although C-type S-potentials are not necessary for colour vision, they are only found in species that have a well-developed colour sense, such as teleost fish (Stell and Lightfoot, 1975) and turtles (Ammermüller et al., 1995).

Spectral tuning of rod and cone visual pigments

Interpreting the visual pigment spectral tuning characteristics of the two species of shovelnose ray examined in this study is hampered by a lack of life-history information, especially with regard to diurnal patterns in activity and vertical migration through the water column. The rod and all three types of cone found in *R. typus* have visual pigment λ_{max} values at longer wavelengths than the corresponding cell types of *A. rostrata*. This is unexpected since the background illumination in the typical coral reef habitat of *R. typus*, especially at moderate depth, is characterized by a relative abundance of short wavelength light compared with the more temperate coastal waters inhabited by *A. rostrata* (Fig. 4A–D). Shifts in cone λ_{max} that are correlated with the spectral radiance of the water have been observed in a number of teleost fish. For example, snappers (*Lutjanidae*) found in the bluer waters of the outer Great Barrier Reef have cone visual pigments with λ_{max} values shifted towards shorter wavelengths compared to congeners occupying the greener waters of inshore reefs and estuaries (Lythgoe et al., 1994).

It is also interesting that identical visual pigments λ_{max} values were found in both reef and coastal *R. typus*. Other species of fish are known to show intraspecific variations in visual pigment λ_{max} depending on the type of water they inhabit (Shand et al., 2002; Jokela et al., 2003). However, because at least one of the visual pigments of *R. typus* would coincide with the wavelength of peak transmission of the water in either habitat, its visual system is perhaps equally well suited to reef and coastal waters.

More information regarding both the spectral properties of habitat light and the behavioural biology of these species is required before further conclusions can be made. Nevertheless, it is evident that the visual ecology of many elasmobranchs is far more complex than once envisaged and is clearly a subject for further investigation. Whether or not the two species of shovelnose ray investigated in this study are capable of using their different cone types for colour discrimination is unknown but behavioural tests are currently underway in our laboratory.

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