A STUDY OF THE VISUAL PIGMENTS FROM THREE FAMILIES OF TELEOST THAT INHABIT ATYPICAL LIGHT ENVIRONMENTS

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Abstract

Over evolutionary time the planet has become populated by a multitude of organisms highly adapted to their own ecological niche. Each individual species relies on a range of modalities that have become fine-tuned to provide accurate and reliable information. Vision is one such sense and shows interspecific variation in both acuity and chromatic sensitivity. One of the prime examples in recent years has been the short-wave shift in sensitivity found in the visual pigments of deep-sea fish (Lythgoe, 1972; Douglas et al., 1998, 2003). In the majority of species a single rod opsin is expressed, which is maximally sensitive to the wavelengths of bioluminescence and down-welling sunlight ($\lambda_{\text{max}} = 470 - 490$ nm). This study examines two deep-sea species that have further adapted their visual sense to aid prey detection, and investigates the notothenioid family of ice fish that live beneath pack ice and inhabit a variety of depths.

The three stomiid genera, Malacosteus, Pachystomias and Aristostomias are unique in emitting far-red light as well as the typical blue bioluminescence. All three utilize a rhodopsin/porphyropsin pigment pair with $\lambda_{\text{max}}$ values around 520/550 nm, approximately 40 and 70 nm long-wave shifted when compared to other deep-sea pigments, to increase sensitivity to long wavelengths (O’Day and Fernandez, 1974; Dartnall, 1975; Bowmaker et al., 1988; Partridge and Douglas, 1995). There is also evidence that Pachystomias and Aristostomias express a second visual opsin, which has a rhodopsin $\lambda_{\text{max}}$ at around 580 nm (Partridge and Douglas, 1995; Douglas et al., 1998). The aim of this study was to identify these two opsins. A single rod opsin has been identified from both Pachystomias and Aristostomias, and there has been no evidence
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for a second opsin. Histology shows a single class of rod photoreceptor in the *Pachystomias* retina.

Another deep-sea species, *Scopelarchus analis*, has highly adapted eyes with seven different retinal specializations. Previously MSP identified three rhodopsins with $\lambda_{\text{max}}$ values at 444, 479 and 505 nm, which show different expression patterns in the main and accessory retinae. It was also demonstrated that there might be a switch in expression from the 505 nm to the 444 nm pigment during maturation (Partridge et al., 1992). This study has identified four retinally expressed opsin genes, an RH2, an SWS2 and two RH1 opsins. The two rod opsins regenerate *in vitro* with $\lambda_{\text{max}}$ at 480 and 490 nm. Both are expressed in adult mRNA, but the 480 nm rod is absent from juvenile mRNA. Further experiments linking the identified genes to the MSP values and examining expression patterns are also shown.

Work has also been undertaken on members of the notothenioid family of ice fish. These live at various depths under thick pack ice in the Antarctic. The light that reaches the water below has been filtered, with long and short wavelengths restricted. This study shows that a number of species express SWS1, SWS2 and RH2 opsins, though the level of SWS1 expression may be low in the majority of species. *In situ* hybridization and histology have helped to localize expression of these genes to different classes of photoreceptor. MSP and *in vitro* regeneration have identified the $\lambda_{\text{max}}$ of these pigments.
Declaration

I declare that this thesis submitted for the degree of Doctor of Philosophy is composed by myself and the work herein is my own or that the author involved is clearly stated.

Marie Anne Pointer B.Sc.
I have enjoyed this project immensely and I could easily have carried on for a further three years! There are lots of people who have helped and supported me along the way. The biggest thank you must go to my supervisor, Prof. David Hunt, who has helped me to develop confidence in my work. Also huge thanks to Prof. Jim Bowmaker, who has been truly generous with time and advice.

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Dedication

This thesis is dedicated to Belle, Rhodri and Greyey.
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λ_{max}  Wavelength of Maximum Absorbance
µg  Micrograms
µl  Microlitre
µm  Micrometer
µM  Micro Molar
A_{260}  Absorbance at 260 nm
A_{280}  Absorbance at 280 nm
cGMP  Cyclic guanosine monophosphate
CIP  Calf Intestinal Phosphatase
dATP  Deoxyadenosine triphosphate
dCTP  Deoxycytidine triphosphate
dGTP  Deoxyguanosine triphosphate
dH_{2}O  Distilled Water
DNA  Deoxyribonucleic Acid
dNTP  Deoxynucleotide triphosphate
dTTP  Deoxythymidine triphosphate
EDTA  Ethylenediaminetetraacetic acid-disodium salt
EtBr  Ethidium bromide
g  Grams
GDP  Guanosine Diphosphate
GMP  Guanosine monophosphate
GTC  Guanidium thiocyanate
GTP  Guanosine Triphosphate
HCl  Hydrochloric Acid
IPTG  isopropyl-1-thio-beta-d-galactopyranoside
kb  Kilobase
kDa  Kilo Dalton
LW  Long-Wave
LWS  Long-Wave Sensitive
m  Metres
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>mg</td>
<td>Milligrams</td>
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<tr>
<td>mins</td>
<td>Minutes</td>
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<td>ml</td>
<td>Millilitres</td>
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<td>mm</td>
<td>Millimetres</td>
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<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
<td>MSP</td>
<td>Mass Spectrophotometer</td>
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<tr>
<td>MW</td>
<td>Middle-Wave</td>
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<tr>
<td>MWS</td>
<td>Middle-Wave Sensitive</td>
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<tr>
<td>ng</td>
<td>Nanograms</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NH₄</td>
<td>Ammonia</td>
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<tr>
<td>nm</td>
<td>Nanometers</td>
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<tr>
<td>OW</td>
<td>Old World</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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<tr>
<td>RIOS</td>
<td>Rod Inner and Outer Segments</td>
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<tr>
<td>RLM-RACE</td>
<td>RNA Ligase Mediated Rapid Amplification of cDNA Ends</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RPE</td>
<td>Retinal Pigment Epithelium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription – Polymerase Chain Reaction</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SW</td>
<td>Short-Wave</td>
</tr>
<tr>
<td>SWS</td>
<td>Short-Wave Sensitive</td>
</tr>
<tr>
<td>TAP</td>
<td>Tobacco Acid Pyrophosphatase</td>
</tr>
<tr>
<td>Tm</td>
<td>Annealing temperature</td>
</tr>
<tr>
<td>U</td>
<td>Enzymatic Unit</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>X g</td>
<td>Revolutions per Minute</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-Bromo-4-chloro-3-indolyl-beta-d-galactopyranoside</td>
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For most vertebrates, information on colour, distance and movement is primarily obtained through the eye and visual processing centres in the brain. However, all vertebrates do not view the world in the same way. Each species has developed a specific spectral sensitivity that correlates to their environment and life style. It is fascinating to try to understand the wide capabilities of the visual sense, and this can be achieved by studying a range of animals that perform various visual tasks. The first stage of visual processing is photoactivation, when a visual pigment isomerises and begins a signal transduction cascade. Individual visual pigments are optimally sensitive to a specific region of the spectrum; this can be shifted to longer or shorter wavelengths by a variety of mechanisms. The study of these pigments or opsins has become a source of some striking examples of adaptive evolution, with peak sensitivity often tuned to enable optimal visual sampling in a particular environment. This study is aimed at investigating the visual pigments of three species of teleost fish, all of which have adapted to inhabit a specific ecological niche.

*Aristostomias tittmani* and *Scopelarchus analis* are both deep-sea species that live at depths as low as 1,200 m. At these depths residual sunlight is absent, though vision still seems to be an important sensory input. It is of interest to characterise the visual pigments and to see how these proteins have been spectrally tuned to adapt to their task.

The family *Nototheniidae* (cod icefishes) contains a number of species that inhabit water under ice sheets in the Antarctic. These teleosts are highly adapted to live in such
extreme conditions. This study is aimed at observing whether the change in light composition caused by the thick sheets of ice has led to a shift in spectral sensitivity. To this end we have tried to identify and isolate the different cone opsin classes that are expressed in representative species.
1.1 The Vertebrate Eye

Figure 1.1 (A) Diagram of a typical vertebrate eye in transverse vertical median section. Adapted from Randall et al., 2000. (B) Diagram of the typical teleost eye in transverse vertical median section. Adapted from Nichol, 1989. Main differences between the two diagrams are that the lens is more spherical in the teleost to confer extra focusing power, and the back of the teleost eye often contains argentea (tapetum), which reflects unabsorbed light back into the retina. Key: cg, Choroid gland; dc, dermal component of cornea; fp, falciparum process; hv, hyaloid vessel of retina; ir, iris; on, optic nerve; ot, ora terminalis; rlm, retractor lentis muscle; sc, scleral cartilage; sl, suspensory ligament of lens; tc, position of tensor choroidae.
The basic structure and image forming mechanisms of the vertebrate eye do not differ greatly between species. In terrestrial species, light rays are first refracted by the cornea (85%) and then by the lens to form an inverted image on the retina. As the refractive index of the cornea is similar to that of water and the aqueous humour, it plays a minimal role in underwater species. The lens needs to be spherical if it is to be useful as the principle point of focus (see figure 1.1). To counteract the consequent distortions produced, the lens is denser in the centre with a gradient of decreasing refractive index towards the periphery (Walls, 1942). Accommodation is achieved by movement of the lens within the eye, rather than from a change in curvature. This movement appears to be nasotemporal (in the pupillary plane) in the majority of teleosts but there are examples of the lens moving forwards and backwards along the pupillary axis (Fernald and Wright, 1985).

In shallow living fish the cornea can sometimes contain a yellow filter, which is often located dorsally and maybe important in reducing the bright down-welling light (Moreland and Lythgoe, 1968). Sometimes complex iridescent reflectors are found within the cornea instead of pigment. This selectively reflects away the bright downwelling rays but allows light from other directions to enter the eye (Lythgoe, 1974).

The pupil is an aperture in the centre of the opaque iris, which controls the amount of light that can enter the eye. In most vertebrates the pupil decreases in diameter when the smooth circular muscles of the iris contract, an increase in pupil size is created by contracting radial muscle fibres. Pupillary constriction in teleosts is still not fully understood. In most teleosts studied the pupil is either static or shows a small response
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to electrical or light stimulation (Nilsson, 1980; Somiya, 1987). However, there is evidence for pupil constriction in a number of species as recently demonstrated in the plainfin midshipman, *Porichthys notatus* (Douglas *et al.*, 1998). Pupillary constriction is particularly common in species with flattened bodies or that spend most of their life on or buried in the sand with only their eyes protruding (Douglas *et al.*, 1998).

1.1.1 The Retina

The retina has two components: the non-neural retinal pigment epithelium (RPE), and the neural retina. The RPE and neural retina are not joined but are very closely apposed. The neural retina is a remote extension of the forebrain and consists of six different neuronal cells; photoreceptors, bipolar cells, horizontal cells, amacrine cells, interplexiform cells and ganglion cells (figure 1.2). The outer nuclear layer is composed of the photoreceptor inner segments. The synapses formed between the photoreceptors and the horizontal and bipolar cells form the outer plexiform layer. The cell bodies of the horizontal, bipolar and amacrine cells are within the inner nuclear layer, with the inner plexiform layer composed of the synapses between bipolar and amacrine cells with ganglion cells (figure 1.2). Interplexiform cells are multipolar neurons with arbors in both the inner and outer plexiform layers. A high degree of neural convergence occurs in the retina; in the human retina for example approximately 130 million receptors converge to about 1 million ganglion cells (Wade, 1991). The degree of convergence is not evenly spaced over the retina. Convergence is least in the area of the retina that requires the highest visual acuity, such as the fovea of mammals. Lateral connections across the retina occur via the horizontal and amacrine cells. This provides summation and colour contrast information before the visual message reaches the ganglion cells. Horizontal cells interact with a number of photoreceptors and bipolar
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cells, forming information networks to converge signals and to fine-tune the information being sent to the brain. The amacrine cells have a similar role but act on the bipolar and ganglion cells. The signal is conveyed to the ganglion cells and then sent to the visual centres in the brain via optic nerve fibres, which leave the eye at the optic disc.
Figure 1.2 The neural retina. The arrows indicate the direction from which light hits the retina. Basically, the rod and cone photoreceptor cells (R) are excited, hyperpolarise and pass a neuronal message to the bipolar (B) and horizontal (H) cells. The message is then relayed to the amacrine (A) cells and ganglion (G) cells before entering the optic nerve fibres. Interplexiform cells are not shown. Key. A, amacrine cell; B, bipolar cell; G, ganglion cell; GCL, ganglion cell layer; H, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; R, receptor cell. Redrawn from Randall et al., 2000.
The role of the horizontal cells includes collecting chromatic opponency information, generating the antagonistic surround of the bipolar receptive field and forming spatial information via gap junctions. These cells relay information via feed-forward and feedback synapses in the outer plexiform layer (Kaneko, 1973; Toyoda and Tonasaki, 1978). Horizontal cells have been extensively studied in the teleost retina. In these vertebrates they are usually densely packed in the outer plexiform layer of the retina. Here they are electrically coupled and provide a feedback loop to the photoreceptors and bipolar cells. In the teleost retina the horizontal cells are the first to generate a spectrally opponent response (Murakami et al., 1972). The horizontal cells of teleosts fall into two broad categories. In mixed rod/cone retinas, there are horizontal cells solely in contact with cone photoreceptors. In species with three or more different chromatic cone types, these horizontal cells can be differentiated into subtypes, with dendrites in contact with the different cone photoreceptors in a specific pattern (Stell et al., 1994). The second category of horizontal cells contact rods exclusively and lack an axon and horizontal processes (Stell, 1967). Rod-connected and three different cone-connected horizontal cells have been identified in cyprinids. H1 cells have the smallest soma are in contact with all spectral classes of cone and produce a general luminosity response. H2 are larger and have lateral ribbon contacts with blue sensitive cones and central contacts with green-sensitive cones. These cells show a biphasic response, they depolarise to long wavelengths and hyperpolarize to short wavelengths. H3 cells have the widest dendritic field and are in contact with blue-sensitive cones only. These cells are triphasic, hyperpolarizing to short, depolarising to middle and hyperpolarizing to long wavelengths. H4 relates to the rod-connected horizontal cells (Stell et al., 1975, 1982; Wagner, 1990; Greenstreet and Djamgoz, 1994; Kamermans and Spekreije, 1995). Gap junctions are also present, electrotonically linking horizontal cells of the
same subtype (Yamada and Ishikawa, 1965). The neurotransmitter most commonly identified in horizontal cells is gamma aminobutyric acid or GABA, and seems to be common between all types (Van Haesendonck and Missotten, 1992).

First determined in teleosts but now recognised in most vertebrate classes is that bipolar cells fall into two main physiological classes: ON-centre and OFF-centre. ON-centre bipolar cells depolarise and OFF-centre cells hyperpolarize in response to central illumination of their receptive fields (Werblin and Dowling, 1969; Kaneko, 1970, 1971). Several more subtypes of bipolar cells have been identified in teleosts, categorised by morphological differences in the photoreceptor contacting synapses (Stell et al., 1977). In these species there is incomplete segregation of the rod and cone pathways within the bipolar cell layer. Only short and middle-wavelength sensitive cones retain a private bipolar cell connection (Stell et al., 1977). The length of the axons from the bipolar cells projecting into the inner plexiform layer is directly related to whether the signal being relayed is ON- or OFF-centre. This can be seen as two separate layers, with the terminals of ON-centre bipolar cells and the dendrites of ON-centre ganglion cells forming the first layer, and the terminals of the OFF-centre bipolar cells and dendrites of the OFF-centre ganglion cells forming the second (Famiglietti et al., 1977). Teleost bipolar cells form mixed rod/cone and specific chromatic information and convey this in antagonistic receptive fields (Kaneko and Tachibana, 1981, 1983). Uniquely, there is no separate scotopic pathway. The dominant neurotransmitter of the bipolar cells is glutamate (Ehinger et al., 1988).

Amacrine cells are the most diverse type of retinal neuron, with 43 different types identified in cyprinids (Wagner and Wagner, 1988). The function they play in teleosts is
not fully understood. They may sharpen the receptive fields of the bipolar cells and/or be involved in chromatic processing (Wagner et al., 1998). In shallow-water species, 80-90% of all amacrine cells contain the neurotransmitters GABA and glycine (Marc, 1982), though a wide number of different transmitters can be found in amacrine cells, with the morphology of the cell related to the neurotransmitter contained within its vesicles.

Interplexiform cells are multipolar cells with arbors in both the inner and outer plexiform layers. Their role is not fully understood but is probably linked with horizontal cell processing. Glycinergic interplexiform cells were first identified in perciformes by Ramón y Cajal in 1892. However, Gallego in 1971 gave the first clear definition of the different interplexiform subtypes in mammals. Glycinergic interplexiform cells have since been identified in the goldfish (Marc and Lam, 1981) as well as many other non-mammalian vertebrates. They have large somas (12 μm across) and are situated in the amacrine layer. Dendrites project into the outer plexiform layer with axonal fields in the inner plexiform layer. Dopaminergic interplexiform cells are particularly large in teleosts. The soma of these cells is again located in the amacrine cell layer, though has dendrites projecting to the inner plexiform layer and axonal fields in the outer plexiform layer (Marc, 1995). Dopaminergic interplexiform cells in other vertebrates are not as large with fewer projections, making them harder to distinguish from amacrine cells. Another class of interplexiform cells, termed GABAergic because they are often found embedded within GABAergic neuropils, have been identified in chondrichthians (Brunken et al., 1986).
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The retinal ganglion cells encode information on the presence or absence of light and the pattern of illumination across an area of visual space. All have concentric receptive fields; this means that the centre and surround are antagonistic. When the centre is excited the surround is inhibited or vice versa. The concentric antagonism can code for luminance or wavelength differences, hence axons sent to the visual centres in the brain encode contrast information. Information reaches the ganglion cells from the bipolar cells via ribbon synapses and from amacrine cells via conventional chemical synapses (Witkovsky and Dowling, 1969). The message is then relayed to the brain along the optic nerve via action potentials. There are two broad categories of ganglion cells distinguished by their size. Giant ganglion cells have a dendritic diameter between 0.4 and 1 mm, and small ganglion cells have fields in the range 0.1-0.3 mm (Wagner, 1990).

Müller cells are the glial cells of the retina. They surround the photoreceptor inner segments and extend to the ganglion layer, sealing the retina from the external environment and contributing to the blood-retina barrier (Tout et al., 1993). The main role is to reduce electrical and chemical interference on retinal neuronal cells. Müller cells express recognition molecules, which may be important in retinal development (Fadool and Linser, 1993).

In teleost fish, body size increases throughout life, with neuron number increasing to maintain neural control, the retina included (Müller, 1952). In the teleost retina new cells are added at the germinal zone at the circumferential margin of the eye and in the outer nuclear layer where new rod photoreceptors are produced. Meanwhile the central retina is stretched, depicted by the density of the retinal neural cells decreasing (Johns,
1977; Johns and Fernald, 1981). The density of rods remains constant though numbers of cone and other retinal neuronal cells decrease during eye enlargement (Johns and Easter, 1977). The proliferation of cells in the retina is under strict control so that vision is not affected. The number of dividing rod precursor cells in the ONL of the retina from a cichlid, \textit{Haplochromis burtoni}, were counted at different time intervals throughout the day and night. These cells were identified with an antibody that is specific for proliferating cell nuclear antigen. The highest number of cell divisions was found at night and was 3 times that found in the day. A rhythm of approximately 24 hours continued when the fish were maintained in constant darkness, which suggests that rod cell proliferation may be controlled by a circadian clock (Chiu et al., 1995).

1.1.2 Photoreceptor Cells

There are two types of photoreceptor, which subserve different visual roles. Rod photoreceptors are necessary for scotopic vision and possess a long cylindrical outer segment. Cone cells underlie colour vision and usually have a cone shaped outer segment, shorter than that of the rods (figure 1.3). Both rods and cones share the same basic structure of an inner and outer segment connected by a cilium. The outer segment contains stacks of discs or lamellae formed by invaginations of the plasma membrane. It is on these discs that the photosensitive pigments are located. In rods the flattened membrane vesicles are found completely enclosed within the outer segment membrane. The precise orientation of the discs is maintained by fibrous elements that link adjacent discs and the surrounding plasma cell membrane. In cones there are fewer vesicles and they remain as continuous projections of the plasma membrane. In both rods and cones new vesicles are constantly forming causing the older discs to migrate towards the distal
end of the outer segment. The oldest discs are in contact with the pigment epithelium and are phagocytically removed. The daily renewal of visual pigment exhibits a circadian rhythm (Korenbrot and Fernald, 1989). The inner segment houses the organelles and forms a synapse with the bipolar cells. The synaptic terminal from the cone photoreceptor cells is more complex than the rods, allowing for the further neural connections required for colour vision. The rod photoresponse is 2-5 times slower than that of a cone but is 100 times more sensitive to light (Baylor, 1987; Yau, 1994). The cone response is terminated more rapidly than that of rods (Tachibanaki et al., 2001), and has a more pronounced adaptation (Normann and Werblin, 1974). At high light levels cones can continue to respond under constant illumination, whereas the rod response is saturated.

In all vertebrate classes the cones are the first photoreceptor to differentiate during retinal development (Raymond, 1995). The rod neuronal pathway is then grafted on to the pre-existing cone pathway.

Animals that need to maximise their visual sensitivity, such as nocturnal species, have higher proportions of rods in their retina. The proportion of rods to cones can therefore vary among species. In the human eye, for example, there are about 100 to 120 million rods and 6 million cones (Farber et al., 1981). Some diurnal vertebrates possess good colour vision and have cone-dominated retinas, i.e. 7.5 million cones to only 1.27 million rods in the ground squirrel, Spermophilus beecheyi (Kryger et al., 1998).
Figure 1.3. A diagram of a typical rod and cone cell. BF, basal filaments; C, cilium; CN, cone nucleus; CP, cyclal processes; COP, cone pedical; COS, cone outer segment; G, golgi apparatus; M, myoid; MF, microtubules and filaments; OLM, outer limiting membrane; RN, rod nucleus; ROS, rod outer segment; RS, rod spherule; SR, synaptic ribbons. Redrawn from Locket, 1999.
In fish, cone cells are present in a variety of forms. These include: single cones, miniature single cones, double cones, triple cones and quadruple cones (Crescitelli, 1972). The more common are the single and double cones. The shorter-wave sensitive pigment is usually contained in the single cone, with the longer-wave pigments in the double cones. Double cones consist of two individual cones fused along their inner segments. The advantage of double cones is not fully understood but has been linked to the perception of polarised light in the green sunfish, *Leponis cyanellus* (Cameron and Pugh, 1991). The morphology of the double cones can vary. The two members can be identical, or there may be differences in size, shape, organelles and/or visual pigment (Engström, 1960, 1963; Levine & MacNichol, 1982). UV sensitive single cones are often present in young salmonid fish, but are absent in the adult. UV cones can only be measured in the brown trout of 1-2 years of age (Bowmaker and Kunz, 1987), though *in situ* hybridisation has identified a sparse population of UV sensitive cones in the dorsal adult retina (Allison et al., 2003). The loss of these cones appears to be via apoptosis and may be linked to a change of diet or habitat, or a hormonal switch during development (Kunz et al., 1994). Most juveniles feed on plankton, and it has been suggested that UV vision aids in foraging (Loew et al., 1993). Older fish generally feed on bigger prey and move down the water column reducing the need for UV vision. Interestingly, UV sensitive cones re-appear in some salmonid species at sexual maturity, when the fish return to their native streams (Beaudet et al., 1997; Novales Flamarique, 2000). This is the only example of cone regeneration during natural development and is linked to thyroid hormone concentration (Alexander et al., 1998; Hawryshyn et al., 2003).
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In many of the teleost retinae studied, the cones are arranged in a highly organised row or square mosaic pattern (Lyall, 1957; Engström, 1960, 1963). The more basic is the row pattern with single and double cones alternating in rows across the retina. The square pattern occurs when four double cones form the sides of a square, with a long single cone at the centre, as in salmonids (Lyall, 1957). In other teleosts, such as the brown trout, short single cones are found in each of the four corners of the double cone square (Lyall, 1957). Figure 1.4 shows the two classic teleost retinal mosaic arrangements pictorially. The mosaic can vary across the retina and in some species changes during development (e.g. salmonids (Lyall, 1957)). The square pattern can be formed from the row pattern, with larval fish often having a row mosaic. In adult fish, especially those with good vision such as predators, the cone mosaic dominates with row mosaics often found in schooling fish (Wagner, 1990). Studies have attempted to link visual pigments to each morphologically distinct cone type. In the goldfish the double cones are composed of a longer, principal member and a shorter, accessory member. The longer member contains the long-wave or red sensitive pigment, while the shorter member contains the middle-wave or green sensitive pigment. Therefore, the double cones are all red/green sensitive. 80% of the long single cones are red sensitive, the rest being green, short single cones are short-wave or blue sensitive and miniature single cones are UV sensitive (Stell, 1975; Marc and Sperling, 1976; Hisatomi et al., 1996). The same relationship between cone type and visual pigment gene expression is found in the killifish (Hisatomi et al., 1997). This is an interesting finding as goldfish and killifish are not close phylogenetically, the similarity in photoreceptor morphology and spectral sensitivity may indicate this is a default arrangement for most teleosts, though there are a number of exceptions.
Figure 1.4. The generalised regular cone mosaic patterns found in the teleost retina. The diagrams show the small UV single cones, the blue single cones and the double green/red cones. The top diagram is the basic row mosaic and the bottom is the square mosaic. Based on Raymond, 1995.
Retinomotor Activity

Retinomotor activity is a mechanical response to differing light intensities seen in the retina of several species of fish, amphibians and birds. Bright light causes the rod outer segments to elongate whilst the cones contract. This positions the cone outer segments into the light path, which enables greater photon absorption as well as rod protection during bright light conditions. The reverse occurs in darkness and the response can be controlled by light or circadian cues (Ali, 1975; Burnside and Ackland, 1984; Burnside and Dearry, 1986; Pierce and Besharse, 1988). The elongation and contraction occurs in the myoid region found between the photoreceptor nucleus and outer segment. There are a number of potential candidates for retinomotor control including cAMP (Burnside and Ackland, 1984; Dearry and Burnside, 1984), dopamine (Dearry and Burnside, 1985; Pierce and Besharse, 1985), melatonin (Pierce and Besharse, 1985) and adenosine (Rey and Burnside, 1999). The RPE also displays a form of migration. The melanin pigment granules migrate into the apical projections of the RPE cells when exposed to light. In the dark the granules move back towards the base of the RPE (Back et al., 1965). The apical projections are positioned around the rod photoreceptor outer segments and therefore during light the melanin granules act as a protective shield and reduce bleaching. The biochemical control for this migration is linked to the retina and a prime candidate is cAMP as for photoreceptor contraction/elongation. Levels of cAMP are high when exposed to darkness, and it is proposed that high levels of cAMP causes aggregation of the pigment granules preventing their movement into the apical processes (Garcia and Burnside, 1994). The actual movement appears to be actin dependent (Burnside et al., 1983).
Phototransduction is the process of converting energy from a photon of light to a neuronal message. Visual pigments consist of two parts: the protein, opsin and the light absorbing chromophore. Rhodopsin, the most common vertebrate visual pigment, has the aldehyde form of vitamin A$_1$ (or 11-cis retinal) as its chromophore. Porphyropsin is formed with the aldehyde of vitamin A$_2$ (or 11-cis 3, 4-dehydroretinal) (Figure 1.5). Birds and mammals only form rhodopsin complexes. Porphyropsin can be found in some fish, amphibian and reptilian species.

![Figure 1.5. The structure of all-trans and 11-cis retinal. The additional double bond in 11-cis-3, 4-dehydroretinal is represented by the dashed line between carbons 3 and 4. Adapted from Bowmaker, 1995.](image)

In the absence of light, retinal adopts the 11-cis configuration. The visual pigment absorbs energy from photons that reach the outer segment of the receptor cell. The energy state of the rhodopsin (or porphyropsin) molecule is raised by increasing the orbital diameter of the electrons found within a reactive double bond. This leads to
retinal changing state to an all-trans configuration (Knowles and Dartnall, 1977). The energy associated with visible light is sufficient to change the energy state of the visual pigment but not volatile enough to cause the molecule to break down. This photoisomerisation step occurs within picoseconds of photon capture (Hayward et al., 1981). The captured energy induces rhodopsin to form an intermediate state, termed metarhodopsin II, by deprotonating the Schiff's base (Longstaff et al., 1986). Metarhodopsin II has a less flexible structure than rhodopsin, preventing movement of the cytoplasmic loops and allowing for protein interactions (Mielke et al., 2002). Rhodopsin is a G-protein coupled receptor and activation creates a binding site for the G-protein, transducin-GDP. Transducin is a trimeric protein, consisting of $\alpha$, $\beta$ and $\gamma$ subunits. The genes encoding these proteins have been identified and all three units differ between cone and rod photoreceptors. The reason for this is not fully understood but may be important for conferring rod or cone type response kinetics. When transducin-GDP binds to metarhodopsin II, it is activated and there is a rapid exchange of the $\alpha$ bound GDP to GTP (Hamm and Gilchrist, 1996). The active $\text{Go}-\text{GTP}$ dissociates from metarhodopsin II and $\text{G}\beta\gamma$ and stimulates a high number of phosphodiesterase (PDE) molecules. PDE is a heterotetramer enzyme. In both rods and cones there are two regulatory $\gamma$ subunits. These act as protein inhibitors of PDE activity and keep non-activated levels low. In rods the catalytic units are nearly identical and named $\alpha$ and $\beta$, whereas in cones there are two identical $\alpha'$ units (Baehr et al., 1979; Deterre et al., 1988; Gillespie and Beavo, 1988). The activation of PDE is due to the binding of the transducin $\alpha$ subunit to the PDE $\gamma$ unit. This removes the constraint on the catalytic PDE $\alpha$ and $\beta$ units. PDE can then hydrolyse cGMP to 5'-GMP, which causes the cytoplasmic concentration of cGMP to be reduced. The role of cGMP is to maintain open cation selective cGMP-gated channels in the plasma membrane, therefore
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when converted to 5'-GMP hundreds of channels are forced to close (Stryer, 1986). This causes the steady inward current of cations, normally Na\(^{+}\) and Ca\(^{2+}\), during the dark state to be halted. The outward movement of K\(^{+}\) and Ca\(^{2+}\) remains constant because the Na\(^{+}\)-Ca\(^{2+}\) exchanger continues to pump these ions out of the cell. This causes a reduction in intracellular cations and the cell becomes hyperpolarized (Matthews et al., 1988; Nakatani and Yau, 1988). This in turn leads to a reduction in the release of the neurotransmitter glutamate from the photoreceptor terminal. The extent of hyperpolarisation is proportional to the cytoplasmic cGMP concentration. When the light stimulus ends, cGMP is regenerated via guanylate cyclase, which is activated when \([\text{Ca}^{2+}]\) is low, such as during hyperpolarisation, and the intermediates metarhodopsin II, G\(\alpha_{r}\)-GTP and activated PDE are rapidly inactivated and restore the cell to the dark state. The activation response is highly amplified, and in rods can elicit a reliable response to single photons. The capture of a single photon by a rod photoreceptor will result in a 2-5% suppression of the cGMP-activated current (Baylor et al., 1979). The two main amplification steps are:

1. formation of 500 Transducin-\(\alpha\)-GTP complexes from one activated rhodopsin,

2. hydrolysis of several thousand cGMP molecules per second by activated PDE.

Restoration of the dark state is attained by a combination of reactions. Firstly, rhodopsin kinase phosphorylates the activated rhodopsin at multiple serine and threonine residues at the C-terminus of the molecule (Kuhn, 1984; Thompson and Findley, 1984), and arrestin (48 kDa protein) binds to these sites (Palczewski et al., 1992; Ohguro et al., 1993; Palczewski, 1994). The activation of rhodopsin kinase may be caused by a decrease in intracellular Ca\(^{2+}\) ions, which removes inhibition by the protein recoverin in rods, or visinin in cones (Kawamura, 1994; Yamagata et al., 1990). Cone and rod specific rhodopsin kinase mRNAs have been isolated from the retina of the Japanese
killifish, *Oryzias latipes* (Hisatomi *et al.*, 1998). The deduced amino acid sequences show only 50% homology to other isolated vertebrate rhodopsin kinase genes. In humans two G protein-coupled receptor kinase (GRK) genes have been isolated and termed RK or GRK1 and GRK7 (Palczewski *et al.*, 1988; Weiss *et al.*, 1998; Chen *et al.*, 2001). GRK1 has been localised to rods in a number of species and cones in humans (Zhao *et al.*, 1998), monkeys (Sears *et al.*, 2000; Weiss *et al.*, 2001) and mouse (Lyubarsky *et al.*, 2000). GRK7 is concentrated in cones in both cone and rod-dominant mammals (Weiss *et al.*, 2001). Arrestin competes with transducin for rhodopsin binding and is essential for PDE inactivity. Mutagenesis experiments have highlighted three residues in rhodopsin that are important for the binding of arrestin, these are Asn73 in cytoplasmic loop I and Pro142 and Met143 in cytoplasmic loop II (Raman *et al.*, 1999). Arrestin differs slightly in cone and rod photoreceptors with the different forms known as S-antigen (rod) and X-arrestin (cone) in human (Yamaki *et al.*, 1988; Murakami *et al.*, 1993) and KfhArr-R and KfhArr-C in killifish (Hisatomi *et al.*, 1997). Before the opsin can absorb another photon the all-trans retinal must be removed and replaced with a molecule of 11-cis retinal. This conversion occurs by different mechanisms in rods and cones. The process is well characterized in rods and is termed the retinoid or visual cycle and occurs within the retinal pigment epithelial cells (Rando, 1992; Chader *et al.*, 1998). The reaction is endothermic utilising a number of retinoid binding proteins and enzymes. It was understood that the same process occurs in cones. However, pigment regeneration after an intense light pulse is four fold faster in cones than in rods (Weale, 1967, 1972; Ripps and Weale, 1969) and cone and not rod opsin can regenerate in isolated frog retinæ separated from the pigment epithelium (Goldstein and Wolf, 1973). Recently Mata *et al.* (2002) discovered that cones regenerate their opsins via an interaction with Müller cells. This pathway is cone specific as rods do not contain the
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enzyme required to oxidise 11-cis retinol to 11-cis retinal. The cone/Müller cycle is 20 times faster than that of the rod/RPE, which is necessary to ensure a continued response under bright light conditions. The pathway was discovered in the chicken and ground squirrel retina, which are both cone dominated species. More needs to be known on whether regeneration via Müller cells is common in species such as humans where cones are not dominant.

The reduction in intracellular [Ca\(^{2+}\)] during hyperpolarisation also increases the sensitivity of the cGMP-gated channels by the uncoupling of calmodulin (Hsu and Molday, 1994). This accelerates the recovery of the dark current. Another Ca\(^{2+}\) sensitive step involves the guanylate cyclase activating proteins (GCAPs). The Ca\(^{2+}\)-free form of GCAP stimulates guanylate cyclase and therefore accelerates the synthesis of cGMP, enabling the reopening of cGMP-gated channels (Palczewski et al., 1994). In zebrafish three isoforms of GCAP have been isolated, GCAP1 and 2 are expressed in rods and short, single cones and GCAP3 is in all subtypes of cone (Imanishi et al., 2002). Recovery is also aided by the GTP molecule bound to the α subunit of transducin slowly hydrolysing back to GDP. This is via the intrinsic GTPase activity of the α unit, though it is aided by the GTPase activity of the γ subunit of PDE, RGS9 (regulator of G protein signalling) and the long splice variant type 5 G protein β subunit (Arshavsky and Bownds, 1992; Cowan et al., 1998; He et al., 1998; Makino et al., 1999). The regulatory γ subunits of PDE are then released from transducin-GTP and can exert their inhibitory effect on the active PDE subunits. The deactivation of transducin and PDE allows the concentration of cGMP to return to the level it had in darkness, for the channels to reopen and the dark current to be restored. A diagram of the main steps in the phototransduction cascade is shown in figure 1.6.
Figure 1.6. A schematic of the vertebrate phototransduction cascade.
All the molecular components of the phototransduction cascade mentioned here have been identified in a variety of vertebrate species, including teleost fish. It seems likely that rod and cone signal transduction molecules diverged before teleosts and tetrapods separated. Interestingly, work by Tokunaga et al. (1999) has demonstrated that in killifish the four different cone classes can all utilise the same isoforms of PDE, arrestin and rhodopsin kinase. This suggests that over a relatively long period of evolutionary time there has been a constraint on divergence between the phototransduction molecules of the different cone classes. Interestingly, a difference in RGS9 concentration is evident between mammalian rods and cones, with cones expressing significantly higher levels (Cowan et al., 1998). The concentration of RGS9 is therefore a potential determinant of the faster response kinetics of cones to rods. This includes faster phosphorylation of the activated rhodopsin, which leads to a faster decay of the photoactivated state (Tachibanaki et al., 2001).

Light information is passed from the photoreceptors to the bipolar and horizontal cells by a graded electrical response. Photoreceptors hyperpolarize in response to light, which causes a reduction in the rate of glutamate release from the synaptic terminals. Bipolar cells detect this (Shiells and Falk, 1990) and the neuronal response continues through the retina and up the optic nerve. Rod and cone terminals consist of synaptic ribbons (Gray and Pease, 1971). These are specialised structures, which can house a large pool of neurotransmitter molecules ready to be released as vesicles. This may be important in supporting the high resting rate of neurotransmitter release.
1.2 Adaptations to Underwater Vision

Water is a monochromator, absorbing long and short-wave light. In clear oceanic water the maximum light transmission is around 470 nm. Light and temperature become greatly limited when travelling down the water column, restricting the number and diversity of species. In coastal waters the level of suspended particles increases and longer wavelength light is more readily transmitted (530 –570 nm). Fresh water is usually rich in phytoplankton and yellow products from vegetable decay (gelbstoffe), these impart a greenish colour to the water (Lythgoe, 1984). This causes the maximal transmission to red shift. Another factor is the level of turbidity, which can have a dramatic effect on the visual range. High turbidity will greatly reduce the distance from which an image is visible. The visual system of fish has evolved to suit these conditions, with a variety of adaptations providing vision in a range of photic environments.

The eyes of aquatic vertebrates are very similar to those found on land (figure 1.1), though there are three main problems specific to underwater vision that need to be solved (Lythgoe, 1980):

1. Using dim light at depth.
2. Coping with the monochromatic nature of the light present.
3. Coping with the directional distribution of the space light.

In dim light, the lack of photons causes discrimination of colour and shape to be severely hampered. To counteract this, light capture and retinal sensitivity need to be increased.
1.2.1 The Deep-Sea Environment

Around 70% of the Earth’s surface is covered by sea, with 60% being classified as deep-ocean, and the remaining 10% as the continental shelf. The unknown quantity makes the deep-sea fascinating. The conditions are known to be extreme: pressures can reach 1000 times that of atmospheric, temperatures are constantly low (around 4°C), and light is minimal (Douglas et al., 1998). The high pressures do not create stress on deep-sea animals because their tissue is permeated with liquid, which is neither more nor less compressible than the surrounding water.

![Diagram showing the relationship between biomass, light penetration, and temperature to depth in oceanic waters. Redrawn from Marshall, 1971.](image)

**Figure 1.7.** Diagram to show the relationship found with biomass, light penetration and temperature to depth in oceanic waters. Redrawn from Marshall, 1971.
Fish with swim bladders alter the tension of gas to allow migration through the water column. Sufficient light for photosynthesis to exceed respiration only penetrates approximately 200 m (euphotic or epipelagic zone) (figure 1.7). Below this and stretching down to around 1000 m, where the last remnants of light are found, is the transition zone. Organisms inhabiting these depths are termed mesopelagic. The fauna here depends on that of the epipelagic zone for nutrition, often ascending at night to feed on the vast amount of plankton. Mesopelagic fish commonly exhibit adaptive features, such as camouflage and bioluminescence, and often have highly specialised eyes. In fact the greatest number and diversity of luminous organs is found in mesopelagic fish (Locket, 1977). From the transition zone to the ocean floor is the aphotic zone, and here there is total darkness apart from chemically produced bioluminescence. The animals that live in the aphotic zone are known as bathypelagic and are either carnivorous, suspension, or detritus feeders. The number of species inhabiting these depths is small. With light from above no longer a factor, countershading and camouflage are not often found. The surface of the seabed is known as the benthal zone. Some species exist swimming just above the ocean floor while others live on or within it. Many of these fish adopt a sit and wait strategy when it comes to finding food (Merrett, 1987). Even though light levels are low, most of the fish caught at depth have well developed eyes and optic tecta. Over 30 species of deep-sea teleost are known to possess foveae. This suggests that vision plays an important sensory role in these deep-sea fish. However, some species examined have small, degenerate eyes, e.g. the aulopiforme Bathymicros regis (Munk, 1966). It may be that as deep-sea fish adapted to the low light environment, some species developed mechanisms to increase visual sensitivity, while others attuned alternative senses, such as hearing and olfaction. Interestingly, it has been observed that ocular degeneration in
deep-sea fish is only found in species that do not possess a luminous organ and inhabit depths lower than that which sunlight can penetrate, i.e. the bathypelagic and benthal zones (Munk, 1966). An observation by Paxton is that the otoliths of luminous fish are often larger than in non-luminous fish (Paxton, 2000). Otoliths are dense calcareous structures located within the ear of teleost fish and are associated with hearing and gravity perception. Paxton speculates that fish that use bioluminescence usually do so because of the poor visual conditions, and therefore may also evolve larger otoliths to compensate for the loss of acute vision.

There are two sources of illumination in the deep-sea: downwelling sunlight and bioluminescence. At a depth of 1000 m, residual sunlight cannot be detected, and fish at this or lower depths rely on bioluminescence (Denton, 1990). Above 1000 m, sunlight is perceptible though the spectral composition decreases with depth. The light becomes more monochromatic, with a spectral range of 470 - 480 nm (figure 1.8). This is due to short and long wavelengths becoming rapidly attenuated with depth (Jerlov, 1951, 1968).
Figure 1.8. Transmission of light through clear oceanic waters. The light becomes increasingly monochromatic and blue as its path length increases. This diagram is not correct for fresh water because of the absorption by organic matter. Redrawn from Levine and MacNichol, 1982.
Bioluminescence

80% of deep-sea fish are bioluminescent (Herring, 1996). These chemical signals are not just produced for illumination but are important for communication and camouflage. Light can be used to startle or attract prey and is an essential part of life in a naturally dark environment. Bioluminescence tends to be blue or blue-green in colour, with a wavelength around 470 nm. This matches the wavelength of sunlight that can penetrate to the mesopelagic realm (Widder et al., 1983) and in the majority of species also correlates with the wavelength of peak sensitivity or $\lambda_{\text{max}}$ of the deep-sea rhodopsins (Lythgoe, 1972; Douglas et al., 1998 discussed in full in section 1.4). The origin of the light can be due to symbiotic bacteria living within the light organ or to a complex photophore containing a specialised structure such as a reflector, filter or lens. The position of the photophore can reveal its main functional role. Many species have photophores located close to the eye. These ocular light organs illuminate the areas that are being directly looked at. A number of species use light as a lure by having it placed at the tip of a barbel hanging over the mouth (deep-sea angler fish, Centrophyrne spimilosa, see figure 1.9) or located within the mouth itself (viperfish, Chauliodus Sp.). Photophores found on other parts of the body are more likely to have a role in camouflage or signalling. An example is counterillumination camouflage.
Counterillumination Camouflage

If an animal is viewed from below, a shadow is formed against the down welling light. A potential predator would easily be able to spot any prey swimming above, especially if waiting on the seabed with upward facing eyes. To counteract this many diurnal pelagic prey have developed an innovative use for bioluminescence. Photophores are found on the ventral surface with emissions matched to the down welling light. Illumination of the ventral photophores allows the fish to merge its silhouette into the down-welling light (Mensinger and Case, 1997). This counterillumination camouflage makes detection by predators more difficult.
1.2.2 Adaptations for Deep-Sea Vision

1.2.2.1 Geometric optics of the eye

The f-number of an eye is the diameter of the lens aperture divided by the focal length (measured from lens to retina). The brightness of an image is proportional to this f-number. Most deep-sea fish possess large eyes with an enlarged pupil (Land, 1990). This increases the f-number and allows light from a wide field to be captured. When light enters the eye at an angle to the optic axis, the effective aperture is reduced as some of the pupil is hidden from the line of light. In some deep-sea fish, especially predators, the pupil is pear shaped leaving a rostral region of the pupil unoccupied by the lens. This enables any light that is falling from above a greater chance of hitting the pupil even though it will not be focused via the lens. The function of the aphakic space is to increase the luminance entering the eye, though there must be a payoff with visual acuity. Rostral aphakic apertures are often associated with the presence of an area temporalis (Bathylagus pacificus) or fovea (Platyctegens mirus) (Munk, 1966). The extra illumination falls on the specialised retinal region increasing sensitivity (Munk and Fredriksen, 1974). Aphakic apertures can also surround the whole pupil (circumlental). This allows for extra illumination to enter the eye from all directions, increasing sensitivity, especially to lateral visual space. Circumlental aphakic apertures have only ever been identified in deep-sea species, this is probably due to problems with reduced visual acuity when abundant light is present. Indeed a correlation has been noted between size of circumlental gap and depth, with larger apertures present in deeper dwelling species of Gonostoma (Marshall, 1954).
1.2.2.2 Tapeta

In nocturnal species and most deep-living fish, a light reflective material or tapetum lucidum can be found sclerad to the photoreceptive layer. The function of the tapeta is to reflect back any light that was not absorbed by the photopigments and to thereby provide a second opportunity for photon gathering. This increases overall illumination but can dramatically reduce resolution. Some genera of deep-sea fish, e.g. *Osmosudis* have individual tapetal reflectors for each photoreceptor, to help maintain resolution (Locket, 1977). Two main tapetal types are known: those found in the choroid and those in the RPE. Examples of both have been found in deep-sea fish (Denton and Nichol, 1964; Somiya, 1980).

### Choroidal Tapeta

The outer layer of the choroid (argenteum) is silver in colour and can act as a tapetum in shallow dwelling fish (Best and Nichol, 1980). True choroidal tapeta normally contain guanine crystals in parallel plates (Nichol *et al.*, 1973). These are arranged at right angles to the incoming light so as to reflect back along the path of the photoreceptor and not to scatter (specular), which helps to reduce losses in visual acuity (Denton and Nichol, 1964). Choroidal tapeta are common in chondrichthyes, though there are only a few reports of their presence in teleosts, one such example being the coelacanth (Locket, 1974).

### RPE Tapeta

These are more common in teleosts than the choroidal type, with light being scattered more diffusely. The light reflective substances vary; lipids, uric acid, pteridine, melanoids and carotenoids can all be used. Some species of teleost e.g. *Scopelarchus*
analisis have RPE tapeta containing guanine, which enables specular reflectance (Locket, 1977).

Tapeta are not always silver, there are examples of blue (*Latemedia*), violet (some mytophids), yellow (catfish), orange (priacanthids) and red (*Malacosteus*, see chapter 3 and figure 3.1) (Locket, 1974; Nichol, 1989; Nichol *et al.*, 1973; Wang *et al.*, 1980; O'Day and Fernandez, 1974). Most deep-sea fish possess tapeta that reflect shorter wavelengths, matching the ambient light and the rod opsin \( \lambda_{\text{max}} \), though some species possess white, silver or golden tapeta that reflect a broader spectrum of light (Douglas, 1998). Red, yellow and orange tapeta are more common in freshwater fish (Lythgoe, 1979).

**1.2.2.3 Tubular Eyes**

Some deep-sea and mesopelagic teleosts possess asymmetric or tubular eyes (Locket, 1977). Marshall identified tubular eyes in 11 different families, indicating that this adaptation has evolved independently in different ancestral lineages (Marshall, 1971). The eyes are located dorsally or rostrally on the head and are cylindrical in shape. The base of the cylinder houses a thick, main retina, and the medial wall contains an accessory retina (Locket, 1977). The eyes have little mobility but there is a large binocular dorsal field, which may aid in judging distances (Wagner *et al.*, 1998). Binocularity is most often found in predators, as it confers a huge advantage for determining the distance of potential prey and hence provides information on when to strike effectively. This is particularly useful for deep-sea predators as there are few monocular clues to aid distance determination as the background is a uniform darkness. The accessory retina subtends a lateral visual field and may in part compensate for the
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restricted field of view. Other adaptations to extend the visual field are often present in tubular eyes, these include an outpocketing of differentiated retina termed the retinal diverticulum (e.g. Winteria, Munk, 1966), a lens pad (e.g. scopelarchids, Locket, 1971b), and optical folds (e.g. evermannellids, Locket, 1977). The advantage of a tubular shaped eye may be that it can house a large lens without an increase in the lateral-medial diameter of the eye, therefore the eye can still fit within a fish of given size. A normal more spherical eye with the same size lens would have a much greater lateral-medial diameter (Franz, 1907 cited from Locket, 1977). A large lens would enable greater light collection than a small lens. Tubular eyes are a common feature of the Scopelarchids, this is reviewed later in chapter 4.

1.2.2.4 Lens Pigmentation

In vertebrates and invertebrates, both on land and in water there are examples of lens pigmentation. On land a pigmented lens is important in high light levels, often lenses contain UV filters that may protect the eye from damaging rays. Short-wave filters are also common in shallow dwelling fish (Douglas et al., 1995). In a deep-sea fish, a pigmented lens will reduce the light reaching the retina and hence limit light sensitivity. This would appear counter productive as light levels are already low. Over 150 species of deep-sea fish have been examined and no short-wave absorbing pigment has been found (Douglas et al., 1995). Lens pigmentation has been detected in 27 species of mesopelagic fish (Douglas and Thorpe, 1992; Douglas et al., 1995), these are species living at depths of 200-1000 m. The proportion of UV/violet light that can reach the retina is reduced and the lenses of these fish are visibly yellow. In Scopelarchus analis for example, there is no transmission through the lens below 450 nm (Douglas et al., 1998). It seems unlikely that mesopelagic fish need UV protection, and other roles for the lens pigments have been proposed (Douglas et al., 1995). Perhaps the most likely
function is to enhance sensitivity to bioluminescence. The peak light emission from photophores is usually in the range 450-500 nm, though often contains longer waves than that naturally found in the water column (Herring, 1983; Widder et al., 1984). A short wave filtering lens will enhance the differences in light composition between the down welling light and bioluminescence thereby providing contrast for objects lit by bioluminescence against a residual sunlight background (Douglas & Thorpe, 1992). An example of where this would be advantageous is for predators trying to discern the outline of prey that utilise counterillumination camouflage. This hypothesis is supported by the observation that fish living at depths lower than 1000 m have clear lenses. If there is no residual sunlight then the bioluminescence will not be confused with the surrounding space light, and therefore the spectral composition does not need to be offset against it (Douglas et al., 1995).

The Pigmented Lens of *Malacosteus niger*

*Malacosteus niger* is a deep-sea stomiid fish with an unusual lens. The absorption profile shows two maxima at 429 and 460 nm (Muntz, 1983). The smaller, and hence younger lens absorbs less at 429 nm than the older, large lens (Muntz, 1983). It seems likely that the lens of *M. niger* contains two filtering pigments, and the proportions change over time, with the pigment absorbing at 429 nm increasing with age. The reason for this has not yet been established.

1.2.2.5 Retinal Morphology

The majority of deep-sea fish possess an outer retina composed solely of rods (Locket, 1977). Rod photoreceptors can take better advantage of the low light levels and its monochromatic nature than cones. The presence of rod only retinas is also found in the
deep-water cottoid fish of Lake Baikal (Bowmaker et al., 1994). The endemic species of Lake Baikal provide an excellent source for the study of visual pigments correlated to depth. MSP data from a number of species have shown that the retinas of the shallow-water fish contain double middle-wave cones and single short-wave cones. In the deep-water species however these cone populations are lost. There are a very small number of single cones containing a middle-wave sensitive pigment, and these are believed to be the remnants of the double cone population (Bowmaker et al., 1994). Throughout all vertebrates, cones are the first type of photoreceptor to differentiate, with rods forming later, grafted on to the pre-existing cone pathway. It is interesting to determine if this occurs in the rod only retinas of deep-sea fish. The central retina of a larval stage deep-sea species (Idiacanthus fasciolatus) has tested positive for the presence of cones, though in 15 other species there was no evidence for cone receptors in the peripheral growth zone (Wagner et al., 1998). This demonstrates that the usual pattern of photoreceptor differentiation still occurs in some species that have rod only retinas, with the cones being lost at maturity. More evidence is required to prove that some deep-sea retinas forgo the cone differentiation pathway.

The amount of rod visual pigment can be increased by extending the rod photoreceptor outer segments or by forming several photoreceptor layers (Locket, 1977), in order to increase the area available for photon capture. The outer segments in deep-sea fish are often much longer than those in shallow living fish. For example the retina of Sternoptyx is 230 μm thick, which is close to the value of 260 μm for the goldfish. The major difference though is that the outer segments of the goldfish are 40 μm in length compared to 95 μm in Sternoptyx (Locket, 1971). Also a high degree of receptor packing can increase the likelihood of a photon hitting an outer segment disc. Partridge
and colleagues have shown that the specific optical densities of visual pigment in deep-sea fish rod outer segments (0.0059-0.0283 $\mu$m$^{-1}$) with a mean at 0.0166 $\mu$m$^{-1}$ (Partridge et al., 1988, 1989) are consistently higher than in shallow-water species (0.01-0.015 $\mu$m$^{-1}$) (Knowles and Dartnall, 1977; Partridge, 1986). This is likely to increase sensitivity as with more visual pigment there is a greater probability of photon capture. The synaptic spherules of the deep-sea rod receptors are generally rod-like with one or two synaptic ribbons and unlike the complicated cone pedicles (Wagner, 1998).

Receptors can also be clumped into optically single units. Light information is summated making it more likely for a firing threshold to be reached. In some deep-sea fish, photoreceptor bundles can be surrounded with tapetal cells, greatly increasing light perception (Locket, 1977).

There are two broad categories of horizontal cells in teleost fish. The first synapses to cones exclusively and the second to rods. Wagner et al., (1998) has examined the horizontal cells in the retina of three species of deep-sea fish. Two tiers of horizontal cells were present in the rod dominated but cone containing retina of Phycis blennoides. However, the pure rod retina of Nematonurus armatus, which contains only one visual pigment contained a single tier of loosely scattered horizontal cells. This suggests that the extra class of horizontal cells present in Phycis blennoides is linked to the presence of cone photoreceptors in the retina. Malacosteus niger was the third species examined. It has a pure-rod retina but with more than one visual pigment. The interesting question was whether there would be more than one type of horizontal cell present. In fact the retina was similar to that of Nematonurus armatus, with only one type of horizontal cell, showing little communication with neighbouring cells. It therefore seems unlikely
that the two visual pigments of *Malacosteus niger* are forming a basic colour vision system. It is more probable that their function is to broaden the wavelength of light that is perceived. Wagner and colleagues also demonstrated that interplexiform cells appear to be absent in rod dominated deep-sea teleosts (Wagner *et al*., 1994). From this observation they suggest that interplexiform cells are solely involved in cone vision, though this may be an over-simplification as cone-rich non-teleosts such as the gar (Family Lepisosteidae) lack dopaminergic interplexiform cells too (Marc, 1996).

The RPE of deep-sea fish is often thin and underdeveloped. As previously mentioned, in some cases the RPE contains reflective crystals and acts as a tapetum, and in these fish melanin can be absent or regionally restricted (Locket, 1977). This is interesting, as it is known that the RPE plays an important role in pigment regeneration (Baumann, 1972). The presence of a reduced RPE suggests that the retina of deep-sea fish has a lower metabolic rate than shallow living species (Locket, 1977). This would not be surprising in view of the low light conditions.

### 1.2.2.6 Multiple Bank Retinae

In some deep-sea fish, the rod inner and outer segments (RIOS) are arranged in multiple banks (figure 1.10). This layer can be several times thicker than the RIOS of shallow dwelling fish and land vertebrates. *Bajacalifornia megalops* has a 28-bank fovea, 750 μm thick (Denton and Locket, 1989) compared to the 28 μm RIOS of the goldfish, *Carassius auratus* (Guerin *et al*., 1993). In species with tapeta the reflected light may excite the distal banks of photoreceptors. It is thought that the multiple banks are a sensitivity-increasing adaptation. The amount of visual pigment is increased and therefore the probability of light capture is also increased. Fish eyes grow throughout
life. New retinal cells originate in the peripheral retina and rod density is maintained in all banks during proliferation (Johns and Easter, 1977; Wagner et al., 1998). At the same time the central retina is stretched (Johns, 1977). Whether the number of RIOSs increases with age is debated (Locket, 1980; Wagner et al., 1998), though it has been suggested that the older RIOSs are displaced towards the back of the eye, leaving the new segments at the front as the retina grows (Denton & Locket, 1989; Fröhlich et al., 1995). In deep-sea teleosts, the evidence suggests that there is no lifelong addition of the RIOS, but that the maximum is reached at around 40% growth (Wagner et al., 1998). Often the larval stages of deep-sea fish inhabit the mesopelagic zones and only migrate down once fully developed (Marshall, 1954). An interesting hypothesis made by Wagner et al. (1998) is that the cue needed for this migration is linked to the acquisition of the final rod inner and outer segment banks. It is only when these are acquired that vision at low depths and low light levels is possible for these fish. The morphology of the rod receptors in the more distal banks differs from typical rods by having an extended myoid region (figure 1.10). The myoid connects the outer segment and mitochondria housing ellipsoid with the inner segment and nucleus. The materials required to form new outer segment discs must travel along these long myoids to reach the outer segment.

A question that still remains unanswered is how the rod outer segments are renewed given that the vitread banks are not in contact with the RPE. In fact ERG tests on conger eels suggested that only the most distal photoreceptors are truly functional (Shapely and Gordon, 1980). However, in a different experiment that labelled the rod opsin in the outer segments with gold particles before viewing with an electron microscope, the result showed that there was no statistical difference in opsin density between the banks.
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(Wagner et al., 1998). This suggests that there is some phagocytic activity in the more vitread banks, even though the RPE is not in close association. An interesting hypothesis is that because deep-sea fish are not in continual contact with light, the amount of pigment being actively bleached would be far less than in typical diurnal retinae (Locket, 1977).

![Diagram of a multibank retina. The vitread bank of rods, 1, is like those in a normal retina. Their ellipsoids, 2, are just sclerad to the outer limiting membrane, 3, their outer segments, 4, do not reach the RPE, 5. The ellipsoid and outer segments of the middle bank of rods, 6, are connected by a slender myoid, 7. This bank also falls short of contact with the RPE. The sclerad bank of rods, 8, has the longest myoids, 9. The outer segment tips of the sclerad bank are in contact with the RPE. 10, ventricular space; 11, radial fibre cytoplasm; 12, rod nuclei; 13, synaptic spherule. Redrawn from Locket, 1977.]

1.2.2.7 Integration Time

Light information can be gathered over a period of time (integration time). This time can be extended for dim-light vision to increase the chance of photon capture. The penalty for this is a loss of temporal detail. An image that moves quickly across the field of view may be undetected, or responded to too slowly. It has been suggested that predator fish with extended integration times may ambush their prey, to prevent an
accumulative effect of its own movement and that of the prey causing confusion (Lythgoe, 1984).

1.2.2.8 Visual Pigments

Usually fish visual pigments exhibit a wide range of $\lambda_{\text{max}}$ values. This is in part due to the presence of rhodopsin / porphyropsin pairs in some species (Lythgoe, 1979; Bowmaker, 1984) but mainly the result of spectral tuning of the opsin, and the presence of more than one class of opsin gene. Fish living in the deep-sea, however, usually contain all rhodopsin in rod only retinae. At the time of writing, the visual pigment absorption spectra have been obtained for some 200 deep-sea fish species. Out of these, the vast majority (176) contain only one visual pigment in their retina. From these species there is no evidence for porphyropsin pigments, with retinal consistently acting as the chromophore (Douglas et al., 2003). The absorption spectra for these single visual pigments shows that 90% have $\lambda_{\text{max}}$ between 475 and 490 nm. This matches the wavelength of the blue bioluminescence, and the residual down-welling light almost exactly and is commonly cited as an example of adaptive evolution.

An aspect of visual pigments that has not been studied in deep-sea fish but is extremely interesting is the regulation of opsin mRNA expression. It has been demonstrated in the toad, Bufo marinus, and the cichlid, Haplochromis burtoni, that the level of rod opsin mRNA fluctuates with a daily rhythm linked to light and circadian cues (Korenbrot and Fernald, 1989). In the tiger salamander this is regulated by dopamine (Alfnito and Townes-Anderson, 2001). When light levels increase there is a rise in dopamine, which in turn leads to increased rod opsin mRNA production. As deep-sea fish will not
encounter major light fluctuations, is opsin transcription controlled by a different mechanism?

1.2.2.9 Pressure Adaptation

The proteins of deep-sea fish show a high resistance to thermal denaturation when compared to shallow living species. This may be down to especially rigid proteins that resist disruption of their tertiary and quaternary structure under high pressure (Somero, 1992a, b). Another adaptation to high pressure is the presence of hydroxyl bearing residues to confer thermal stability. In a study on deep-sea rod opsin sequences, the deepest living species investigated, *H. bathybius* and the deep sea eel showed the highest proportion of hydroxyl residues; 18.4 and 18.8% respectively compared to the shallow species *P. minutus* with 15.7% (Hope *et al.*, 1997), though there is little evidence of consistent changes in opsin amino acid composition (Hunt *et al.*, 2000).

1.2.2.10 Non-visual adaptations in deep-sea fish

It has been demonstrated that the muscles of deep-sea fish contain elevated levels of trimethylamine oxide (TMAO) (Kelly and Yancey, 1999; Treberg and Driedzic, 2002). In the skate, the levels of TMAO increased with depth of capture. In light of these results it was proposed that TMAO may be important in counteracting the effects of high pressure on protein function. If this hypothesis is true then it would be expected that TMAO would be high in all tissues. Treberg and Driedzic (2002) showed that TMAO is elevated in heart, brain, liver and kidney as well as muscle in seven deep-sea teleosts and two deep-sea elasmobranchs. TMAO is known to increase protein interactions and help to decrease the hydration shell of the protein. This may be important for protein reactions that involve an increase or decrease in volume, such as
the polymerisation of G-actin to f-actin (Yancey et al., 2001). TMAO seems to function by limiting the stress created by a volume change under high pressure. Interestingly, there is evidence that during metarhodopsin II formation there is an increase in volume of the opsin protein (Lamola et al., 1974). It could be hypothesised that TMAO may have a role in photoactivation when it occurs at depth.
1.3 Visual Pigments

Visual pigments consist of a protein, opsin, with a chromophore embedded in a pocket within it. The rhodopsin chromophore is retinal (vitamin A$_1$), and 3-dehydroretinal (vitamin A$_2$) is the corresponding molecule for porphyropsin. Rhodopsins and porphyropsins are found in fish, amphibians and reptiles, whereas birds and mammals only synthesise rhodopsins. As explained previously, the extra double bond in the terminal ring of 3-dehydroretinal causes the maximal absorbance of porphyropsin to be long-wave shifted. A protonated retinylidene Schiff's base alone in solution absorbs maximally at 440 nm. An absorbance shift from this value either to longer or shorter wavelengths is due to interactions with the attached opsin molecule, and is known as the 'opsin shift' (Kito et al., 1968; Applebury and Hargrave, 1986). Rhodopsin can be found in both rod and cone photoreceptors. However, different rhodopsins can be sensitive to different areas of the electromagnetic spectrum. This specific spectral sensitivity is determined by direct and indirect interactions between the chromophore and one of five classes of visual opsins (Sakmar and Fahmy, 1996).

Rod visual pigments are most sensitive to wavelengths of light around 500 nm, but this varies in different vertebrates, particularly in teleosts. The $\lambda_{\text{max}}$ (wavelength of maximum sensitivity) values of fish rhodopsins are known to range from 467 nm (Bathylagus microphthalmus) to 526 nm (Aristostomias scintillans), with corresponding porphyropsins at 502 nm and 551 nm (Lythgoe, 1972; O’Day and Fernandez, 1974).

Cone opsins can be optimally sensitive to different regions of the spectrum. In fish, birds and reptiles, the four cone classes SWS1 (UV/violet sensitive), SWS2 (blue
sensitive), RH2 (green sensitive) and MWS/LWS (green/red sensitive) have been identified (Okano et al., 1992; Kawamura and Yokoyama, 1993, 1995, 1996; Johnson et al., 1993; Histomi et al., 1996). Mammals have lost the SWS2 and RH2 cone classes and are therefore dichromatic. The exception of mammalian trichromacy found in primates and humans is discussed later.

1.3.1 Opsin Protein Structure

Opsins are members of the G protein-coupled receptor family of proteins. This protein family is involved in signal transmission across membranes in a wide range of systems. Different members are activated by different ligands, including acetylcholine, dopamine and other peptides. In the case of opsin however, the ligand is usually retinal, but activation requires a photon of light. The protein is approximately 350 amino acids in length, encoding seven transmembrane α-helices linked by extracellular loops (figure 1.11). These form a transmembrane protein that sits in the lamellae of photoreceptor cells. Each α-helix is composed of 20-32 amino acids, with the central 16-18 residues embedded in the cell plasma membrane (Baldwin, 1993; Palczewski et al., 2000). The crystal structure of bovine rhodopsin has been elucidated (Palczewski et al., 2000) and has proven particularly useful in identifying amino acids, which lie close to the retinal binding pocket and may interact with the chromophore (figure 1.12). The helices are arranged sequentially in a clockwise fashion. The N-terminus of the protein is on the extracellular side of the membrane and the C-terminus is intracellularly located (Applebury and Hargrave, 1986). When rod opsin protein sequences are compared variability is found in the length of the six inter-helical loops (Baldwin et al., 1997), with intracellular loops generally shorter than the extracellular loops. The seven transmembrane domains form a pocket within which the chromophore sits. The
chromophore binds to the opsin via a Schiff's base linkage with lysine at position 296 found in helix VII (numbering as in bovine rod opsin). The intracellular side of rhodopsin is involved in interactions with transducin. In particular, regions at the ends of helices III, V and VI, and intracellular loops III-IV and V-VI are important (Baldwin, 1994).

Figure 1.11. Schematic depiction of the opsin protein. The 7 transmembrane helices are indicated in roman numerals. Some of the essential amino acids that are conserved in all vertebrate opsins to date are shown in colour (adapted from Baldwin, 1993).
Figure 1.12. (A) Ribbon drawing of rhodopsin based on the crystal structure parallel to the plane of the membrane. (B) Schematic of the side chains surrounding the chromophore 11-cis retinal. (C) Schematic of the residues within close proximity of the retinal molecule. Diagrams from Palczewski et al, 2000.
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There are several conserved residues, which are vital for the protein structure, these include: the Schiff's base (Lys 296) (Dratz and Hargrave, 1983), the counterion to the Schiff's base (Glu 113) (Sakmar et al., 1989; Zhukovsky and Oprian, 1989), the cysteine residues at positions 110 and 187 that form a disulphide bond (Karnik and Khorana, 1990), the cysteine residues at positions 322 and 323 for palmitoylation (Ovachinnikov et al., 1988) and the glutamate residue at position 134, which stabilizes the inactive opsin (Cohen et al., 1993). In the resting state the Schiff's base is protonated and stabilised by the negatively charged glutamate at 113 (Nathans, 1990). Photoactivation of the visual pigment and the conformational change it creates leads to deprotonation of the Schiff's base and the formation of metarhodopsin I, which in turn isomerises to metarhodopsin II (Zyvaga et al., 1994; Shieh et al., 1997). Weitz and Nathans have demonstrated that the three residues Asp83, Glu134 and Arg135 of bovine rod opsin act to stabilize metarhodopsin I (Weitz and Nathans, 1993), and help to activate transducin more efficiently (Sakmar et al., 1989; Nakayama and Khorana, 1991). As the response kinetics of the rod and cone phototransduction cascades differ in the activation and decaying steps it is interesting to determine if the visual pigment itself is in part responsible. A common property of cone opsins is the presence of many basic residues, whereas in rods there are more acidic amino acids (Okano et al., 1992). A mutagenesis study on the chicken rod and green cone opsins showed that site 122 is responsible for the efficiency of activating transducin (Imai et al., 1997). In rod opsin there is a glutamate residue at position 122. When this is substituted with a glutamine or isoleucine residue as found in the cone opsins, the regeneration rate was much faster and was much more typical of a cone response than of a rod. The amino acid at site 122 may therefore play a vital role in determining the kinetics of the visual pigment response to light.
1.3.2 Opsin Genes

In 1983 the first rod opsin gene was identified when Nathans and Hogness published the cDNA sequence of bovine rhodopsin (Nathans and Hogness, 1983). Subsequently, Nathans and colleagues identified the human long-wave, middle-wave and short-wave sensitive cone opsins (Nathans, et al., 1986). With this starting information it has been relatively easy to identify a multitude of opsins from a wide range of species. The $\lambda_{\text{max}}$ of some of these opsins has been elucidated by MSP. With these two pieces of information, interesting comparisons can be made between species, giving clues to evolutionary trends.

Opsin Classes

Evolution of wide-ranging spectral sensitivity in the animal kingdom necessitated the generation of five separate classes of retinal visual pigments. These classes are designated RH1 (rod opsin), SWS1 (UV/violet cone opsin), SWS2 (blue cone opsin), RH2 (green cone opsin) and MWS/LWS (red cone opsin) (Yokoyama, 2000). There are also a number of non-retinal opsins. Yokoyama suggests that there have been five gene duplication events, which have given rise to retinal and non-retinal opsins. After each duplication event one of the genes accumulated nucleotide substitutions while the other retained its original structure and function. In time the separate opsin classes were formed. During evolution visual pigments within the same class from different species have diverged, creating differences in spectral sensitivity. The following section looks at the five opsin classes and at some interesting examples of spectral tuning.
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RH1 Cluster - The Rod Opsins

The $\lambda_{\text{max}}$ value of rod opsin pigments usually lies between 490 and 510nm. The vertebrate rod opsins share about 85% identity. The $\lambda_{\text{max}}$ of rod opsins do not vary greatly in the majority of species (exceptions discussed later).

RH2 Cluster – The MWS Cone Opsins

The MWS cone opsin genes found in birds, reptiles and fish share much higher identity with rod opsin than with any of the other cone classes. Even so the protein is found in cone-like photoreceptors and not in rod cells. RH2 opsins are not found in mammals, which suggests that the gene was lost at some point in mammalian evolution (Jacobs, 1993). The $\lambda_{\text{max}}$ varies quite widely in this group from 467 nm in the gecko, *Gekko gekko* (Crescitelli, 1977) to 516 nm in the cottoid fish, *Limnocottus eurystomus* (Bowmaker *et al.*, 1994). In fact this range can be increased further because many species utilise the chromophore 3-dehydroretinal to form long-wave shifted porphyropsin complexes. In some fish, distinct RH2 genes are present in the genome, e.g. goldfish (Johnson *et al.*, 1993) and zebrafish (Vithelic *et al.*, 1999). In the goldfish there are two RH2 genes, which form proteins that when regenerated *in vitro* with 11-cis retinal have $\lambda_{\text{max}}$ at 505 and 511 nm (Johnson *et al.*, 1993). The difference in $\lambda_{\text{max}}$ is likely due to an amino acid substitution at site 217, with alanine present in the 511 nm pigment and threonine in the more short-wave 505 nm pigment (Bowmaker, 1995). In the zebrafish four separate RH2 genes have been isolated. These pigments have been regenerated *in vitro* with with $\lambda_{\text{max}}$ values at 467 nm (RH2-1), 476 nm (RH2-2), 488 nm (RH2-3) and 505 nm (RH2-4). RT-PCR has demonstrated that RH2-2 retinal expression is significantly higher than the other three RH2 genes (Chinen *et al.*, 2003).
LWS/MWS Cluster – Red Cone Opsin

This cluster of visual pigments encompasses $\lambda_{\text{max}}$ values ranging for $A_1$ pigments from 508 nm in the mouse, Mus musculus (Jacobs et al., 1991), to 570 nm in the chicken, Gallus gallus (Okano et al., 1989). In some species more than one visual pigment from this group are expressed, as with the RH2 gene. An example is the cavefish, Astyanax fasciatus. This species has three LWS/MWS genes in its genome, two being green sensitive, and the third more red sensitive (Yokoyama and Yokoyama, 1990). This parallels the situation found in Old World monkeys, which is discussed later.

The LWS/MWS class of opsins differ from all the other opsins with respect to anion sensitivity (Kleinschmidt and Harosi, 1992). The long-wave tuning of the visual pigment when bound to its chromophore may involve the binding of a chloride ion near to the protonated Schiff’s base (Kleinschmidt and Harosi, 1992). For example, when iodopsin is in Cl⁻ depleted medium, its $\lambda_{\text{max}}$ is short-wave shifted to 520 nm. When this pigment is measured in a typical medium containing Cl⁻ ions, the $\lambda_{\text{max}}$ is at 562 nm (Knowles, 1976). Chloride ions are necessary to shift the sensitivity into the longer wavelengths. Two residues, histidine at 197 and lysine at 200, are conserved in all LWS opsins and mutagenesis work has demonstrated they are necessary for Cl⁻ binding (Wang et al., 1993). His197 is on the extracellular side of the membrane where Cl⁻ concentration is high and not limited in vivo, ensuring that the long-wave shift of the LWS/MWS pigments is maintained. His197 and Lys200 are absent in all rod and short-wave opsins identified to date. This suggests that the evolutionary branch of LWS pigments was formed when an ancestral opsin acquired the ability to bind chloride ions, by the acquisition of a chloride binding site (Wang et al., 1993).
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**SWS1 Cluster – UV/Violet Cone Opsin**

The $\lambda_{\text{max}}$ of these pigments ranges from 360 nm as found in mouse (Jacobs et al., 1991) and zebrafinch (Yokoyama et al., 1997) to 455 nm in the cow (Jacobs et al., 1998). Representatives from this cluster are found in all vertebrate classes, including mammals. UV vision is possible in many birds (e.g. pigeon $\lambda_{\text{max}}$ 359 nm (Yokoyama et al., 1998)), reptiles (e.g. American chameleon $\lambda_{\text{max}}$ 358 nm (Kawamura and Yokoyama, 1998)), fish (e.g. goldfish $\lambda_{\text{max}}$ 358 nm (Yokoyama et al., 1998)) and small mammalian species (e.g. mouse $\lambda_{\text{max}}$ 358 nm (Jacobs et al., 1991)). MSP work on the UV pigments has shown that sensitivity may reach to wavelengths as short as 280 nm in some fish (Lythgoe and Partridge, 1989). Vision in the very short-wave end of the spectrum is reduced in humans probably because of the yellowing of the lens and absorption by the pre-retinal media (Wyszecki and Stiles, 1967). Experiments on the mouse have demonstrated that the SWS1 opsin is the first to be expressed in the retina during embryogenesis (Szél et al., 1993). It may be that there is a default pathway for immature cones to develop as short-wave sensitive. Thyroid hormone receptor $\beta_2$ (TR$\beta_2$) has been linked to the induction of LWS/MWS opsin expression (Ng et al., 2001).

The $\lambda_{\text{max}}$ of the UV SWS1 pigments is so short-wave shifted because the Schiff's base is deprotonated during the dark state (Yokoyama and Shi, 2000; Fasick et al., 2002) becoming protonated during the photobleaching cascade, a situation not found in the other opsin classes (Vought et al., 1999; Dukkipati et al., 2002).

**SWS2 Cluster – Blue Cone Opsin**

This group of pigments cover a wide spectral range from 416 nm in the zebrafish, *Danio rerio* (Chinen et al., 2003), to 474 nm in the Japanese common newt, *Cynops*
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This group of pigments cover a wide spectral range from 416 nm in the zebrafish, *Danio rerio* (Chinen et al., 2003), to 474 nm in the Japanese common newt, *Cynops pyrrhogaster* (Takahashi and Ebrey, 2003). The gene duplication event responsible for this cluster occurred before the *mammalia* lineage split, but is not present in modern day mammals. Short-wave sensitivity in mammals therefore relies on the SWS1 class only. Many species of bird, reptile and fish utilise both SWS1 and SWS2 opsins for greater chromatic sensitivity in this region of the spectrum.

**Non-Visual Opsins**

Non-visual opsins have been identified in a variety of vertebrate species, including fish. The role of these opsins is likely to be linked to the generation and entrainment of circadian rhythms, camouflage and colour change and seasonal changes in photoperiod. Here the focus is limited to non-visual opsin genes identified in teleosts. Vertebrate ancient (VA) opsin has been isolated from salmon and zebrafish, and has been localised to a subset of horizontal and amacrine cells in the salmon retina (Soni and Foster, 1997; Soni et al., 1998). A longer isoform of VA opsin, termed vertebrate ancient-long (VAL) opsin has been isolated from zebrafish and localised to deep brain and retinal horizontal cells (Kojima et al., 2000). Both VA and VAL opsins have been expressed in human embryonic kidney cells and regenerated with 11-cis-retinal. VA opsin regenerated with a $\lambda_{\text{max}}$ of 451 nm (Soni et al., 1998), while VAL opsin gave a $\lambda_{\text{max}}$ close to 500 nm (Kojima et al., 2000). Melanopsin has been identified in zebrafish (Bellingham et al., 2002) and two isoforms of this photopigment has since been isolated from Atlantic cod, *Gadus morhua*, and their expression localised to the inner retina, skin and the suprachiasmatic nucleus and habenula centres of the brain (Drivenes et al., 2003). Exorhodopsin or extra-retinal rod-like opsin has been isolated from the pineal gland of
zebrafish and salmon and from the brain of puffer fish (Mano, *et al*., 1999; Philp, *et al*., 2000). P opsin (pineal gland-specific opsin) has been cloned and characterised from the marine lamprey, *Petromyzon marinus* (Yokoyama and Zhang, 1997). Interestingly, the lamprey P opsin is 90 amino acids longer due to an extended fifth exon, than the orthologous genes of chicken, pigeon and American chameleon. Parapinopsin has been isolated from catfish with *in situ* hybridisation localising expression to parapinealocytes and pineal photoreceptors (Blackshaw and Snyder, 1997). Parapinopsin is the most divergent member of the vertebrate opsin family.

There has been much debate over which opsin class represents the ancestral pigment. Work by Okano *et al.* (1992) on the phylogenetic relationship of the chicken, human and fish opsins, identified the ancestral pigment as being cone-like. This pigment diverged to form the different cone classes including the RH2 green sensitive opsins. A duplication of this class then led to the RH1 rod opsins. As the vertebrates diverged, opsin classes were lost. Fish, reptiles and birds still contain all five visual opsin classes, though all are not expressed in every species. An extreme case is the rudd, *Scardinius erythrophthalmus*, which expresses 10 different visual pigments. This is due to the expression of five classes of opsin, all forming pigments with both chromophores vitamin A1 and A2 (Loew and Dartnall, 1976; Whitmore and Bowmaker, 1989). In placental mammals the SWS2 and RH2 cones have been lost (Jacobs, 1993). This means most mammals have dichromatic colour vision, far less ‘colour’ sensitive than the tetrachromatic vision of some lower vertebrates. For marsupials there is evidence for trichromatic vision based on long-wave, middle-wave and UV sensitivity due to recent work on the honey possum, *Tarsipes rostratus*, and the fat-tailed dunnart, *Sminthopsis crassicaudata* (Arrese *et al*., 2002).
Comparisons between the opsin classes show many similarities and a few important differences. The gene lengths can vary considerably between species, mostly due to differences in intron length. Teleost fish are unique as they contain intronless rod opsin genes, which are about 1 kb in length (Fitzgibbon, 1995). The mechanism accounting for this loss is not known, though it has been hypothesised that it may be due to a retrotransposition event (Fitzgibbon et al., 1995; Bellingham et al., 2003). The extraretinal rod-like opsin gene (errlo) of teleosts contains introns. It has been suggested that errlo is the original rod opsin and that the ocular rod opsin gene (rho) is the result of an errlo RNA transcript becoming incorporated into the genome. Retrotransposition events are not uncommon but most acquire mutations and are transcriptionally silent. If rho is the product of a retrotransposon event then it must have been immediately advantageous. It may be that for genes transcribed in high numbers as found with opsin, it is metabolically beneficial to lose the introns (Bellingham et al., 2003). Interestingly if this hypothesis is correct, the insertion event must have occurred close to a rod specific promoter. The probability of this occurring by chance must be very low. An alternative possibility is an homologous recombination event between a processed cDNA transcript and the Rh gene (Fitzgibbon et al., 1995). This would preserve the position of the intron-less Rh with regards to a specific promoter. The mechanism has been reported previously for the NADH dehydrogenase subunit 4 gene of lettuce, Lactuca sativa (Geiss et al., 1994). With the exception of teleost rod opsin and the LWS/MWS opsin class all visual pigment genes contain five exons (Nathans and Hogness., 1983; Nathans et al., 1986a; Yokoyama and Yokoyama, 1993). The LWS/MWS opsin genes contain an extra exon (Nathans et al., 1986a; Kawamura and Yokoyama, 1993) at the 3' end of the gene. This was demonstrated by
comparative studies on the opsin genes of the pigeon. Here it was shown that introns 1-4 of the RH1, RH2, SWS1 and SWS2 genes interrupt their coding sequences at precisely the same positions as introns 2-5 in the LWS/MWS gene (Kawamura et al., 1999). The second intron of the pigeon P opsin gene is displaced 15 nucleotides towards the 3' end of the gene (Max et al., 1995; Kawamura and Yokoyama, 1996). To date there is only one example of two isoforms of an opsin gene due to alternative splicing. This is found in the previously mentioned vertebrate ancient opsin of zebrafish (Kojima et al., 2000). The VAL-opsin isoform has a cytoplasmic tail 67 amino acids longer than the second isoform, VA-opsin. The former is typical in length though both can form a functional protein as demonstrated by in vitro regeneration with 11-cis retinal (Soni et al., 1998; Kojima et al., 2000).

Recently Ma et al. (2001) demonstrated an exceptional situation in the tiger salamander. Immunohistochemistry, spectrophotometry and RT-PCR were all able to show that the same SWS2 pigment is expressed in blue sensitive cones, and also in morphologically distinct rod photoreceptors. It was demonstrated that the SWS2 pigment can bind efficiently to both cone and rod α-transducin and elicit a photosensitive response. The response kinetics for both cell types is cone-like which suggests that it is the visual pigment rather than the cellular machinery that determines the kinetics of the rod and cone response (Ma et al., 2001).
1.4 Adaptive Evolution and Spectral Tuning of Visual Pigments

Visible light only composes a small section of the electromagnetic spectrum (figure 1.13). Visually detectable light has wavelengths ranging from <400 nm to close to 700 nm. Light with a shorter wavelength is in the ultraviolet region and is high in energy. Light that has a longer wavelength is in the infrared range of the spectrum and contains less energy.

In nature colour has multiple roles. Camouflage is important for prey and predator alike. Mimicry allows a defenceless animal to appear poisonous or dangerous. This is a technique often used by invertebrates. Colourful sexual displays are common in vertebrates, especially in birds and fish. With colour playing such vital roles it is not surprising that over time different species have evolved spectral sensitivities to suit their own particular requirements. The diversity of visual pigments is not closely correlated...
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with phylogenetic relationships (Crescitelli, 1972; Lythgoe, 1972; Munz and McFarland, 1973) but more to light environment and behaviour.

1.4.1 Colour Vision in Mammals

The colour vision of mammals is interesting because it includes gene deletion, opsin polymorphism and examples of relatively recent evolution events. The majority of mammals are dichromats, containing a LWS/MWS pigment (usually 530-570 nm) and a violet sensitive pigment (420–440 nm) as in primates (Bowmaker et al., 1991) or an ultraviolet pigment (≈365 nm) as in rats and mice (Jacobs et al., 1991). The reason for the loss of the RH2 and SWS2 cone classes is thought to be the result of a nocturnal period of evolution. During this time the green (480-520 nm) and blue (440-460 nm) pigments were lost. Mammalian eyes are sensitive to a wide spectral range though chromatic vision is limited.

In Old World primates (including humans), trichromacy has been restored by a duplication event of the LWS/MWS pigment gene found on the X chromosome, maybe as recently as 30-40 million years ago (Nathans et al., 1986a,b; Yokoyama and Yokoyama, 1990). A slight deviation of the amino acid sequence has caused one form of the gene to encode a middle-wave sensitive opsin (Ala180, Phe277, Ala285), while the other codes for a long-wave sensitive pigment (Ser180, Phe277, Thr285) (Nathans et al., 1986a; Neitz et al., 1991; Ibbotson et al., 1992; Deeb et al., 1994; Dulai et al., 1994; Yokoyama and Yokoyama, 1990). The work of Yokoyama and Yokoyama (1990) proposes that the LWS opsin has arisen via a duplication of an original MWS pigment. This differs from the inferred ancestral primate opsin, which is believed to be long-wave
long-wave sensitive (Nei et al., 1997). Analysis suggests that the ancestor contained the three critical amino acids for long-wave tuning; Ala180, Tyr277 and Thr285. If this is true then the ancestor of humans and Old World monkeys had the LWS gene and the duplication event produced the MWS pigment (Nei et al., 1997). Interestingly the same three substitutions are found in the MWS and LWS genes of the cave-fish, *Astyanax fasciatus*, and are now a classic example of convergent evolution (Yokoyama and Yokoyama, 1990). The only New World primate with two LWS/MWS cone classes is the howler monkey, the duplication event occurring independently of that in the Old World monkeys maybe as recently as 13 mya (Jacobs et al., 1996; Hunt et al., 1998).

Trichromacy is possible for some Platyrrhine species as the MWS/LWS gene is polymorphic, encoding three spectrally distinct pigments (Jacobs, 1984; Bowmaker et al., 1985; Travis et al., 1988; Neitz et al., 1991; Williams et al., 1992; Hunt et al., 1993). Males only possess one of the three allelic variations, females however have two X chromosomes and can therefore possess one or two of the three. Hence a female, which expresses two distinct forms of the MWS/LWS gene and the SWS1 gene, will be trichromatic. Males are all dichromatic as only a single MWS/LWS allele is expressed with the SWS1 gene. It is suggested that trichromatic vision is an advantage for the Old World and individual platyrrhine monkeys because it enables easier detection of ripe fruit or young leaves against a mature foliage background (Polyak, 1957; Mollon, 1989; Sumner & Mollon, 2000a, b; Dominy and Lucas, 2001).

The mouse retina contains SWS1 and MWS/LWS opsins as typical in other dichromatic mammals but with an unusual expression pattern. In the majority of cones the SWS1 and MWS/LWS opsins are co-expressed. The level of SWS1 opsin remains relatively constant for all cones but the level of MWS/LWS expression varies from dorsal (high)
to ventral (low) retina (Applebury et al., 2000). Overall the total mRNA levels of SWS1 opsin exceed those of MWS/LWS opsin approximately 3-fold. This is unusual as for most mammals MWS/LWS cones far outnumber SWS1 cones (Mollon and Bowmaker, 1992; Szél et al., 1996, 2000). In fact murine cone receptors have been shown to be more sensitive to UV light than green when the relative sensitivity of the two systems are measured (Jacobs et al., 1991; Lyubarsky et al., 1999). The high expression of SWS1 opsin in the murine retina may be linked to an increased importance for ultraviolet sensitivity. Co-expression has also been demonstrated in the rabbit (Rölich et al., 1994) and guinea pig retina (Parry and Bowmaker, 2002).

An interesting observation has recently been made concerning marine mammals. The blue cone pigment has been lost in whales, dolphins and seals (Fasick et al., 1998; Peichl et al., 2001). In all species studied, only LWS/MWS and rod opsin expression was detected. SWS1 pseudogenes have been identified in several species of baleen (mysticete) and toothed (odontocete) cetaceans confirming loss of SWS1 function (Levenson and Dizon, 2003). Closely related terrestrial mammals have also been analysed and the short wave pigment is present. It seems curious that an animal living in a mostly blue environment should have poor vision in this part of the spectrum. Peichl and co-workers suggest that the reason behind the blue pigment loss is an adaptation to coastal waters early in evolution. Later these mammals began to inhabit deeper waters but the lost blue pigment could not be regained (Peichl et al., 2001). The rod opsins seem to have partly compensated for this loss. In deep foraging marine mammals, the rod opsins are blue shifted, with the deepest foraging animals possessing the most short wave shifted pigments (Lythgoe, 1972; Fasick et al., 1998; Fasick and Robinson, 2000).
Loss of the SWS1 gene is not limited to marine mammals but has also occurred in some nocturnal terrestrial species. This was first reported in two nocturnal monkeys, the owl monkey, *Aotus trivirgatus*, and the greater bushbaby, *Galago garnetti* (Wikler and Rakic, 1990; Jacobs *et al.*, 1993). Both species still contain the SWS1 gene in the genome but it is non-functional due to acquired mutations (Jacobs *et al.*, 1993, 1996b). Since then absence of short-wave sensitivity has been identified in a number of nocturnal rodents and carnivores (Jacobs and Deegan 1992; Szél *et al.*, 1994, 1996; Calderone and Jacobs, 1995; Peichl and Moutairou, 1998).

### 1.4.2 Colour Vision and Visual Pigments of Fish

When considering the evolution of the visual system, fish provide vital clues. Relatives of ancestral fish may show us the origins of spectral tuning. It is from these ancient opsins that the adapted visual pigments of today are based.

![Hierarchy of Higher Categories of Fishes](image)

**Figure 1.14.** Phylogenetic tree illustrating the five classes of extant fish: myxini, cephalaspidomorphi, chondrichthyes, sarcopterygii and actinopterygii. Lampreys are members of the jawless cephalaspidomorphu. Coelacanths are members of the sarcopterygii as are all tetrapods. All other teleost species discussed are from the actinopterygii class. Redrawn from Nelson, 1994.
Figure 1.14 shows the classes that contain all known jawed (gnathostoma) and jawless (agnatha) fish species. The cyclostomes (lampreys and hagfish) have arisen directly from the ancient agnatha. These are jawless, fish-like vertebrates that separated from the jawed vertebrates during the Cambrian period and lampreys are members of the cephalaspidomorphi class. The visual pigments of lampreys are interesting as they are difficult to class as rods or cones. Examination of the lamprey retina in two northern hemisphere species (Petromyzon and Lampetra) shows two types of photoreceptor, one with a long outer segment and the other with a short outer segment. Also, both contain discs formed from infoldings of the plasma membrane, as seen in modern day cone receptors (Govardovskii and Lychakov, 1984; Negishi et al., 1987; Tamotsu et al., 1994). Both receptors contain rhodopsin/porphyropsin mixtures, with shorter outer segments most sensitive at 517 and 525 nm, and longer segments long-wave shifted with $\lambda_{\text{max}}$ at 550 and 600 nm (Govardskii and Lychakov, 1984; Hárosi and Kleinschmidt, 1993). Physiological experiments show these photoreceptors to have basic cone properties, such as the pigment reacting to hydroxylamine and being anion sensitive (Govardskii and Lychakov, 1984). More recent work has shown an extra class of cone-like receptors in the retina of the southern hemisphere lamprey, Geotria australis (Collin et al., 1999). In this species there are two morphologically distinct cone cells, with MSP identifying their $\lambda_{\text{max}}$ at approximately 610-615 nm and 515 nm, as well as a rod-like receptor at 500-506 nm (Collin et al., 2003a). A rod-like opsin sequence has been isolated from the two northern hemisphere lamprey species, and is believed to be the pigment expressed in the shorter more rod-like receptors. Recent work on G. australis, has identified SWS1, SWS2 and LWS/MWS genes, as well as two rod-like genes that are equally related to RH1 and RH2 genes of jawed vertebrates (Collin et al., 2003b). The data suggests that the common ancestor of jawed and jawless
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fish possessed three cone classes and a single RH gene, which later evolved in to the RH1 and RH2 genes of jawed vertebrates. The molecular events giving rise to the SWS1, SWS2 and MWS/LWS genes must have taken place prior to the separation of jawed and jawless fish, at least 600 million years ago (mya). It seems likely that the ancestral visual system was cone based, and only later when the RH1 gene had evolved was full scotopic vision possible (Nathans et al., 1986a; Collin et al., 2003b).

It is interesting to compare the results for the early teleosts to the molecular work on the coelacanth, Latimeria chalumnae. Once thought extinct, two coelacanths were found in the waters off the Comoro Islands in 1972 (Dartnall, 1972) and more recently in Indonesia (Holder et al., 1999) and again in the Comoros archipelago in the western Indian Ocean (Fricke and Hissmann, 1990). The coelacanth is a member of the sarcopterygii class (figure 1.14) as are tetrapods and was thought to be the missing link between water and land living species because of its fleshy, manoeuvrable fins. It is now believed that it was probably not the coelacanth that took the first step to land but more likely a close relative. The coelacanth lives at a depth of about 200 m, receiving a narrow band of light at about 480 nm. Molecular genetic work has shown that two opsins are expressed in the retina: an RH1 pigment (\(\lambda_{\text{max}}\) 485 nm) and an RH2 pigment (\(\lambda_{\text{max}}\) 478 nm) (Dartnall, 1972; Yokoyama et al., 1999). These sensitivities are both short-wave shifted approximately 20 nm from typical teleost rod opsins and correspond to the narrow range of light found in the coelacanth’s natural environment. The tuning sites for the RH1 and RH2 pigments are proposed as Glu122Gln/Ser292Ala and Glu122Gln/Met207Lys respectively (Yokoyama and Tada, 2000). Presumably, the SWS1, SWS2 and MWS/LWS cone classes have been lost. Yokoyama hypothesises that this is due to an adaptation to the deep-sea environment about 200 million years ago.
(Yokoyama and Tada, 2000). The early coelacanths in the Carboniferous period lived in rivers and swamps (Maisey, 1996) and then for reasons not fully understood the species migrated to deeper depths. In this different light environment the SWS and LWS cones may have become defunct and lost. It is interesting that a green pigment was retained and shifted rather than maintaining a short wave opsin.

Bayliss et al. (1936) made the hypothesis that the visual pigments of deep-sea fish should be shifted towards a narrow band in the blue end of the spectrum to correlate with the down welling light. It was also predicted that these fish would have evolved rod only retinæ, as colour vision would not be possible in such low levels of light intensity. In contrast, fish living in shallower water will be in contact with high light intensity of broad irradiance, and therefore chromatic sensitivity would be highly advantageous. It is now known that these hypotheses were mostly correct. In 1973, Munz and McFarland examined a large number of tropical marine fish and categorized them into three sample groups; surface (0-5 m), intermediate (1-30 m) and deep-living species (20-200 m). The \( \lambda_{\text{max}} \) values for the rod opsins were determined by retinal extract and shown to correlate with depth (Munz and McFarland, 1973, see figure 1.15). The pigments became more short-wave sensitive when fish inhabited deeper waters. This correlation was not affected by the activity pattern of the fish, with the samples taken from diurnal, crepuscular and nocturnal species. A similar pattern has also been demonstrated in the cichlid flock of Lake Malawi (Muntz, 1976).
Figure 1.15. Frequency distribution of the visual pigments of tropical marine fishes. The species are grouped by depth of habitat, each square representing one species. Rhodopsin based pigments are shown in blue, porphyropsin in pink. Redrawn from Munz and McFarland, 1973.

Many fish living at depth possess pure rod retinae (Munk, 1966; Bowmaker et al., 1994). In deep-water species the $\lambda_{\text{max}}$ of the rod photoreceptors show a correlation with the maximum transmission qualities of the water almost perfectly (figure 1.16) (Lythgoe, 1972; Douglas et al., 1998b). Out of 200 deep-sea fish examined, 176 have retinae containing a single rhodopsin pigment (Douglas et al., 2003). The $\lambda_{\text{max}}$ of these pigments fall within a narrow range of wavelengths, between 475 and 490 nm, with
very few exceptions. This matches the attenuated down-welling sunlight and chemically produced bioluminescence almost exactly. However, there are 24 deep-sea species that are known to have more than a single visual pigment, with the majority possessing at least one pigment with maximum sensitivity outside of the typical 460-490 nm. Some of these species are listed in table 1.1. In most cases these multiple pigments are not due to rhodopsin/porphyropsin pairs, but to the expression of different opsin genes. (Douglas et al., 2003). In all species except Scopelarchus analis (see chapter 4) the pigments are found in separate photoreceptors (Partridge et al., 1988, 1989, 1992). Utilisation of multiple pigments can broaden sensitivity to a wider range of wavelengths.
<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>$\lambda_{max}$</th>
<th>Method</th>
<th>References</th>
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<tr>
<td>Alepocephalus bairdii</td>
<td>Alepocephalidae</td>
<td>467, 481 nm</td>
<td>Wholemount</td>
<td>Douglas et al., 1995</td>
</tr>
<tr>
<td>Diretmus argenteus</td>
<td>Diretmidae</td>
<td>484, 500 nm</td>
<td>Wholemount</td>
<td>Denton and Locket, 1989.</td>
</tr>
<tr>
<td>Howella sherboni</td>
<td>Howellidae</td>
<td>463, 492 nm</td>
<td>MSP</td>
<td>Partridge et al., 1989.</td>
</tr>
<tr>
<td>Lepidion eques</td>
<td>Moridae</td>
<td>476, 484 nm</td>
<td>Extract</td>
<td>Douglas and Partridge, 1997; Partridge et al., 1995.</td>
</tr>
<tr>
<td>Malacocephalus laevis</td>
<td>Macrouridae</td>
<td>477, 485 nm</td>
<td>MSP</td>
<td>Douglas et al., 1995; Partridge et al., 1988.</td>
</tr>
</tbody>
</table>

Table 1.1. List of selected deep-sea species that are believed to have multiple visual pigments. * represents a rhodopsin/porphyropsin pair.
Bioluminescence from different species can differ in long and short-wave composition (Herring, 1983; Widder et al., 1983), and sensitivity to these differences from downwelling sunlight may be advantageous. There may even be the possibility of basic chromatic sensitivity if the appropriate neuronal circuitry is available.

**Other Factors that may affect Opsin Function in the Deep-sea Environment**

Light may not be the only factor driving evolutionary adaptations in the deep-sea rod opsins. A key factor may be the necessity to reduce the signal to noise ratio. A noise response is due to spontaneous dark activation of the visual pigment, which mimics that of a typical photon response. If photons are few in number then a noisy receptor could lead to reduced acuity. If receptor noise shows a correlation with $\lambda_{\text{max}}$ then in a dim light environment it would be advantageous to have a $\lambda_{\text{max}}$ positioned where noise is kept to a minimum, though still produces a relatively high signal. Experiments and thermodynamic theory suggest that long-wave sensitive pigments are more prone to spontaneous dark noise (Barlow, 1957; Firsov and Govardovski, 1990), except between A1/A2 pigments, as porphyropsins are more prone to thermal activation (Koskelainen et al., 2000). The hypsochromatic deep-sea rod opsins may be tuned as much for noise reduction as for sensitivity (Douglas et al., 1998).

Another factor that may restrain the $\lambda_{\text{max}}$ of deep-sea opsins is pressure. The opsin protein has a complex tertiary structure, which may be sensitive to external pressure. It maybe that opsin can only function under high pressure when tuned to around 480 nm. MSP and retinal extraction experiments are performed at atmospheric pressure. It is possible that the $\lambda_{\text{max}}$ obtained in these experiments is not the true $\lambda_{\text{max}}$ when the protein is subjected to pressure.
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The correlation between the $\lambda_{\text{max}}$ of the rod opsins of deep-sea species and maximum transmission of water does not hold so perfectly for shallower living species (Lythgoe, 1972; Lythgoe, 1980). In fact in species higher up the water column the rod opsins are short-wave shifted from the maximum transmission of the water. It may be that a perfect correlation between available light and scotopic sensitivity is not necessary when there is greater background illumination. A more direct correlation can be seen in the cone pigments. Fish living at intermediate depths ($< 200$ m) in the green coastal waters of the English Channel and more tropical waters tend to rely on two cone pigments for photopic vision (Levine and MacNichol, 1979). The majority of species have single short-wave sensitive cones ($\lambda_{\text{max}}$ 440-470 nm) and double middle-wave sensitive cones ($\lambda_{\text{max}}$ 520-540 nm) (Levine and MacNichol, 1979; Lythgoe, 1984). The double cones can have the same pigment in both members, or two different pigments, though both are green sensitive (Lythgoe, 1984; Lythgoe et al., 1994). UV sensitive and true long-wave sensitive cones have been lost from most midwater species. Interestingly, snappers living in mangrove and estuarine waters have long-wave shifted double and single cones, whereas closely related snappers living on the outer edge of the Great Barrier Reef have maintained more blue-green sensitivity. For example the mangrove snapper, *Lutjanus johnii*, has $\lambda_{\text{max}}$ at 543 and 567 nm in double cones and a $\lambda_{\text{max}}$ of 458 nm in singles, while the reef snapper has doubles with $\lambda_{\text{max}}$ at 487 and 518 nm and singles at 430 nm (Lythgoe et al., 1994). Hence, there appears to be a correlation with the $\lambda_{\text{max}}$ found in double and single cones and the spectral composition of the water (Lythgoe et al., 1994). In the snappers of the Great Barrier Reef, the $\lambda_{\text{max}}$es of the double cones match the ambient light relatively well. The single cones do not show such a good match but a short-wave shift is still evident, though a single snapper species, *Lutjanus malabaricus*, contains a population of violet sensitive single cones.
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(\lambda_{\text{max}} 408 \text{ nm}). Short-wave shifts correlated to depth are also present in the middle-wave sensitive double cones and the short-wave sensitive single cones of the endemic cottoids of Lake Baikal (Bowmaker et al., 1994), though cone receptors are sparse in species inhabiting depths below 400 m (Bowmaker et al., 1994). The blue shift in sensitivity does not match the available light, as found in deep-sea fish, because the waters of Lake Baikal are yellowish, with maximum transmission at around 550 to 600 nm. This is discussed later with regards to spectral tuning of the cottoid rod opsins.

Midwater teleosts that live in freshwater environments often possess true long-wave sensitive cones with \( \lambda_{\text{max}} \) in the range 580-620 nm as well as middle-wave and short-wave sensitive cones (Bowmaker, 1995). There are also examples of ultraviolet-sensitive receptors with \( \lambda_{\text{max}} \) in the range 355-390 nm (Bowmaker et al., 1994). Overall, sensitivity is often long-wave shifted due to the production of porphyropsin pigments. This may be an adaptation to living in turbid waters, and/or because phytoplankton are at high densities. In a number of species it has been documented that the rhodopsin/porphyropsin composition can change during life. This is also discussed later.

Diurnal fish living near the water surface often have a high proportion of cones and in some species, such as the goldfish, all four cone classes have been found (Bowmaker et al., 1991; Neumeyer, 1992). An interesting feature of several tropical surface-living fish is the possession of a filter within the inner segments of the double cone photoreceptors. The filters or ellipsosomes, are composed of modified mitochondria, and reduce transmission of short-wave light to the outer segments (MacNichol et al., 1978). Short-wave filters are also often found in the lens and/or cornea of fish that inhabit ‘blue’ waters (Moreland and Lythgoe, 1968). The function of short-wave filters may be to
reduce the amount of scattered blue light reaching the visual pigments, which may enhancing acuity by reducing background noise. It was first thought that visual pigments were always tuned to match environmental light. It is in fact much more complicated with opsins being tuned for greatest sensitivity, which is often offset from the space light \( \lambda_{\text{max}} \).

1.4.3 Variation of Spectral Sensitivity

There are five mechanisms by which colour vision can be varied at the photoreceptor level. Chromophore and/or opsin substitution can occur at a particular stage of an organism’s lifecycle. Different species can show different opsin expression patterns. Within a species there can be opsin polymorphism, and visual pigments can evolve so that the \( \lambda_{\text{max}} \) is shifted to confer greater sensitivity in a different part of the visual spectrum.

1.4.3.1 Chromophore Substitution

This mechanism was mentioned previously when the structure of rhodopsin and porphyropsin was discussed. When the opsin moiety is bound to 3,4 dehydroretinal its sensitivity is long-wave shifted. The degree of shift depends on the original \( \lambda_{\text{max}} \) of the opsin. At long wavelengths, the shift could be as much as 60 nm (Dartnall and Lythgoe, 1965). Most animals use rhodopsin. Porphyropsin is generally found in the retinas of non-surface dwelling freshwater fish (Lythgoe, 1979). Chromophore substitution is the process of switching between these two chromophores. This occurs not infrequently in species that inhabit differing light environments during their lifecycle. Studies in the rudd, *Scardinius erythrophthalmus*, and salmon, *Salmo salar*, show that during winter
the fish move to deeper water and the proportion of porphyropsin in the retina increases (Dartnall et al., 1961; Muntz and Mouat, 1984). This suggests that greater sensitivity to longer wavelengths of light confers a visual advantage in winter. Another example is the Atlantic eel, *Anguilla anguilla*. Immature elvers live in freshwater and utilise porphyropsin. However, during adolescence the chromophore is switched to 11-cis retinal in preparation for migration to the sea (Carlisle and Denton, 1959). The switch in chromophore is controlled hormonally, linked to cues from seasonal changes such as photoperiod, temperature and diet. In fact the shift in $\lambda_{\text{max}}$, which correlates to the switch from porphyropsin to rhodopsin expression, can be induced experimentally with the administration of gonadotropin (Wood and Partridge, 1993). The distribution of the rhodopsin and porphyropsin pigments in the photoreceptors during a switch in expression is not fully understood. In the rainbow trout, the levels of rhodopsin were increased experimentally. MSP showed that porphyropsin and rhodopsin were distributed homogeneously throughout the rod outer segments, suggesting that the conversion of pigment is occurring independently of the basal replacement of discs (Liebman, 1972). However, when the rudd is maintained in continuous light for four weeks to induce rhodopsin production, porphyropsin is limited to the apical tips of the photoreceptors (Loew and Dartnall, 1976), which correlate to the basal replacement theory. However, the mechanism controlling porphyropsin/rhodopsin expression is more complicated than this as there can be intraretinal differences. In the rudd and two species of trout there is less porphyropsin expression in the dorsal than the ventral retina (Muntz and Northmore, 1971), requiring regional control of chromophore production. In *Aristostomias* there may even be individual photoreceptor control (Bowmaker et al., 1988). How this occurs is not fully understood, but work by Bridges and Yokoyama (1970) has demonstrated that control may not only be systemic. One eye of the rudd
was covered, to simulate continual darkness, while the uncovered eye was exposed to rhodopsin-favouring light conditions. After four weeks the two eyes showed substantial differences in rhodopsin/porphyropsin composition, suggesting that the rhodopsin expression was controlled locally in each eye. It seems likely however, that there is dual control, both local and systemic. It is accepted that all-trans retinol is isomerised to 11-cis retinal in the RPE, before being transported to the outer segments (McBee et al., 2001. Review). How the retina controls which chromophore is taken up by the outer segments needs to be addressed.

### 1.4.3.2 Opsin Substitution

In some organisms there is a switch in opsin gene expression, an example being the Atlantic eel, *Anguilla anguilla*. As mentioned previously the immature elver inhabits shallow freshwater, expressing a single rod opsin forming a rhodopsin with a $\lambda_{\text{max}}$ at 501 nm and a porphyropsin at 523 nm. Once sexually mature, the eel descends to the deeper waters of the Sargasso Sea to breed (Tesch, 1977). Prior to its descent, the eel undergoes a series of physiological changes to prepare for mating; the eyes increase in size, the gonads swell and the gut and swim bladder shrink (Bridges, 1972). In conjunction with the change in chromophore previously discussed, the original rod opsin expression is switched off and a more short-wave sensitive rod opsin gene is turned on. This pigment is only found as rhodopsin and has a $\lambda_{\text{max}}$ at 482 nm, similar to other deep-sea fish (Archer et al., 1996; Hope et al., 1998). A similar pattern of rod opsin expression has been identified in the Japanese eel, *Anguilla japonica* (Zhang et al., 2000). However, this does not only occur with rod opsins. In the Pollack, SWS2 opsin expression is switched from a 420 nm to a 460 nm sensitive protein at maturity (Shand et al., 1988). In the winter flounder, *Pseudopleuronectes americanus*, MSP has
identified different spectral absorbances in the pre- and postmetamorphic stages (Evans et al., 1993). In the juvenile only one type of cone photoreceptor was identified with a $\lambda_{\text{max}}$ of 519 nm. After metamorphosis the retina contains single and double cone as well as rod photoreceptors. The single cones are maximally sensitive at 457 nm, and the doubles either contain identical pigments sensitive at 531 nm, or two different halves sensitive at 531 and 547 nm. The rod photoreceptors have a $\lambda_{\text{max}}$ of 506 nm. It appears that there is complete reorganisation of the retinal visual pigments during metamorphosis. The juvenile pigment is not apparent in the adult retina, which suggests that its expression is turned off or at least severely down regulated.

Sometimes a morphologically distinct cone photoreceptor that is found during juvenile stages is lost at maturity rather than changing the opsin that is expressed within. A common occurrence in teleosts is when the corner single cones of a square mosaic disappear at maturity (Lyall, 1957). Before the age of 2 years the brown trout retina contains a population of SWS1 expressing cones, these are then lost, which matches the time course for loss of corner single cones (Bowmaker and Kunz, 1987). The same has been shown in the rainbow trout and has been linked to an increase in thyroxine hormone (Browman and Hawryshyn, 1992). Removal of the UV cones is probably via apoptosis (Kunz et al., 1994). It has been suggested that UV vision in juveniles may aid contrast detection of zooplankton (Bowmaker and Kunz, 1987; Leow et al., 1993). Once mature, the fish usually lives further down the water column and feeds on larger prey. UV vision at this stage would be less advantageous.
1.4.3.3 Differential Opsin Gene Expression

In some related species it has been demonstrated that a similar set of opsin genes are present and intact in the genome, though the pattern of expression differs between these species. The cichlid fish of Lake Malawi are well described examples of this phenomena (Carleton and Kocher, 2001). These fish inhabit two main ecological niches. There are the camouflaged sand dwellers that feed on other fish (e.g. *Dimidiochromis compressiceps*) and the brightly coloured rock dwellers that feed on plankton (e.g. *Metriaclima zebra*). In both species the genome contains five cone opsin genes, one from each of the LWS/MWS, RH2 and SWS1 classes, and two from the SWS2 (termed A and B) class. The sequences for the corresponding genes in both fish are highly similar, suggesting little divergence since speciation. The expression levels of the five genes were calculated by real-time RT-PCR and were found to differ considerably between the two species. The sand dweller expressed the SWS2A, RH2 and LWS/MWS genes, while the rock dweller expressed the SWS1, SWS2B and RH2 genes in its retina (Carleton and Kocher, 2001). The overall sensitivity of the rock dweller is considerably blue shifted when compared to the sand dweller. It was proposed that the brightly coloured *M. zebra* requires UV sensitivity to find conspecifics and to aid foraging (Carleton and Kocher, 2001).

1.4.3.4 Opsin Polymorphism

Opsin polymorphism has been noted within a variety of species, with the most well characterised case in the LWS/MWS cone opsins of New World monkeys. Opsin polymorphism has also been identified in the guppy, *Poecilia reticulata*, again in the LWS/MWS class of cones. The polymorphic cone opsin genes code for proteins with $\lambda_{\text{max}}$ values of 533 and 572 nm, with some cones optimally sensitive at 548 nm,
probably due to a mixture of the 533 and 572 nm rhodopsins (Archer et al., 1987). In this case the polymorphic genes do not appear to be sex linked, with there being no differences between males and females in the distribution of the three long-wave classes (Archer and Lythgoe, 1990). The advantage of these polymorphisms being retained in the population is not understood.

1.4.4 Molecular Basis of Spectral Tuning

Sequence analysis and site-directed mutagenesis experiments have demonstrated that amino acid changes at particular sites can shift the $\lambda_{\text{max}}$ to longer or shorter wavelengths. Twelve sites have been identified where substitutions can cause a shift of greater than 5 nm in $\lambda_{\text{max}}$. Eleven out of twelve of these are located in the transmembrane domains of the opsin protein. Evidence suggests that spectrally important substitutions in opsin occur in regions that are in close proximity to the chromophore, and usually involve the loss or gain of a polar residue. The proposed mechanism for a short-wave shift in $\lambda_{\text{max}}$ is the recruitment of a charged or polar amino acid residue near to the protonated Schiff’s base. This leads to preferential stabilization of the ground state of the chromophore over the excited state and therefore greater energy (shorter-wave light) is required to excite the rhodopsin molecule (Mathies and Stryer, 1976). Another proposed mechanism for spectral tuning is the addition of large residues, constricting the chromophore binding pocket (Shieh et al., 1997).

1.4.4.1 The Clustering Effect

Dartnall and Lythgoe (1965) surveyed the visual pigments of some 83 species of teleost. The $\lambda_{\text{max}}$ values of these species were not uniformly distributed but tended to cluster at
particular wavelengths separated by approximately 8 nm. In 1989, Partridge and colleagues looked at 52 species of deep-sea fish. These rhodopsins also tended to cluster at intervals of 6-10 nm, with the majority at around 477 and 483 nm. Clustering has also been noted in the cottoid fish of Lake Baikal (Bowmaker et al., 1994; Hunt et al., 1996) and in the cone pigments of mammals (Bowmaker, 1990; Jacobs, 1993).

Clustering Effect On Primate Colour Vision

In primates there appear to be five spectral clustal points for red-green opsins. In situ the \( \lambda_{\text{max}} \) values are located at about 535, 542, 550, 556 and 563 nm. Tuning depends primarily on amino acid substitutions at three main sites in the opsin transmembrane regions. These sites numbered as for bovine rod are:

- i. serine to alanine at position 164 in helix IV
- ii. phenylalanine to tyrosine at position 261 in helix VI
- iii. threonine to alanine at position 269 also in helix VI.

For the opsin to be tuned to a \( \lambda_{\text{max}} \) of 535 nm (the most short-wave sensitive), it must contain non-hydroxyl bearing amino acids at all three positions. When tuned to 563 nm (the most long-wave sensitive), all three sites contain an hydroxyl-bearing amino acid. The \( \lambda_{\text{max}} \) values in between these two extremes contain different combinations of hydroxyl and non-hydroxyl residues (Nathans et al., 1986a,b; Merbs & Nathans, 1992b; Asenjo et al., 1994). However, this is an over simplification, as there are five further potential tuning sites, which may cause small additional shifts in sensitivity (Asenjo et al., 1994).
The Cottoid Fish of Lake Baikal

As previously mentioned, the rod visual pigments of 11 freshwater cottoid fish caught in Lake Baikal cluster at specific $\lambda_{\text{max}}$ points: 516, 505, 495, 489 and 482 nm (Bowmaker et al., 1994; Hunt et al., 1996). The most blue shifted rod opsins have $\lambda_{\text{max}}$ values around 482 nm and are found in the abyssal species. The short-wave shift decreases as the water column is ascended, with littoral species sensitive at around 516 nm (table 1.2). This difference is not due to a change in chromophore as all species only synthesise rhodopsin pigments (Bowmaker et al., 1994). The rod opsins studied all shared considerable identity (>93%), and only twelve candidate tuning sites were found (figure 1.17). The criteria for a potential tuning site was tightened to include only non-conserved amino acids that face into the chromophore binding pocket, with a loss or gain of an hydroxyl group. This reduced the number of sites to three: at positions 83, 261 and 292.

Based on this Hunt et al. (1996) proposed the following tuning mechanisms to explain the blue-shift in the cottoid rod opsins:

- All those with a $\lambda_{\text{max}}$ of 505 nm or below have a tyrosine to phenylalanine substitution at position 261.
- The spectral shift from 505 nm to 495 nm is caused by a substitution of aspartate with asparagine at position 83.
- To shift from 505 nm to 489 nm requires an alanine to serine change at position 292.
<table>
<thead>
<tr>
<th>Spectral Class</th>
<th>Species</th>
<th>Family</th>
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<th>$\lambda_{\text{max}}$</th>
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<td>3</td>
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<td>Supra-abyssal (50-450 m)</td>
<td>495 nm</td>
</tr>
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<td>Supra-abyssal (100-500 m)</td>
<td>490 nm</td>
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<td>Abyssal (300-1000 m)</td>
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<td>Abyssal (100-1000 m)</td>
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</table>

Table 1.2. Rod visual pigment $\lambda_{\text{max}}$ correlated to depth distribution for 11 species of cottoid fish from Lake Baikal. The $\lambda_{\text{max}}$ values were obtained by MSP on fresh or fixed tissue (Bowmaker et al., 1994).
Figure 1.17. Three dimensional model of opsin indicating the 12 potential tuning sites identified in the Lake Baikal fish. Positions 83, 261, and 292 (in pink) are the most likely candidates. The model is derived from Baldwin (1993) and redrawn from Hunt et al., (1996).

The most blue-shifted opsin studied absorbs maximally at 482 nm. In this pigment all three substitutions are present (Hunt et al., 1996). Sites 261 and 292 have already been identified as tuning sites in primate LWS/MWS (Hunt et al., 1996) and SWS opsin genes (Lin et al., 1998) respectively. The substitutions Asp83Asn and Ala292Ser have since been identified as responsible for the short-wave shifted rod opsins of marine mammals, with an additional Ala299Ser substitution (Fasick and Robinson, 1998, 2000). The tuning sites in the opsins of mammals and cottoid fish are an example of convergent evolution. It seems likely that there are a limited number of sites in the opsin protein that are involved in spectral tuning.
1.4.5 Further Examples of Spectral Tuning

1.4.5.1 Spectral Tuning of SWS1 Pigments

The spectral tuning of the violet and ultraviolet sensitive visual pigments is fairly well characterised. In birds, the shift from violet sensitivity as found in chicken (\(\lambda_{\text{max}} = 418\) nm) to ultraviolet found in budgerigar (\(\lambda_{\text{max}} = 372\) nm) is nearly fully explained by the substitutions Cys90Ser, Thr93Val and Ala118Thr (Wilkie et al., 2000; Yokoyama et al., 2000) with the largest long-wave shift of 35 nm caused by the cysteine to serine change at position 90. This substitution is present in all avian UVS (ultraviolet sensitive) pigments characterised to date but is not present in the UVS pigments of other vertebrate classes.

The violet SWS1 pigments found in cow and pig differ from those of fish, reptiles, amphibia and rodents by possessing the substitution Phe86Tyr. Site directed mutagenesis experiments have shown that Phe86 short-wave shifts bovine SWS1 opsin into the ultraviolet (Cowing et al., 2002a; Fasick et al., 2002), though this is not the mechanism in primates and amphibian violet SWS1 pigments. Earlier work by Lin et al. (1998) compared the human SWS1 and rod pigments. Twelve candidate sites were identified and the simultaneous substitution of nine of these (Met86Leu, Gly90Ser, Ala117Gly, Glu122Leu, Ala124Thr, Trp265Tyr, Ala292Ser, Ala295Ser and Ala299Cys) in bovine rod opsin shifted the \(\lambda_{\text{max}}\) from 500 to 438 nm. This short-wave shift of 62 nm accounts for 80% of the difference in \(\lambda_{\text{max}}\) between the human rod and SWS1 pigments, though the reverse mutations in human SWS1 opsin at sites 90 and 292 did not induce the proposed long-wave shift (Fasick and Robinson, 2000). Site 292 may also explain the approximate 11 nm difference in \(\lambda_{\text{max}}\) between the SWS1 pigments...
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### 1.4.5.2 Spectral Tuning of SWS2 Pigments

Less information is known on the tuning of SWS2 pigments than on MWS/LWS and SWS1 pigments. Site directed mutagenesis work by Yokoyama and Tada (2003) has demonstrated that the $\lambda_{\text{max}}$ of the ancestral SWS2 pigment was approximately 440 nm. There has been a long-wave shift in the chicken ($\lambda_{\text{max}} = 455$ nm) and pigeon ($\lambda_{\text{max}} = 448$ nm) SWS2 pigments, which is likely to be accounted for by Ile49Ala and Ala269Ser/Thr substitutions. The SWS2 pigments from the salamander ($\lambda_{\text{max}} = 431$ nm) and bullfrog ($\lambda_{\text{max}} = 443$ nm) are short-wave shifted from the red shifted Ile49Ala pigment by Thr93Val and Leu207Ile substitutions respectively (Yokoyama and Tada, 2003). An interesting exception is the newt SWS2 opsin, which has a $\lambda_{\text{max}}$ of 474 nm. Site directed mutagenesis has isolated seven candidate long-wave shifting substitutions including Ser91Pro, Ala94Ser, Met122Ile, Pro261Tyr, Ser292Ala, Cys211Ser and Ser127Cys, with the long-wave shifting residue written proceeding the site number (Takahashi and Ebrey, 2003).

The only study on the SWS2 opsins of teleost fish is on the cottoid flock of Lake Baikal mentioned earlier with regards to their rod opsins. The SWS2 opsins are short-wave shifted, with a 9 nm shift linked to the Ala269Thr substitution and a 20 nm shift caused by Ala/Gly118Thr (Cowing *et al.*, 2002b).
1.4.6 Spectral Tuning in Deep-Sea Fish

The deep-sea places an unusually high constraint on light sensitive pigments with the limited down-welling light and chemically produced bioluminescence both considerably short-wave in composition. The identified rod opsins of several species of deep-sea fish are spectrally tuned to enhance sensitivity to these wavelengths.

Hope et al. (1997) studied the rod opsin sequences from four species of teleost fish and two species of eel to find potential candidate tuning sites (figure 1.17). All the deep-sea species have short-wave shifted rod opsins when compared to shallower species. Twelve sites were identified that faced into the chromophore pocket, which followed the same criteria as previously mentioned in the work by Hunt et al. (1996). Substitutions Asp83Asn and Ala292Ser as seen in the Lake Baikal fish were repeatedly present in the deep-sea species. The rod opsins of the sand goby, P. minutus ($\lambda_{\text{max}} = 501$ nm) and the freshwater eel, A. anguilla ($\lambda_{\text{max}} = 502$ nm) did not contain these substitutions, while the deep-sea opsins studied ($\lambda_{\text{max}}$ 484, 483, 482, 477, and 468 nm) all contained these changes. The eel, H. bathybius, has a rod opsin shifted to a $\lambda_{\text{max}}$ of 477 nm. The substitutions at 83 and 292 account for a shift to 480 nm. The additional 3 nm blue-shift is proposed to be due to a substitution of serine by alanine at position 168 (Hope et al., 1997). Though this may not be correct as the deep-sea eel opsin also contains this substitution but has a $\lambda_{\text{max}}$ at 480 nm. The rod opsin of C. laticeps has the most blue-shifted $\lambda_{\text{max}}$ found in Hope’s study (468 nm), the mechanism for this is not fully understood but maybe due to an Ala124Ser substitution (Hope et al., 1997).
Hunt and co-workers (2001) extended the study to 28 species, to determine if the same rules applied to members of the aulopiformes, beryciformes, gadiformes, myctophiformes, ophidiformes, osmeriformes and stomiiformes orders. The latter is discussed in chapter 3. Rod genomic DNA sequences including the coding region for all seven α-helical regions were amplified from all species. The translated sequences were compared, with special attention paid to amino acids facing the retinal binding pocket that involved a loss or gain of an hydroxyl group. In total there appear to be five key sites and four less common ones that are important for spectral tuning. The five common substitutions are found at positions 83, 122, 124, 261 and 292, and the four less common at 132, 208, 299 and 300 (figure 1.17) (Hope et al., 1997; Hunt et al., 2001).
2001). From the studies by Hope et al. and Hunt et al. a pattern emerges for the 83 and 292 substitutions. The deep-sea rod opsins with $\lambda_{\text{max}}$ at or below 480 nm contain an uncharged residue at 83 and a hydroxyl bearing polar residue at 292. In contrast, rod opsins with a $\lambda_{\text{max}}$ above 480 nm have a charged residue at 83 and a non-polar residue at 292. Amino acid positions 83, 292, 299 and 300 are especially close to the protonated Schiff’s base and negative counterion (Palczewski et al., 2000). Substitutions at these positions may lead to either stabilisation or destabilisation of the Schiff’s base depending on the change in charge of the residue. Sites 122, 124, 132 and 261 are all close to the polyene chain of retinal, and site 208 may well interact with the $\beta$-ionone ring of retinal. Residues 122 and 261 are components of the cytoplasmic face of the chromophore binding pocket and site 122 is also important in the interaction between helix 3 and the $\beta$-ionone ring of retinal (Palczewski et al., 2000). In several of the deep-sea rod opsins there is a Glu122Gln substitution. Imai et al. (1997) introduced this mutation into chicken rod opsin and found that the mutant pigment had a significantly faster rate of metarhodopsin II decay than the wild type. The presence of this mutation in a number of rod opsins from different deep-sea species may suggest that a faster decay time is beneficial in low light conditions. Site 83 has also been highlighted in kinetic experiments. Bovine rhodopsin containing the Asp83Asn mutation shows increased metarhodopsin I stability after photic excitation, leading to an increased efficiency in transducin activation (Sakmar et al., 1989; Weitz and Nathans, 1993). Interestingly, the Asp83Asn mutation has been inserted into bovine rod opsin, and the resulting $\lambda_{\text{max}}$ is short-wave shifted 8.5 nm from wild type (Nathans, 1990). It may be that this substitution is present in deep-sea rod opsins for two purposes, to short-wave shift the sensitivity of the pigment and to increase the efficiency of the phototransduction cascade in low light conditions. The similar tuning mechanisms at
phototransduction cascade in low light conditions. The similar tuning mechanisms at sites 83, 261 and 292 for the cottoid and deep-sea fish is interesting because the light environments are quite different. The freshwater of Lake Baikal contains more long-wave light than oceanic waters, and bioluminescence is absent. The upper layers of the lake are yellowish, and the deeper waters have maximum transmission above 500 nm (Dovgij, 1977; Sherstjankin, 1975, 1979 cited from Hunt et al., 1996). It may be that substitutions at sites 83, 261 and 292 not only short-wave shift the $\lambda_{\text{max}}$ but also confer other advantages for deeper dwelling fish, such as restraining against increasing pressure or lowering susceptibility to thermal excitation (Govardovski, 1972; Donner et al., 1990).

In deep-sea species that contain more than one visual pigment, the majority express more than one opsin gene, all binding to 11-cis retinal. A list of the multiple visual pigments in a variety of deep-sea species is shown in table 1.1. The exception is in three genera of stomiid fish; *Aristostomias*, *Pachystomias* and *Malacosteus*, where rhodopsin/porphyropsin pairs have been identified with $\lambda_{\text{max}}$ values around 520 and 540 nm, with additional long-wave pigments in *Aristostomias* and *Pachystomias* at around 590 nm (Table 1.3). These fascinating fish are examined more closely in chapter three. The only other deep-sea genus that contains three visual pigments in its retina is *Scopelarchus*; this is examined in chapter four. A retina with more than a single visual pigment, either a rhodopsin/porphyropsin pair or the expression of more than one opsin gene, could theoretically provide a basic colour visual system. In most cases only rod photoreceptors are present. If these are to play a chromatic role there would need to be some integration of neuronal output. Further study is needed on the inner nuclear layers of the retina to determine if interactions do occur.
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<table>
<thead>
<tr>
<th>Species</th>
<th>Absorbance Peaks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>517*, 552* nm</td>
<td>Dartnall, 1975. Digitonin extract.</td>
</tr>
<tr>
<td><em>Aristostomias xenostoma</em></td>
<td>514*, 551* nm</td>
<td>Knowles and Dartnall, 1977b. Digitonin extract.</td>
</tr>
<tr>
<td></td>
<td>515*, 543* nm</td>
<td>Bowmaker <em>et al.</em>, 1988. MSP.</td>
</tr>
<tr>
<td></td>
<td>515*, 543* nm</td>
<td>Bowmaker <em>et al.</em>, 1988. MSP.</td>
</tr>
<tr>
<td></td>
<td>521*, 538* nm</td>
<td>Partridge <em>et al.</em>, 1989. MSP.</td>
</tr>
<tr>
<td></td>
<td>517*, 541* nm</td>
<td>Douglas <em>et al.</em>, 1999. Digitonin extract and outer segment suspension</td>
</tr>
</tbody>
</table>

Table 1.3. The $\lambda_{max}$ values identified by MSP, retinal wholemount and digitonin extract for three genera of stomiids; *Aristostomias, Pachystomias* and *Malacosteus*. * denotes a probable rhodopsin/porphyropsin pair.
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1.5 Aims

The main aims of this study are as follows:

1. To identify, isolate and characterise the visual pigments of the deep-sea stomiids, 
   *Aristostomias* Sps and *Pachystomias microdon*.

2. To identify, isolate and characterise the visual pigments in the deep-sea 
   scopelarchid, *Scopelarchus analis*.

3. To identify, isolate and characterise the visual pigments in ice fish species from the 
   notothenioidae, that live under pack ice through which available sunlight has been 
   filtered.

4. To discover important spectral tuning sites in these opsins and to find evolutionary 
   patterns between these and opsins of other teleost fish.
Chapter 2 - Materials and Methods

2.1 Materials

2.1.1 Kits

- QuickPrep® Micro mRNA Purification Kit (Pharmacia Biotech).
- SuperScript™ First-Strand Synthesis System for RT-PCR (GibcoBRL Ltd, Life Technologies).
- FirstChoice™ RLM-RACE Kit (Ambion).
- pGEM®-T Easy Vector System (Promega).
- QIAGen® Miniprep kit (Qiagen).
- QIAGen® Maxiprep kit (Qiagen).
- Ready-To-Go™ DNA Labelling Beads (dCTP) (Amersham Biosciences).
- DIG RNA Labelling Kit (SP6/T7) (Roche).
- DIG Nucleic Acid Detection Kit (Roche).

2.1.2 General Reagents and Solutions

- 10 x NH₄ Reaction Buffer: 160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8), 0.1% (w/v) Tween 20 (Bioline).
- 50 mM MgCl₂ (Bioline).
- dNTP mix containing: dTTP (10 mM), dGTP (10 mM), dCTP (10 mM), dATP (10 mM) (Promega).
- Ethidium bromide (Fluka).
- Phenol liquefied washed in Tris buffer (Fisher Scientific UK Ltd.).
- Ethanol (100%) (BDH Laboratory Supplies).
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- Chloroform (100%) (BDH Laboratory Supplies).
- SDS (BDH Laboratory Supplies).
- 20X SSC (3 M NaCl, 0.3 M sodium citrate).
- NE Buffer (100 mM NaCl, 25 mM EDTA pH 8.0).
- PBS (phosphate buffered saline) (Oxoid).
- Ammonium acetate (8 M).
- Potassium acetate (2.5 M)
- Glycogen solution (5-10 mg/ml).
- Biotaq™ DNA Polymerase 5 U/µl (Bioline).
- EcoR1 12 U/µl (Pharmacia).
- Proteinase K (fungal) (BDH Laboratory Supplies).
- Ampicillin 50 mg/ml.
- X-Gal 20 mg/ml.
- IPTG 100 mM.
- 1 Kb DNA Ladder 1 µg/µl (GibcoBRL, Life Technologies).
- 1 Kb + DNA Ladder 1 µg/µl (GibcoBRL, Life Technologies).

2.1.3 Primers

Except for the RACE primers supplied in the FirstChoice™ RLM-RACE Kit (Ambion), all oligonucleotide primers were synthesised by Sigma-Genosys Ltd.

2.1.4 Electrophoresis Reagents

- Molecular Biology Certified Agarose (Bio-Rad laboratories).
- 1X TAE (0.4M Tris-acetate (BDH Laboratory Supplies); 10 mM EDTA (Eastman Kodak Company)).
• Orange G (6X Loading Buffer: 0.25% (v/v) xylene cyanol FF, 30% (w/v) glycerol in water).

2.1.5 Culture Media

• S.O.C: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose. (GibcoBRL, Life Technologies).
• LB broth: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl.
• LB agar: LB broth, 1.5% (w/v) bacteriological agar.

2.1.6 Bacterial Strains

• Subcloning Efficiency™ DH5α™ Competent Cells (GibcoBRL, Life Technologies).
• JM109 competent cells (Promega).

2.1.7 Additional Reagents for Southern Blotting

• 50X Denhardt's reagent (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin in 1 litre dH₂O).
• Hybridisation Buffer (0.05 M PO₄, 4x SSC, 5x Denhardt's reagent, 5 mg/ml denatured, fragmented salmon sperm DNA, 0.3% (w/v) SDS, 0.15% (w/v) NaPPi in dH₂O).
• ProbeQuant™ G-50 Micro Columns (Amersham Biosciences).

2.1.8 Tissue Culture Cells, Media and Reagents

• Human embryonic kidney (HEK) 293T human fibroblast cell line (European Collection of Cell Cultures, Salisbury, Wiltshire)
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- Dulbecco’s Modified Eagle Medium with GlutaMAX I™. Contains 4.5 g/l D-glucose (GibcoBRL, Life Technologies).
- Foetal calf serum (First Link (UK) Ltd).
- Penicillin (1000 iu/ml)/Streptomycin (100 mg/ml) solution (GibcoBRL, Life Technologies).
- Trypsin-EDTA: per litre Modified Puck’s Saline A 0.5 g Trypsin, 2 g EDTA (GibcoBRL, Life Technologies).
- Genejuice (Invitrogen).

2.1.9 Additional Reagents for Reconstitution of Opsin with 11-cis retinal

- Dodecyl maltoside: 2,1 and 0.1% (w/v) in 1X PBS.
- Phenylmethylsuphonyl fluoride (50 mg/ml) in propan-2-ol.
- 11-cis retinal (donated by Dr. Rosalie Crouch)
- Peptide I: Asp-Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala (Sigma Genosys).
- Anti-1D4 mouse monoclonal antibody (NIH cell culture centre, USA).
- CNBr-activated Sepharose 4B (Pharmacia).

2.1.10 Additional Reagents for In Situ Hybridisation

- pBluescript vector (Stratagene).
- 10X One-Phor-all buffer (Pharmacia).
- Diethyl Pyrocarbonate (DepC) (Sigma).
- T3 RNA polymerase and transcription buffer (Promega).
- Ethylenediaminetetraacetic acid-disodium salt (EDTA) (BDH).
- TES (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1% (w/v) SDS).
5 M LiCl (BDH).

Formamide (Sigma).

Polyoxyethylene-Sorbitan monolaurate (Tween\(^{1} 20\)) (Sigma).

N-lauryl sarcosine (Sigma).

Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5).

Washing buffer (Maleic acid buffer, 0.3% (v/v) Tween\(^{1} 20\)).

Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5).

Colour Substrate Solution (10 ml Detection buffer, 200 μl from 50X stock NBT/BCIP solution (18.75 mg/ml NBT, 9.4 mg/ml BCIP in 67% (v/v) dimethyl formamide)).

Hybridisation buffer (50% (v/v) formamide, 5X SSC, 2% (v/v) blocking solution, 0.1% (w/v) N-lauryl sarcosine, 0.02% (w/v) SDS).

4% (w/v) Paraformaldehyde.

25% (w/v) Sucrose in PBS.

PBST (PBS + 0.1% (w/v) Tween\(^{1} 20\)).

Tissue-Tek\(^{\circledR}\) O.C.T. embedding medium (Sakura).

2.1.11 Additional Reagents for Histology

2% (w/v) Paraformaldehyde.

2% (v/v) Glutaraldehyde.

Technovit Embedding Resin (Heraeus).

1% (w/v) Toluidine Blue.
2.2 Methods

2.2.1 Nucleic Acid Extraction

2.2.1.1 mRNA isolation

To extract an adequate amount of mRNA from limited tissue, the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) was utilised. This method isolates mRNA directly without requiring the synthesis of total RNA first. Procedures were carried out in accordance with manufacturer’s instructions. Briefly, up to 0.1 g of tissue was homogenized in an extraction buffer containing a high concentration of guanidium thiocyanate (GTC). This ensured the rapid inactivation of endogenous RNases. The extract was then diluted three fold with an elution buffer (10 mM Tris-HCL (pH 7.5), 1 mM EDTA) to reduce the GTC concentration. The suspension was spun down to produce a clear homogenate. The mRNA isolation was achieved by passing the extract though a suspension of oligo(dT)-Cellulose in extraction buffer, which binds poly(A)$^+$ RNA. This pellet was washed several times in high and low salt buffers before the mRNA was eluted in 0.4 ml elution buffer at 65° C.

2.2.1.2 Quantification of mRNA

mRNA concentration was determined by spectrophotometry using the formula below:

$$[\text{mRNA}] = A_{260} \times 40 \, \mu\text{g/ml.}$$
2.2.1.3 Precipitation of mRNA

To precipitate the mRNA, a 1/10 volume of potassium acetate solution (2.5 M), 10 µl of glycogen (5-10 mg/ml), and 1 ml of 95% (v/v) ethanol were added. The sample was placed at -20°C overnight. Precipitated mRNA was collected the following day by centrifugation and solubilised in 20-50 µl of elution buffer depending on the spectrophotometry reading.

2.2.1.4 Reverse transcription- Polymerase Chain Reaction (RT-PCR)

cDNA synthesis from an mRNA template was achieved by RT-PCR with the Superscript First-Strand Synthesis System (GibcoBRL). This procedure is able to convert 1-5 µg of total RNA or 50-500 ng of mRNA into first-strand cDNA. The manufacturer’s protocol was followed. The mRNA was targeted with an oligo (dT)\textsubscript{12-18} primer (0.5 µg) and cDNA synthesis was performed by the Superscript II RT enzyme (50U) at 42°C for 1 hour. Finally, RNase H was added to digest the RNA, leaving cDNA useful as a template for PCR.

2.2.1.5 Extraction of gDNA from Liver

Liver samples had been stored in absolute ethanol at -80°C. The tissue was weighed and ground up in liquid nitrogen using a pestle and mortar. The powdered tissue was then resuspended in 5 ml of ice-cold NE buffer (100 mM NaCl, 25 mM EDTA, pH 8.0) and spun at 4000 X g for 7 mins. The supernatant was removed and the pellet resuspended in another 5 ml NE buffer before being spun again. This was repeated another three times with the sample finally being resuspended in 5 ml NE buffer with proteinase K (400 µg/ml) and 500 µl of 10% (w/v) SDS, mixed gently and then placed at 50°C overnight. The following morning, an equivalent volume of buffered phenol was added, the sample shaken for 20 mins and then spun at 200 X g for 10 mins to
separate the aqueous and organic layers. The top aqueous layer was removed by aspiration and fresh phenol added, discarding the lower organic layer. This was repeated until the aqueous layer was clear. An equal volume of chloroform was then added and the sample spun again. The top layer was again removed and placed into a fresh tube.

DNA was precipitated by the addition of two volumes of absolute ethanol, and by inverting the sample for 2 mins. This was recovered either by spooling on to a sterile loop or by centrifugation. In either case, the DNA was resuspended in 1 ml dH₂O and left at 4°C overnight to ensure that the DNA had completely gone into solution. The DNA was reprecipitated the following day with 500 μl ammonium acetate (8M) and 6 volumes of absolute ethanol. The sample was kept at -80°C for 1 hour and then spun at 3000 X g for 10 mins. The resulting pellet was washed in 70% (v/v) ethanol, spun at 3000 X g for another 10 mins, and the supernatant removed. The pellet was left to air dry for 2-3 hours and then resuspended in 1 ml dH₂O.
2.2.2 DNA Amplification

2.2.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were performed to selectively amplify regions of DNA from gDNA or cDNA templates.

The standard reaction contained 0.4 mM dNTPs, 1.5 – 3.0 μM MgCl₂, 0.4 μM each of the forward and reverse primers, 2.5 U Biotaq DNA polymerase (Bioline) and approximately 30 ng cDNA or 100 ng gDNA in a total volume of 50 μl. Cycling conditions were an initial denaturation at 94°C for 3 mins, then 35 cycles of denaturing at 94°C for 30 s, annealing for 45 s and extension at 72°C for 45 s. This was followed by a final extension for 7 mins at 72°C. The annealing temperature was calculated with the formula, Tm (° C) = [2(No. of bases A/T) + 4(No. of bases G/C)] – 4. However, the Techne gradient PCR machine was used over a range of annealing temperatures to increase the likelihood of primer annealing. Negative controls were run in parallel, containing the same reagents but omitting the template. On completion, 40 μl was run on a 1-2 % (w/v) agarose gel containing ethidium bromide at 1 μg/ml. A DNA 1 kb plus ladder (GibcoBRL Ltd) was also run to allow approximate size determination of DNA fragments. The gel was then viewed on a UV transilluminator and a digital image taken using a digital camera (SONY).

2.2.2.2 DNA Extraction from Agarose Gel

When a band was visible under UV light, it was excised using a sterile razor blade and spun through a Wizard™ column (Promega) for 20 mins at 4°C. The columns allow for
DNA elution but prevent agarose moving through the filter. The eluted DNA was then either directly sequenced (see section 2.2.4) or cloned (see section 2.2.3).

### 2.2.2.3 Walking PCR

Walking PCR on genomic DNA is a useful method to obtain sequence information beyond a previously sequenced region of a gene. The protocol used was adapted from Dominguez and Lopez-Larrea, 1994.

#### 1st Round PCR

50 µl reactions contained 100 ng genomic DNA, 10 X NH₄ buffer, 2 µl of 12.5 µM UNI33 primer (5'-TTTTTTTTTTTTTTTTGTTTGTTGTGGGGGGGTT-3'), 10 mM dNTPs and 1.5 or 3.0 mM MgCl₂ in dH₂O. Each reaction was then divided in half to form two 25 µl reactions and denatured at 94°C for 1 min. 1 µl of 1/10 diluted Taq DNA polymerase (Bioline) was then added and the reactions continued at 80°C for 30 seconds, 15°C for 2 mins, 25°C for 10 mins, 72°C for 60 s and then 90°C for 60 s. At this point 1 µl of 12.5 µM gene specific outer primer was added. The following steps were then performed for 35 cycles, 94°C for 10 s, 62°C for 60 s and 72°C for 60 s. The final extension step followed at 72°C for 60 s.

#### 2nd Round PCR

1 µl of the first round PCR was added to 1 ml of dH₂O to form a 1:1000 dilution and this was used as the template for the second round PCR. The 50 µl reactions contained 2 µl of template DNA (1/1000 1st round PCR), 10X buffer, 2 µl of 12.5 µM UNI17 primer (5'-TTTTTGTTTTGTTGTGGG-3'), 2 µl of 12.5 µM inner gene specific primer, 10 mM dNTPs, 1.5 mM MgCl₂ in dH₂O. The solution was again divided in half to
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produce two reactions each of 25 μl. The solutions were denatured at 94°C for 10 s. Then 1 μl of a 1/20 dilution of taq DNA polymerase was added to each 25 μl reaction and the following program run for 35 cycles, 94°C for 5 s, 55°C for 5 s and 72°C for 30 s. A final extension at 72°C for 2 mins was then performed.

20 μl of both the first and second round reactions were viewed by gel electrophoresis on a 1.5% agarose gel containing EtBr (1 μg/ml). If a product had been amplified it was excised from the gel, cloned and sequenced (see sections 2.2.3 and 2.2.4).

2.2.2.4 Rapid Amplification of cDNA Ends (RACE)

Rapid amplification of cDNA 5’ and 3’ ends was achieved by RACE using the FirstChoice™ RLM – RACE Kit (Ambion). Methods were carried out in accordance with the manufacturer’s instructions.

5’ RACE

This method is optimal for 250 ng of poly(A)+ RNA. The mRNA was treated with calf intestinal phosphatase (CIP) to remove free 5’-phosphates, and Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure. This left a monophosphate at the 5’ end of the RNA. An RNA adapter oligonucleotide was ligated to the RNA using T4 RNA ligase. Reverse transcription was achieved by priming with random decamers to synthesize cDNA. An outer PCR was performed using the kit supplied 5’ RACE Outer Primer and a gene-specific reverse primer designed to previously identified sequence. A 50 μl reaction contained 1 μl of the RT reaction, 10X buffer, 10 mM dNTP mix, 10 μM of both forward and reverse primers, 1 U taq polymerase with the final volume made up with dH2O. The cycling parameters were 3 mins at 94°C, followed by 35 cycles at 94°C.
for 30 s, 60°C for 30 s and 72°C for 30 s. The final extension step was at 72°C for 7 mins. On completion the reaction was put on ice, and 1 µl was used as the template for a nested PCR. The inner reaction contained the same reagents as the outer reaction, except for the kit supplied 5’ RACE Inner primer and a primer designed to previously identified sequence that lies 5’ of that used in the outer PCR. The cycling parameters were the same as described for the inner PCR.

3’ RACE
First strand cDNA was synthesized from mRNA using the 3’ RACE Adapter, a poly(A) primer. Nested PCR was again performed though this time the kit supplied the 3’ RACE Outer and Inner primers and gene specific forward primers were designed. Cycling conditions were identical to those used for 5’ RACE.

Gel Analysis
40 µl of the 3’ and 5’ inner and outer PCR reactions were run on 2% (w/v) agarose gels (EtBr 1 µg/ml) and viewed by electrophoresis. Any products of roughly the predicted size were gel excised and spun through a wizard column. If the PCR amplified a single clean product then the DNA was excised and sequenced directly (see section 2.2.4). In the majority of cases however, the fragment was cloned prior to sequencing (see section 2.2.3).
2.2.3 Cloning of PCR Products

To enable individual amplified fragments of DNA to be sequenced, PCR products were ligated into a DNA vector, transformed into bacterial cells and plasmid DNA extracted.

2.2.3.1 Ligation

DNA fragments were ligated into the pGEM T-Easy vector (Promega). This vector contains the ampicillin resistance gene and a multiple cloning site, which is found within the coding sequence of β-galactosidase (lacZ operon) (see figure 2.1). Successful ligations will interrupt this sequence and prevent its transcription. Overnight reactions were kept at 4°C and contained 50 ng pGEM-T-Easy, 3U ligase, 2X rapid ligation buffer and between 15 – 30 ng of amplified DNA.

![Figure 2.1. Circular map of pGEM-T Easy. Amp R is the ampicillin resistance gene and lacZ is the β-galactosidase gene.](image)

2.2.3.2 Transformation

JM109 High Efficiency Competent Cells (Promega) were transformed with the ligation reaction in accordance with manufacturer’s instructions. Frozen JM109 cells were
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removed from storage at -80°C and thawed on ice. Once thawed, 3 µl of the ligation reaction was added to 25 µl of competent cells and the reaction left on ice for 20 mins. The cells were heat shocked for 50 s at 42°C and then chilled on ice for 2 mins. 300µl of SOC medium (GibcoBRL, Life Technologies) was added, the cells were shaken for 1.5 hours at 37°C and then spread on to LB agar plates containing 50 µg/ml Ampicillin, 20 µg/ml IPTG, and 20 µg/ml X-Gal. The cells were left to replicate overnight at 37°C. The following morning the plates were placed at 4°C for at least 30 mins. The IPTG present in the agar induces β-galactosidase production, which when present breaks down X-Gal producing a stable dark blue product. Colonies that contain an insert could not produce the enzyme and therefore appeared white.

2.2.3.3 LB Broth Culture

White colonies were selected and cultured overnight in 5 ml LB broth, containing 50 mg/ml ampicillin at 37°C.

2.2.3.4 Plasmid Isolation from Competent Cells

The QIAprep® Miniprep kit (Qiagen) was used to extract the pGEM-T-Easy plasmid containing inserted DNA from transformed cells. The protocol was performed as according to manufacturer’s instructions. Briefly the bacterial cells were lysed by alkaline lysis, the lysate was then neutralized and adjusted to high-salt binding conditions. The lysate was loaded onto a filter and cellular components removed by filtration. The plasmid DNA was absorbed by a silica gel membrane in high-salt conditions but could be eluted in low-salt conditions. Any endonucleases were removed by washing with the provided PB Buffer (Qiagen), and the PE Buffer (Qiagen) removed
any salts. The plasmid DNA was eluted in 50 μl dH₂O to give an approximate yield of 150 ng/μl.

2.2.3.5 Restriction Enzyme Digest

EcoR1 restriction digests were used to identify which miniprep cultures contained DNA inserts of the correct size. The pGEM-T-Easy vector contains EcoR1 sites (GAATTTC) on either side of the multiple cloning site (MCS), allowing the DNA inserted to be excised and its size determined. 10 μl reactions contained 12U EcoR1, 5 μl miniprep DNA (∼ 750 ng), 10X H buffer (Promega) in dH₂O. Reactions were incubated at 37°C for 1 hour and run on a 1.6% (w/v) agarose gel containing EtBr (1 μg/ml). Digestion products were then viewed on a transilluminator. If the insert was of the correct size then it was sequenced (see section 2.2.4).
2.2.4 **BigDye® Terminator v3.1 Cycle Sequencing**

PCR products and plasmid DNA were sequenced on an ABI 3100. To prepare samples for sequencing, the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) was used. The kit provided the reagents to perform a fluorescence-based cycle sequencing reaction using gene specific primers or ones designed to the vector sequence, i.e. pTAG 3' and 5'.

Sequencing reactions contained:

- Ready Reaction Mix 4 μl
- 5 x BigDye Sequencing Buffer 2μl
- Miniprep DNA or PCR fragment 3 μl
- pTAG 3' primer (10 μM) 3.2 μl
  
  \[(S'\text{- TTGTAAAACGACGGCCAGTGAA-3'})\]

  or

- pTAG 5' primer (10 μM) 3.2 μl
  
  \[(5'\text{- TTGGCGTAATCATGGTCATAGC-3'})\]

- dH₂O 7.2 μl

This gave a final reaction volume of 20 μl. The samples were placed in the Thermal Cycler (Perkin Elmer) and run through 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 30 s. On completion, the DNA was precipitated by the addition of 5 μl 125 mM EDTA and 60 μl 100% ethanol. The sample was vortexed and left to stand for 20 mins before being spun for 20 mins at 13,000 rpm. The supernatant was then removed carefully by aspiration and 60 μl of 70% (v/v) ethanol added. Centrifugation was
repeated for 15 mins, the supernatant carefully removed and the sample left to air dry. The pellet was then resuspended in 11 μl of deionized formamide and then loaded into a sequencing 96 well plate and run on the ABI 3100.
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2.2.5 Southern Hybridisation

Southern blotting is a method for identifying the presence of known DNA sequences (genes) in DNA. The DNA to be probed is digested with restriction enzymes, denatured and bound to a membrane. This is then probed with a radioactive DNA fragment. If the sequence is present in the bound DNA, it binds the radioactively-labelled probe and can be viewed after washing by exposure to photographic film. On developing, dark bands indicate where the probe has bound.

2.2.5.1 Digestion of Genomic DNA

Approximately 15 µg of gDNA was digested in a 50 µl reaction, containing 5 U of a restriction enzyme (EcoRI, BamHI or HindIII) and 5 µl 10X buffer (Promega), at 37°C overnight. The following day the whole 50 µl was separated on a 0.8% (w/v) agarose gel (containing EtBr) by electrophoresis overnight at 35 V.

2.2.5.2 Capillary Transfer of DNA to Membrane

The gel was submerged in a denaturing solution (1.5M NaCl, 0.5M NaOH) on a shaking platform for 45 mins and then transferred to a neutralising solution (1 M Tris (pH 7.4), 50 mM EDTA, 1.5 M NaCl) for a further 45 mins. A tray was then filled 1 inch deep with 20X SSC and a gel tray placed upside down in the SSC to act as a support for the DNA transfer. A piece of Whatman 3MM paper was wetted in 20X SSC, laid onto the gel tray and pressed flat. The edges of the Whatman paper overhung at least 1 cm into the SSC on all sides. The gel was laid carefully on top of the Whatman paper. A piece of nitrocellulose membrane was cut to the same size as the gel, wetted in distilled water and 20X SSC, and then laid on top of the gel. The position of
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the lanes was marked on the membrane with pencil. Three more pieces of Whatman paper (all pre-wetted in 20X SSC) were placed on top below a large number of dry paper towels. A glass plate was put on top of the whole stack under a weight of \( \approx 500 \) g. The apparatus was then left overnight to allow for capillary transfer of DNA to the membrane.

2.2.5.3 Fixing DNA onto Membrane

After 24 hours, the apparatus was dismantled and the gel discarded. The membrane was rinsed in 2X SSC, patted dry and then baked at 80°C for 2 to 3 hours.

2.2.5.4 Synthesis of Radioactive Probes

The probe was made using Ready-To-Go™ DNA Labelling Beads (-dCTP) (Amersham Biosciences). The protocol required 5 ng of DNA (usually a PCR product) in a total volume of 45 \( \mu l \) dH\(_2\)O. The DNA was denatured at 95°C for 3 mins and then chilled on ice for 2 mins. The denatured DNA was added to a tube containing a reaction mix bead (containing buffer, dATP, dGTP, dTTP, 7-12U FPLCpure™ Klenow fragment, random oligodeoxyribonucleotides), and 1.85 MBq of [\( \alpha ^{32}P \)] dCTP. The labelling reaction proceeded at 37°C for 15 mins. Labelled probes were cleaned by spinning through ProbeQuant™ G-50 Micro Columns (Amersham Biosciences) to remove any unincorporated [\( \alpha ^{32}P \)] dCTP’s.

2.2.5.5 Hybridisation with Radioactive Opsin Probes

The nitrocellulose membrane was prehybridised at 65°C for 3 hours in 25 ml hybridisation buffer (0.05 M PO\(_4\), 4X SSC, 5X Denhardt’s reagent, 5 mg/ml denatured, fragmented salmon sperm DNA, 0.3% (w/v) SDS, 0.15% (w/v) NaPPi in dH\(_2\)O).
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Denhardt’s reagent and salmon sperm DNA are necessary to block non-specific attachment of the probe to the surface of the membrane. The radioactive probe was denatured at 94°C for 2 mins, chilled on ice for 2 mins and then added to the hybridisation buffer used to prehybridise the membrane. The hybridisation reaction was left overnight at 60°C, or lower if the probe was non-specific.

2.2.5.6 Removal of Non-specific Binding

The blot was washed twice in 6X SSC, 0.5% (w/v) SDS, and twice in 3X SSC, 0.5% (w/v) SDS. Washes were carried out for 10 mins at 65°C or lower if the probe was non-specific.

2.2.5.7 Visualisation of Blot

After washing, the membrane was wrapped in cling film and placed in a film cassette containing intensifying screens, against X-ray film. The cassette was left overnight at -80°C. Later the film was developed and further washes were performed if the background signal was too strong. These were carried out at 1X SSC, 0.5% (w/v) SDS concentrations. Length of film exposure to the blot varied depending on signal intensity to ensure an optimal result.
2.2.6 Reconstitution of Recombinant Opsin with 11 cis-Retinal

When the full coding sequence of an opsin gene had been identified, an expression construct was made and recombinant proteins were expressed using a mammalian cell line. The opsin protein were then isolated and regenerated in the dark with 11-cis retinal. The visual pigment was extracted and a dark scan taken from 200 to 700 nm by spectrophotometry. Followed by bleaching by exposure to light. Absorbance and bleached spectra were taken and the in vitro $\lambda_{\text{max}}$ determined.

2.2.6.1 Amplification of full length opsin with modified primers

Modified primers were designed to include restriction enzyme sites to enable cloning into the expression vector pMT4 (a kind gift from Dr. Phylis Robinson). The forward primer contained an EcoR1 site and the reverse primer a Sal1 site (both shown in red).

5’ primer: GCGCGAATTCCCACCATG $\rightarrow$ 18-20 bp opsin sequence from ATG

3’ primer: CGGCGTCGACGC $\rightarrow$ 18-20 bp reverse opsin sequence from the penultimate codon (i.e. not including the stop codon).

These primers were used in a standard polymerase chain reaction containing the proof reading polymerase pfu (Promega) with 3 mins denaturation at 94°C, and 35 cycles of 30 s at 94°C, 45 s at 60°C and 45 s at 72°C. the final extension was at 72°C for 10 mins. The product was visualised by gel electrophoresis, and then excised and isolated using a Wizard™ column.

2.2.6.2 Cloning of Full Length Opsin

The opsin DNA was first cloned into the pGEM T-Easy vector to be fully sequenced before being sub-cloned into the expression vector pMT4. The PCR fragment and
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pGEM T-Easy were both digested with EcoRI and SalI enzymes to provide complementary ‘sticky ends’. This is because amplification with pfu creates blunt ended fragments rather than the A overhangs as added by taq polymerase. Blunt ended fragments cannot be inserted between the vector T overhangs. The digestion reactions contained approximately 5 U of both EcoRI and SalI, 10X D buffer (Promega) and 5 μl miniprep DNA. The inserts were sequenced and if the nucleotide sequence was correct the opsin gene was digested from the plasmid using the EcoRI and SalI restriction sites. Aliquots of the pMT4 expression vector were digested with the same restriction enzymes. The opsin digested out of the pGEM-T Easy vector was ligated into the pMT4 vector. This was then transformed into JM109 cells and grown up in LB broth as described previously. From the 5 ml miniprep culture, 500 μl was taken to inoculate a 500 ml overnight culture. A maxiprep kit could then be used to extract large amounts of DNA. This procedure follows exactly the same principles as the miniprep kit but can be used on larger culture volumes. The concentration of eluted DNA was quantified by spectrophotometry.

2.2.6.3 Growth and Transfection of HEK 293T Cell Line

All tissue culture work was carried out in a microflow safety cabinet. HEK 293T human fibroblast cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma) with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin for three nights in a flask at 37°C until confluency. The medium was aspirated and the cells washed in 4 ml 1X PBS. This was removed and 1 ml trypsin/EDTA was added to the flask and left to incubate for 5 mins at room temperature to dislodge the attached cells. The volume in the flask was made to 10 ml with DMEM (containing foetal calf serum and antibiotics) and 5 ml DMEM (with foetal calf serum and antibiotics) was also added to 10 petri
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dishes (60 mm). 1 ml of the homogeneous cell suspension was then added to each of these dishes. The cells were incubated for 24 hours at 37°C. Three flasks of cells were needed per transfection experiment, therefore 30 dishes were prepared as above. To transfect 30 plates, 610 µl of Genejuice (Invitrogen) was added to 21 ml DMEM (without foetal calf serum and antibiotics). This mixture was vortexed and left to incubate for 5 mins at room temperature. After incubation 210 µg of maxiprep expression construct was added and the sample incubated for a further 15 mins. The 30 plates were then removed from the incubator and the media aspirated. 720 µl of the transfection mix was added to each plate and then incubated at 37°C. The cells were harvested after two days. The media was aspirated and the cells washed in 5 ml PBS. This was also removed and 2 ml fresh PBS added to each dish. The cells were scraped off the plates using a cell scraper. The cells were placed in a falcon tube and spun for 10 mins at 2,000 g. The supernatant was removed and 10 ml fresh PBS added. Centrifugation was repeated and the supernatant removed. The cell pellets were stored at -80°C.

2.2.6.4 Preparation of the 1D4 Column

To purify the opsin, an anti-rod opsin 1D4 monoclonal antibody was coupled to CNBr-activated sepharose. 6 mg of the antibody was dissolved in a 10 ml solution containing 0.1 M NaHCO₃ pH 8.3 and 0.5 M NaCl. 2 g of sepharose was added to 12 ml 1 mM HCl for 15 mins. The sepharose was then transferred to a glass filter aspirator and washed with 400 ml 1 M HCl, and then quickly with 100 ml ice-cold 0.1 M NaHCO₃/0.5 M NaCl solution. The sepharose gel was scraped into a falcon tube containing the 1D4 antibody and placed on a rotary mixer at 4°C overnight. The next day the tube was spun at 4,000 rpm for 10 mins and the supernatant removed. The
sepharose gel was washed twice in 40 ml 0.2 M glycine pH 8.0 and spun at 4,000 rpm for 10 mins. Another 40 ml of 0.2 M glycine was added and the solution incubated on a rotary mixer for 2 hours. The sepharose gel was then transferred to the glass filter again and washed twice with 40 ml 0.1 M NaHCO₃/0.5 M NaCl, twice with 40 ml 0.1 M Na acetate/0.5 M NaCl and twice with 40 ml PBS/0.02% (w/v) sodium azide. The sepharose was scraped into a fresh tube and resuspended in 6 ml PBS/0.02% (w/v) sodium azide. The column was stored in this state at 4°C until required.

2.2.6.5 Reconstitution of Recombinant Opsin with 11-cis Retinal

The following method is based on that used by Oprian \( et \) al., (1987). 11-cis retinal was prepared by dissolving a single crystal in 400 μl anhydrous ethanol. A 1 in 500 dilution was placed in the spectrophotometer and an absorbance reading taken at 380 nm. The retinal concentration was then determined by Baer’s Law:

\[
C = \frac{\text{Absorbance at } 380 \text{ nm}}{25,000 \text{ pathlength nmoles/μl}}
\]

The cell pellet was thawed on ice and resuspended in 10 ml 1X PBS. From this point work was done in the dark to prevent bleaching of the pigment. 11-cis retinal was added to the cell suspension to a final concentration of 40 μM. The tube was then wrapped in foil and placed on a rotary mixer for 1 hour at 4°C. After incubation the cells were spun down for 5 mins at 3,500 rpm in a chilled centrifuge. The supernatant was discarded and 5 ml of a solution of PBS and phenylmethylsulphonyl fluoride (PMSF) (0.2 mg/ml) added. The pellet was resuspended and 5 ml 2% (w/v) Dodecyl maltoside (DDM) added. The sample was placed back on the rotary mixer for a further hour again kept at 4°C. At this point 500 μl of the 1D4 coupled sepharose column was washed with 1 ml 1% (w/v) DDM, and spun at 3,000 rpm for 1 min. This was repeated three times to remove traces of sodium azide and to equilibrate the column. After the final spin a little
supernatant was left to prevent the column from drying out. After the hour incubation
the cells were spun at 3,500 rpm for 5 mins. The supernatant was removed and added to
the prepared 1D4 coupled column. The sample was incubated for 2 hours on the rotary
mixer at 4°C. Once the protein had bound to the column it was eluted using a peptide
that competes for the 1D4 antibody. The column was spun at 3,500 rpm for 5 mins and
all but 1 ml of the supernatant removed. The pellet was resuspended in this 1 ml and
then added to a 1 ml syringe, which was plugged at the end with glass wool. The
syringe was placed in a falcon tube and spun for 15 s at 3,000 rpm. The column was
washed with 1 ml 0.1% (w/v) DDM, spun for 15 s at 3,000 rpm and the flow-through
removed. This was repeated a further eight times. The bottom of the syringe was then
covered with nescofilm™ and 200 μl of peptide I added. Peptide I competes for the 1D4
antibody and promotes dissociation of the opsin. The column was incubated for 30 mins
on ice. The film was removed and the syringe spun in a falcon at 3,500 rpm for 30 s.
Another 200 μl was then added and the syringe spun again. The eluate contained the
visual pigment and was analysed spectrophotometrically.

2.2.6.6 Spectrophotometric Determination of Visual Pigment $\lambda_{\text{max}}$

To determine the $\lambda_{\text{max}}$ of the regenerated opsin, the eluate was placed in a Unicam UV-
visible spectrophotometer. A scan range from 200-700 nm was used with readings taken
every 1 nm. After a dark spectrum had been obtained, the sample was exposed to light
for 15 mins and then scanned again. Dark and bleached readings were taken three times.
A difference spectra was then formed by deducting the bleached from the dark spectra.
A standard Gavordovskii rhodopsin A$_1$ template with a bleached retinal curve
subtracted was then fitted to the data using an excel spreadsheet and the $\lambda_{\text{max}}$
determined.
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2.2.6.7 Regeneration Under Pressure

Some of the deep-sea opsins were regenerated in a high pressure chamber to simulate deep-sea conditions. The protocol was exactly the same as just described except when 11-cis retinal was added. The total volume was reduced to 3 rather than 10 ml, and the hour long incubation proceeded in a fluid filled, air tight chamber. The pressure within was raised to 100 bar. The remaining washing and elution steps had to be performed at room pressure as manipulation of the sample within the chamber was impossible.
2.2.7 In Situ Hybridisation

In situ hybridisation is a technique that involves hybridising RNA probes to sections of fixed tissue, in this case sections of retina. The probes were the antisense sequence of the opsin RNA of interest, which binds to the sense RNA that is expressed in cells. Sense probes were used as a control, as these should not bind to sense RNA. Hybridisation was visualised by binding a secondary antibody linked to alkaline phosphatase, which produces a blue-purple colour when exposed to 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) in the dark.

2.2.7.1 Synthesis of RNA Probes

RNA antisense and sense probes were generated using the DIG RNA Labelling Kit (SP6/T7) (Roche). This enabled RNA labelled with digoxigenin-UTP to be synthesised from an insert in a DNA double stranded plasmid, by in vitro transcription using an RNA polymerase. The DNA insert was flanked by two different restriction enzyme sites, so that the plasmid could be linearised and RNA transcription run in either direction across the insert (see Figure 2.2). If the plasmid was linearised with EcoR I then RNA transcription was run from the T3 promoter to produce a sense probe. If the plasmid was linearised with Xho I then transcription was directed from the T7 promoter to generate the antisense probe.
Figure 2.2. Circular map of the pBluescript vector. The DNA template was inserted into the vector between the EcoR I and Xho I restriction sites. RNA transcription can be run from the T7 or T3 promoters.

2.2.7.2 Cloning of DNA fragment into pBluescript

The probe sequence was amplified from cDNA in a standard polymerase chain reaction using primers flanked with restriction enzyme sites. The forward primer contained a Xho1 site and the reverse primer an EcoR1 site (shown in red).

Forward 5' primer: 5' - GCGCCTCGAG - specific sequence - 3'
Reverse 3' primer: 3' - CGGCGAATTC - specific sequence - 5'

PCR products were separated by electrophoresis on a 1.4% (w/v) agarose gel containing EtBr and visualised on a UV transilluminator. The amplified product was excised and spun through a Wizard™ column. The vector and DNA fragments were digested with both Xho1 and EcoR1. The digestion reaction contained 30 µl eluted DNA, 10X one-phor-all buffer (Pharmacia), 1 U EcoR1 and 1U Xho1 in a final volume of 30 µl. The reaction was maintained at 37°C for two hours. The sample was then run out on a 1.4% (w/v) agarose gel and the digested fragment excised and eluted through a Wizard™
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column. The DNA fragment was ligated into the pBluescript vector and then transformed into JM109 competent cells (see section 2.2.3). Colonies were selected by ampicillin screening and cultured.

2.2.7.3 Plasmid Linearisation and Phenol/Chloroform Extraction

The vector was linearised with either Xho I for the antisense probe or EcoR I for the sense as explained previously. The digestion reactions contained 10 µl linearised plasmid, 10X buffer (Promega) and 4U restriction enzyme in a 100 µl total volume, and were incubated for two hours at 37°C. After this time 5 µl were run out on a 1.4% (w/v) agarose gel to check that digestion had run to completion. Since the plasmid DNA needed to be free of any proteins that may interfere with RNA transcription, in particular RNases, the remaining 95 µl of the digest reaction was extracted with 100 µl of phenol. The sample was shaken for 15 mins and spun at 200 rpm for 10 mins. The top aqueous layer was aspirated and added to a fresh tube and the organic phase was discarded. This step was repeated with phenol/isoamyl alcohol/chloroform in a ratio of 16:3:1, and then with 100% chloroform, each time keeping the aqueous layer. To precipitate the DNA, 100 µl 100% ethanol was added, the sample was briefly vortexed and then stored at -80°C for 30 mins. After incubation the sample was spun at 13,000 rpm for 30 mins and the supernatant removed. This was replaced with 70% (v/v) ethanol and the tube spun at 13,000 rpm for 5 mins. The supernatant was removed and the pellet left to air dry. When dry the DNA was resuspended in 11 µl DepC H₂O and stored at -20°C. All reagents from this point were DepC treated to prevent RNases contaminating the samples.
2.2.7.4 Transcription and Precipitation of Riboprobes

All reagents apart from the linearised plasmids and the T3 RNA polymerase were supplied with the DIG RNA Labelling Kit (SP6/T7) (Roche). The protocol generated digoxigenin labelled single stranded RNA from either the T3 or the T7 promoter. Approximately 1 μg of linearised template DNA was made to a final volume of 13 μl with DepC H₂O. The following were then added on ice: 2 μl 10X NTP labelling mixture, 2 μl 10X transcription buffer, 20 U RNase Inhibitor, 20U RNA polymerase (T3 or T7). The reagents were gently mixed, spun briefly and then incubated for two hours at 37°C. To remove the DNA template, 20U DNase I was added and the sample incubated for a further 15 mins at 37°C. To quench the reaction 2 μl of 0.2 M EDTA (pH 8.0) was added. The probes were precipitated by adding the following reagents: 300 μl 100% ethanol, 80 μl TES (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1% (w/v) SDS), 15 μl DepC H₂O and 8 μl 5 M LiCl. The sample was kept at −20°C overnight. The following day the sample was spun at 13,000 rpm for 5 mins and the supernatant carefully aspirated and discarded. The above precipitation was repeated. The supernatant was again discarded and the sample allowed to air dry before resuspension in 100 μl TES. The probes were stored at −20°C.

2.2.7.5 Detection and Quantification of Probe on an RNA Blot

The probe was roughly at a concentration of 10 ng/μl at this stage, but the success of riboprobe synthesis needed to be confirmed. In order to address this a series of dilutions of the probes were made and dotted on to a nylon membrane. The dilutions made from the original probe (taken as 10 ng/μl) were; 1 ng/μl, 10 pg/μl and 3 pg/μl, with 1 μl of each added to the membrane. A control riboprobe of known concentration was supplied.
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with the kit and could be added on the blot to allow comparison with the synthesised probes. The blot was then baked for 30 mins at 120°C.

The membrane was then subjected to immunological detection with anti-digoxigenin-alkaline phosphatase conjugated antibodies. The antibody binding was visualised by an enzyme-catalysed colour reaction with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT). The reaction produces an insoluble blue precipitate due to the enzymatic activity of phosphatase. Most of the reagents necessary for this were supplied with the DIG Nucleic Acid Detection Kit (Roche). The membrane was placed in a blocking solution made from a stock of blocking reagent (from kit) diluted 1 in 10 with Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5). The blot was agitated for 30 mins. The blocking solution was then removed and replaced with fresh blocking solution containing a 1:5000 dilution of anti-digoxigenin-AP (150 mU/ml). The blot was left shaking for another 30 mins. Any unbound antibody was removed by two 15 min washes with washing buffer (Maleic acid buffer, 0.3% (v/v) Tween^1 20). The blot was equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5) prior to being detected in colour substrate solution (10 ml detection buffer, 200 μl from 50X stock NBT/BCIP solution (18.75 mg/ml NBT; 9.4 mg/ml BCIP in 67% (v/v) dimethyl formamide)) in the dark. This was left at room temperature until a dot appeared (~30 mins) and the reaction was quenched with a couple of washes in DepC H₂O.

2.2.7.6 Pre-treatment of Retinal Tissue for Whole-mounts

Eyecups were dissected from the fish on capture. After the lens was removed, the eye was fixed in 4% (w/v) paraformaldehyde in 1X PBS overnight (~ 12 hours). The
following day the paraformaldehyde was removed, the retina dissected away from the RPE and cut into 1 cm wide strips. These strips were placed photoreceptor layer side up on glass slides under weighted down cover slips to ensure that they remained flat. The slides were then left in 25% (w/v) sucrose in 1X PBS overnight to cryoprotect the tissue, and the following day the cover slips were removed and the slides stored at -80°C.

2.2.7 In situ Hybridisation of Retinal Whole-mount

Day 1
The slides were removed from the -80°C freezer and allowed to thaw for 30 mins. They were then rinsed in 1X PBS and left to dry over a couple of hours. The slides were processed in a plastic slide holder with a total volume of approximately 20 ml. The whole-mounts were washed 5 times for 5 mins in PBST (PBS + 0.1% (w/v) Tween 20). The tissue was fixed again with 4% (w/v) paraformaldehyde in 1X PBS for 20 mins and then washed a further 5 times in PBST. The whole-mounts were then left in pre-warmed hybridisation buffer to prehybridise for an hour at 60°C. The probe was denatured at 80°C for 5 mins and then added to fresh pre-warmed hybridisation buffer to form a final concentration of ~ 0.5 µg/ml (roughly 5 µl transcription reaction in 200 µl buffer). To probe each slide separately, the edge of the slide was marked with a PAP pen, which created a hydrophobic barrier. This allowed for the hybridisation buffer containing the riboprobe to be placed on to the slide surface without any running off the slide. The slides were laid flat in a sealed box, lined with 3 mm Whatmann paper wetted in 50% (v/v) formamide, and left overnight on a shaking platform at 60°C.
Day 2

The hybridisation buffer was removed and the slides washed in the following:

- 66% (v/v) Hyb buffer/ 33% (v/v) 2X SSC for 5 mins at 60°C
- 33% (v/v) Hyb Buffer/ 66% (v/v) 2X SSC for 5 mins at 60°C
- 2X SSC for 5 mins at 60°C
- 0.2X SSC, 0.1% (w/v) Tween^ 20 for 20 mins at 60°C
- 0.1X SSC, 0.1% (w/v) Tween^ 20 for 20 mins at 60°C twice
- 66% (v/v) 0.2X SSC/ 33% (v/v) PBST for 5 mins at room temperature (RT)
- 33% (v/v) 0.2X SSC/ 66% (v/v) PBST for 5 mins at RT
- PBST for 5 mins

The wash was stringent to remove any background labelling as the cleaner the whole-mount then the easier it is to see specific positive labelling. The background was blocked with the supplied blocking solution (Roche) or 10% (v/v) sheep serum for 1 hour at RT to reduce non-specific antibody binding. After an hour this was replaced with fresh blocking solution containing a 1:5000 dilution of the anti-digoxigenin antibody. The samples were left shaking overnight at 4°C.

Day 3

The antibody solution was discarded and the slides washed 5 times for 15 mins in PBST at RT. The whole-mounts were then washed 4 times for 5 mins in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5). The colour substrate solution was made by adding 200 μl of the NBT/BCIP stock solution (18.75 mg/ml NBT, 9.4 mg/ml BCIP) to 10 ml detection buffer. This was added to the whole-mounts which were left in the dark for the blue precipitate to develop. The slides were viewed under a light microscope to monitor the colour reaction. The reaction was quenched by washing in DepC H₂O. The
slides were then coverslipped in 1X PBS and digital images taken with a digital camera (Nikon) attached to a light microscope.

2.2.7.8 Pretreatment of Tissue for Cryostat Sectioning

Probing whole flat mounted retinae can prove problematic if the probe cannot penetrate deep enough into the tissue. In this instance sections of tissue can be cut, creating thinner sections, which allows easier access. The retina can either be cut into transverse sections to include all retinal layers, or en face, to slice through the retina taking sections through single layers. Either way the processing of the tissue is identical, the only difference being the orientation of the retina when it is embedded into the resin.

The eye was dissected from the fish, the lens removed and the eyecup fixed in 4% (w/v) paraformaldehyde in 1X PBS overnight. The following day the retina and RPE were dissected from the eye and placed in 25% (w/v) sucrose for 24 hours. A small (1 cm diameter) foil cup was made and half filled with O.C.T medium. The retina was placed into the cup, manoeuvred into the desired orientation and then quickly placed on dry ice. The cup was filled with O.C.T. medium to ensure the tissue was completely embedded. The medium froze solid around the tissue and formed a hard block, this was cut into sections (2 μm thick) on a cryostat.

Eyes that had not previously been fixed in paraformaldehyde were treated differently. The tissue was slowly thawed in Carnoy’s solution (60% (v/v) chloroform, 30% (v/v) absolute ethanol and 15% (v/v) glacial acetic acid). Once raised to room temperature the cornea was removed from the eye and the remaining tissue dehydrated in ascending
alcohol concentrations. The retina and RPE were then dissected, cryoprotected and fixed as described above.

2.2.7.9 In situ Hybridisation on Cryostat Sections

Day 1

The slides were removed from the −80°C freezer and thawed on the bench for 30 mins. The tissue was refixed by incubating in 4% (w/v) paraformaldehyde in 1X PBS for 15 mins and then washed in 1X PBS twice for 10 mins. The slides were prehybridised as described previously for 1 hour in hybridisation buffer at 60°C. The probes were denatured and made up to 200 µl with hybridisation buffer. The slides were placed in a box lined with 3 mm Whatmann paper pre-wetted in 50% (v/v) formamide. The probe was added to the top of each slide and a thin piece of plastic laid on top. This prevented the slide from drying out but created less suction pressure than a conventional cover slip, which can disturb the tissue when removed. The slides were incubated overnight at 60°C.

Day 2

The plastic was removed carefully and the slides washed in the following:

- 2X SSC for 10 mins at RT
- 2X SSC for 45 mins at 60°C
- 1X SSC, 50% (v/v) formamide, 0.1% (w/v) Tween 1 20 for 1 hour at 60°C
- 0.5X SSC for 1 hour at RT
- PBST for 15 mins at RT three times.
Chapter 2 – Materials and Methods

The samples were then incubated in the blocking solution for 1 to 2 hours. This was replaced with fresh blocking solution containing a 1:5000 dilution of anti-digoxigenin antibody and left shaking overnight at 4°C.

Day 3

To remove any unbound antibody the slides were washed 5 times in PBST for 15 mins. This was followed with 4 washes for 5 mins in detection buffer. The slides were then incubated in the dark in the colour substrate solution. Once the desired colour reaction had occurred the slides were washed in DepC H$_2$O and cover slipped in 1X PBS. Digital images were taken as before.
2.2.8 Basic Histology of Retinal Sections

To identify the different photoreceptor cell types present in a retina and to view how these receptors are laid out with relation to each other some basic histological techniques were employed.

2.2.8.1 Pre-treatment of Retinal Tissue and Formation of Block

Once the eyes were dissected from the fish they were fixed in 2% (w/v) parafomaldehyde, 2% (v/v) glutaraldehyde in 1X PBS. The tissue could be stored in this state for a significant period of time. The retina and RPE were dissected from the eye and were gradually dehydrated through ascending ethanol concentrations (50% (v/v), 75% (v/v), 90% (v/v), 100%), with 25 mins in each. The ethanol was then removed and the tissue very gently tissue dried. Any remaining attached vitreous or choroid was removed to reduce the risk of air bubbles, which would prevent complete penetration of resin. The retina was then placed in historesin for 1-5 nights at 4°C. The retina was placed in a block, embedded in technovit embedding hardener (Heraeus) and left overnight at RT. The following day any peripheral resin was cut away with a hacksaw to create as small a block as possible.

2.2.8.2 Production of Slides

The block was placed in a microtome and cut with either a tungsten steel or a glass blade. 0.5-2.5 μm thick sections were cut. These were floated on 100% ethanol, manoeuvred onto a slide and left on a heated block to dry. The cells were stained with 1% (w/v) toluidine blue, rinsed in dH_2O and then left to dry again. The slides were then
cover slipped in depX and viewed under a light microscope. Images were captured using a digital camera (Nikon) attached to a light microscope.
Chapter 3 - The Visual Pigments of *Aristostomias* and *Pachystomias* Sp.

3.1 Introduction

The bioluminescence produced by most deep-sea fish matches almost perfectly the spectral composition of down welling sunlight, and is therefore restricted to wavelengths between 480 and 490 nm. As described earlier the rod pigments in the retina of these fish are maximally sensitive in this range. The deep-sea stomiids; *Malacosteus niger, Aristostomias* Sp. and *Pachystomias microdon* are unique in emitting far-red light (>700 nm) from suborbital photophores as well as the typical blue (≈ 471 nm) from post orbital light organs (figures 3.1 and 3.2) (Denton *et al.*, 1970; Widder *et al*, 1984; Denton *et al.*, 1985).

*Figure 3.1.* Photograph of *Pachystomias microdon*. The large sub orbital photophore emits red to far-red light, while the post orbital photophores emit blue. Photograph courtesy of Prof. D. M. Hunt.
Figure 3.2. Photograph of the anterior half of *Malacosteus niger*. A large, red photophore is situated just beneath the eye. Post orbitally is a smaller photophore that emits blue light, though appears white under the conditions this photograph was taken. The eye looks red because of a long-wave reflecting tapetum found at the back of the eye. Photograph courtesy of Prof. Ron Douglas, taken by Dr. Peter Herring and modified by Dr. Julian Partridge.

Light that is far-red in composition is practically undetectable by the majority of deep-sea fish. However MSP and retinal extract techniques have shown *Aristostomias* (figure 3.3) and *Pachystomias* to be sensitive to long wavelengths. O’Day and Fernandez (1974) identified a rhodopsin (526 nm) / porphyropsin (551 nm) pair in *Aristostomias scintillans* by partial bleaching. The proportion of these two pigments (rhodopsin/porphyropsin) was calculated as 29:71 (O’Day and Fernandez, 1974). MSP on individual rods showed that these two visual pigments are segregated into two distinct photoreceptor classes, with one class dominated by rhodopsin and the other by porphyropsin (Bowmaker et al, 1988). This is an interesting finding because it is
Figure 3.3. (a) Bioluminescence of *Aristostomias tittmani* is indicated by the dashed line (Widder *et al.*, 1984). Black lines show best-fit sensitivity templates for the visual pigments proposed by MSP, a rhodopsin/porphyropsin pair ($\lambda_{\text{max}}$ 520/ 551 nm), and a rhodopsin ($\lambda_{\text{max}}$ 588 nm). The grey line shows the predicted sensitivity if the opsin responsible for the 588 nm rhodopsin pigment instead contained vitamin A2 as its chromophore and formed a porphyropsin pigment.

(b) Bioluminescence of *Malacosteus niger* is again shown by a dotted line (Widder *et al.*, 1984). The best-fit templates for the rhodopsin/porphyropsin pair ($\lambda_{\text{max}}$ 515/ 540 nm) are shown by black lines. The grey line shows the absorption spectrum of the putative photosensitizer (Douglas *et al.*, 1999).
generally accepted that levels of the two chromophores 11-cis retinal and 3, 4 dehydroretinal are under RPE control, with proportions relatively constant across the retina (Bridges, 1972). However, the mechanism for selection of chromophore in these stomiid species must reside in individual rod receptors. Aristostomias and Pachystomias also contain a rhodopsin with a $\lambda_{\text{max}}$ of $\approx$588 nm as identified by MSP and fresh whole-mount recording (Partridge and Douglas, 1995, Douglas et al., 1998). It is reasonable to propose that there might also be a porphyropsin pigment based on the same opsin with an approximate $\lambda_{\text{max}}$ at 670 nm, especially as the work by O'Day and Fernandez showed that porphyropsin was more commonly found than rhodopsin. Surprisingly, there was no evidence for this proposed pigment, even though it would confer greater sensitivity to the long-wave bioluminescence (Partridge and Douglas, 1995; Douglas et al, 1998).

The closely related Malacosteus niger expresses a single rod opsin, which forms a rhodopsin/porphyropsin pair with $\lambda_{\text{max}}$ values at 517 and 542 nm as determined by MSP (Bowmaker et al., 1988; Partridge et al., 1989) and by outer segment suspension and retinal extract recording (Douglas et al, 1999) (figure 3.3). Refer back to table 1.3 found at the end of chapter one for a list of pigments identified in Aristostomias, Pachystomias, and Malacosteus. The $\lambda_{\text{max}}$ values measured from the same species differ in some cases. This may be due to incomplete segregation of rhodopsin and porphyropsin into distinct photoreceptors. If so, slightly different proportions of the two pigments would create variations in $\lambda_{\text{max}}$.

A single rod opsin gene was isolated from M. niger genomic DNA by PCR using degenerate rod opsin primers (Douglas et al., 1999). To confirm the presence of a single rod gene, digested genomic DNA was probed with a 259 bp fragment of M. niger rod
opsin at low stringency conditions. A single band was labelled, identifying the presence of a single opsin (Douglas et al., 1999).

The 542 nm pigment is long-wave shifted compared to typical deep-sea opsins but would not confer maximum sensitivity to light emitted from the suborbital photophores. Retinal extracts and MSP have shown that within the outer segments there are pigments absorbing maximally at 670 nm (Bowmaker et al., 1988; Partridge et al., 1989). These have been identified as derivatives of bacteriochlorophylls c and d, and it has been hypothesised that these molecules act as photosensitizers increasing the sensitivity of Malacosteus niger to far-red light (Douglas et al., 1999). The proposed mechanism is that the photosensitizing pigment absorbs long-wave light, and indirectly causes the visual pigments to bleach (Bowmaker et al., 1988, Douglas et al., 1998). In fact, when retinal extracts were bleached with either 670 or 640 nm light, there was 50 times greater pigment absorbance with the longer wave light, indicating an interaction with the photosensitizer as this could not be explained by the pigments alone (Bowmaker et al., 1988). The origin of these bacteriochlorophylls is unknown although it is most likely to be through diet. Bacteriochlorophylls c and d are known to occur in green photosynthetic bacteria, though these have never been identified in the open ocean. How these become incorporated in the food chain, which eventually leads to Malacosteus, is at present unknown. The only clue being the unusual diet of this fish when compared to its close relatives. The usual stomiid diet is deep-sea lantern fish (myctophids), whereas Malacosteus feeds on euchaetid and aetideid copepods (Sutton & Hopkins, 1996). Copepods are usually thought to feed on phytoplankton that contain chlorophylls c and d and not on bacteriochlorophylls. Spectra were taken from homogenates and methanol extracts of whole copepods caught in the Gulf of Main
where *Malacosteus* are often found. The spectra were very similar to those of the retinal sensitizers, though it is not known if this was due to chlorophylls or bacteriochlorophylls (Douglas *et al.*, 2000). Sensitivity to long-wave light is further enhanced in *Malacosteus* by the possession of a bright red tapetum that is constructed from the carotenoid, astaxanthin (Locket, 1977; Somiya, 1982; Bowmaker *et al.*, 1988).

*Aristostomias*, *Pachystomias* and *Malacosteus* all possess yellow lenses, which reduce the level of short-wave light reaching the retina (Douglas and Thorpe, 1992). It has been proposed that this may help to distinguish between blue down-welling light and red light emitted from photophores by increasing the relative brightness of long-wave light (Douglas and Thorpe, 1992).

Sensitivity to long wavelengths would allow for a discrete form of camouflage in the deep seas. Bioluminescence is essential for many species living in a dim light environment but can create problems with detection. The stomiid dragonfish use long-wave bioluminescence, which is invisible to most other deep-sea fish. This enables intraspecific communication without alerting predators, and prey illumination without detection. Recently, retinal extracts were made from *Myctophum nitidulum*, a species of myctophid that are commonly eaten by stomiids. Partial bleaching identified two pigments, a rhodopsin with a $\lambda_{\text{max}}$ at 466 nm and a porphyropsin with a $\lambda_{\text{max}}$ at 521 nm (Douglas *et al.*, 2003). These values are too far apart to be considered a pigment pair, and it is more likely that there are two different opsins being expressed, which are binding to different chromophores. The same situation appears to also be true for another myctophid, *Bonapartia pedialiota*, which has a rhodopsin with a $\lambda_{\text{max}}$ at 471 nm and a porphyropsin with a $\lambda_{\text{max}}$ at 514 nm, again too separated to be formed from the
same opsin (Douglas and Partridge, 1997). These species appear to have long-wave shifted their visual sensitivity, perhaps to enable detection and escape from stomiid predators.

**Potential Long-Wave Tuning Sites**

The rod opsin gene for *Malacosteus niger* has been sequenced and potential tuning sites identified (table 3.1). Three sites that may be responsible for the long wave shift to 517 nm are: Phe208Tyr, Phe261Tyr, and Ser292Ile. Substitutions at positions 292 and 261 were mentioned previously with respect to Lake Baikal and other deep-sea fish. A rod opsin sequence has also been isolated from *Aristostomias tittmanni*. Phylogenetic analysis indicates that this sequence is the pigment absorbing maximally at around 520 nm. The Phe261Tyr and Ser292Ile substitutions are present though there is no change at position 208. This suggests that this substitution is not long-wave shifting, but in fact shifts the pigment of *Malacosteus niger* 5 nm to the blue (Hunt et al, 2001). However, a second opsin sequence has not been isolated from any stomiid species.
<table>
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<th>Residue found in <em>M. niger</em></th>
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<td>Tyr → Phe</td>
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<td>16 nm blue shift</td>
<td>Ile</td>
<td>Ile</td>
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</table>

Table 3.1 The tuning sites previously discovered in deep-sea fish, and the corresponding residues in *Aristostomias tittmanni* and *Malacosteus niger*. The substitutions at positions 124, 261 and 292 in *Aristostomias* and positions 261 and 292 in *Malacosteus* are to amino acids with similar properties to the original occurring amino acids before blue shifting. However, at position 83, the stomiids possess the blue tuning asparagine rather than aspartate. Data from Hunt *et al.*, 2001.
3.2 Aims

1. Identification of opsin genes expressed in the retina and/or present in the genome of

   *Aristostomias tittmanni* and *Pachystomias microdon*.

2. Estimation of the $\lambda_{\text{max}}$ of these pigments by *in vitro* regeneration.

3. Examination of retinal histology to ascertain if the retina contains more than one

   morphologically distinct class of photoreceptor.
3.3 Results

3.3.1 Isolation of Aristostomias tittmanni and Pachystomias microdon Rod Opsin Sequences

Messenger RNA was extracted from a single Aristostomias tittmanni eye using the QuickPrep® Micro mRNA Purification Kit (Pharmacia Biotech). DNA was synthesised from this RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (GibcoBRL Ltd, Life Technologies). This cDNA was then used as the template for a degenerate PCR with primers designed to conserved regions of teleost rod sequence. These were primers FRHO B (5'-CTTCCYRTCACTTCCCTAC-3') and FRHO B-(5'-TGCTTGTTCAWGCAGATGTAG-3'), which had both been used previously to amplify a fragment between nucleotide positions 196 and 956 (Hunt et al., 2001). The PCR reaction was repeated containing 4 mM MgCl₂, and 12.5 μM of both the forward and reverse primers. An initial denaturation of 3 mins at 94°C was followed by 35 cycles with denaturing, annealing (56°C) and extension steps of 30 s. A negative control was also run (as for all subsequent PCRs) at the same time, which contained all the reagents minus template DNA. A fragment of approximately the right size was amplified (figure 3.4 A). The product was excised, spun through a Wizard™ column, cloned into the pGEM-T-Easy vector (Promega) and sequenced from the 3' and 5' ends. The resulting sequences when aligned just overlapped though the accuracy of the sequence in this region was low. All sequences were the same with no evidence for more than one rod opsin being expressed.
To obtain better sequence information for the middle region, specific primers were designed, ROD437+ (5'-CCACCTCCTGGATTTATGGC-3') and ROD607- (5'-GCAGAAGATGACGATCATAGG-3'). A standard PCR was run at three annealing temperatures, 57°C, 58°C and 59°C. When viewed by gel electrophoresis, the reactions at 57°C and 59°C had been more successful in amplifying the expected product (figure 3.4 B). These were excised and cloned. The resulting sequence confirmed the nucleotides within the region of reduced accuracy in the middle of the opsin sequence.

The FRHOB/B- fragment was used to design 3' RACE outer and inner gene specific primers (Outer 3'-TGATGGTGGTCTCTTACCTGG-5', Inner 3'-ACCAATCTTCATGGGTCTCC-5'). 3' RACE was performed to manufacturer's instructions and both outer and inner PCR reactions amplified clearly identifiable products. The outer PCR amplified a fragment of approximately 650-700 bp in size (figure 3.4 C), and the inner PCR produced a fragment of approximately 500 bp (figure 3.4 D). Both were excised, cloned and sequenced and provided sequence information that enabled completion of the 3' end of the opsin cDNA fragment. To consolidate, a further PCR was performed with primers amplifying between nucleotide positions 808 and 1024 bp (AROD808+ 5'-TGTGCTGCGAAAGAACC-3', AROD1024- 5'-GCAAACTGTCTTTAGGTCG-3'). The PCR conditions were standard with a 58°C annealing step. The fragment amplified was of the expected size (figure 3.4 E) and was cloned and sequenced to confirm the 3' end.

5' RACE was unsuccessful in amplifying the 5' end of the rod opsin sequence from cDNA. Genomic DNA was extracted from a whole body (3.11 g stored at -80°C) using a standard phenol/chloroform procedure. Approximately 0.3 μg of this DNA was used
Chapter 3 – Aristostomias and Pachystomias

as the template for walking PCR (see methods 2.2.2.3). The inner and outer walking PCR primers were designed to the identified sequence (WKAROD outer 3’-GCAACAGCCAGGTTCCAGCAGG-5’, WKAROD inner 3’-CTCGATGGTAACGTAGAG-5’). Both the outer and inner walking PCRs amplified fragments of expected size as detected by gel electrophoresis (figure 3.4 F). These were cloned and sequenced. This resulted in the completion of the 5’ end of A. tittmanni rod opsins. The cDNA sequence from the start codon to the poly (A) tail is shown in figure 3.5. Approximately 200 bp of 5’ upstream sequence is shown in figure 3.6. The translated sequence shares only 91% identity with that obtained by Hunt et al. (2001).
Figure 3.4. Gel images of the degenerate PCR, 3’ RACE and genomic walk reactions used to amplify *Aristostomias tittmanni* rod opsin. A, Degenerate PCR with FRHOB+ and FRHOB- primers. B, PCR with specific primers (437+/607-) to confirm the middle section of isolated sequence. C, Outer and D, Inner 3’ RACE reactions. E, PCR with specific primers (808+/1024-) to confirm 3’ sequence. F, Second round genomic walk PCR performed at 1.5 and 3.0 mM MgCl₂ concentration. Negative controls contain the same reagents minus template DNA and are run on the same PCR programme in conjunction with the samples.
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Figure 3.5. Complete cDNA sequence for Aristostomias tittmanni rod opsin. The translation start and stop codons are highlighted in blue. The poly(A) tail is shown in red. The coding sequence is 1036 bp.

Figure 3.6. Approximately 200 bp of 5’ upstream sequence from Aristostomias tittmanni rod opsin. The start codon is shown in blue.
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The Pachystomias rod opsin sequence was amplified from genomic DNA. A PCR was performed on approximately 0.6 μg gDNA using primers designed from the start and stop codons of the Aristostomias isolated sequence, AristoRodF (3’-GCGCGAATTCCACCATGAACGGCACGGAG-5’) and AristoRodR (3’-CGGCGTCGACGCCGCCGGTGACACGGAG-5’). An amplified product of approximately 1 kb in size was identified when the 50 μl reaction was separated by gel electrophoresis (figure 3.7). The product was excised, cloned and then sequenced from both the 3’ and 5’ ends. The resulting reads produced around 700 bp of good sequence, providing information on the whole amplified fragment. A rod opsin sequence has therefore been isolated from Pachystomias microdon genomic DNA, complete except for the first and last 20 bp, which are derived from non-specific primers (figure 3.8). The genomic sequence contains no exons as expected for a teleost rod opsin (Fitzgibbon et al., 1995).

Figure 3.7. Gel picture of product amplified from Pachystomias microdon gDNA using Aristostomias specific primers. The approximately 1 Kb product was excised, cloned and sequenced.
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ATGAACGGCA CGGAGGGACC AGACTCTATAT GTTCCAATGC TAAATACCA
AGGCATTGTG AGGAGCCTCT ATTGTATGCC TCAGTACTAC CTTGTCAGCC
CAGTGGCATA CTTCATACTG GGAATCTACA TGTTCTCTCT CATCTTCACC
TGCTTCCCAG TCAACTTCCT GACCCCTGTAT GTTACCATGG AGAACAAGAA
GCTGAGGACC GCCCTTACTG ACATCTCCTCT GAACTCTGCT GTTGCATTACC
TCTTCATGTT GTTCGCTGGA TTACACGACCA CGGTATACAC TTGCATGAA
GGCTACTTCC TCTTAGGAGC GTTAGGCTGC AACCTTGAGG GGTTCCTCGC
TACCTCAGGT GGTGAGATTTG CCCCTGTTGTC CTCTGCTGTTG CTGGGTATGC
AGAGGTATCT GTTGCTGCTGC AACCTATTCA CCAACTTCGG CTTCCGGGAG
AACACACGCC TCATTGGATT GGCCTTCTCC TGGGTGATGG CTCCTACGCT
TTCCGTGCCG CCTCTGTTGG GATGTTGCCG TTACATCCCA GAGGCTTCC
AGGTGCTCTG CGGATTCGAC TACTACACCC GCCAGAGCAGG TTACACAAAC
GAGTCCTTGG TCATCTACAT GTTCATACCA CACCTTACAA TTCCCATTAG
CATCATCACC TTCTGTATAT GCCGCTTCTG GTGCCGGCTA AAGGAGCGG
CCGGCCCCC GCAGGAGTCT GAGACCCACG AGAAGGCGCA GAAGGAGTTG
ACTCAGCGCC AGACGCTTGT GGGATCTCTG AAACAGGATG TCGGTATCC
GCCAACCTCT CATGCTATTG CCGATCTTCT TCGCCAAGAG CTGGGGTCCC
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GGCTAACCAC GTGTGGCCAG GCAGGACTCAG AAGCAGGACG GAGCAGAGG
CCGGCAAGAC CGAGGGCTTG TCCGGCTCTT CGGTGCACC GGC

Figure 3.8. *Pachystomias microdon* rod opsin sequence, complete except for the very 3’ and 5’ ends, which are non-specific primer sequences (shown in blue).

The translated *Aristostomias* and *Pachystomias* sequences are shown aligned in figure 3.9. Table 3.2 shows percentage nucleotide and amino acid identities between the rod opsins identified from *Aristostomias tittmanni*, *Pachystomias microdon* and *Malacosteus niger*, the latter from Hunt et al. (2001). The highest identity (86% amino acid; 91% nucleotide) is between *Aristostomias* and *Pachystomias*, though all three species share approximately 90% nucleotide identity.
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**Figure 3.9.** *Pachystomias microdon* and *Aristostomias tittmanni* translated rod opsin sequences. Conserved residues are blocked in grey. The amino acids highlighted in purple at the start and end of the *Pachystomias* sequence are from primers and are therefore not *Pachystomias* specific. Key residues are also highlighted. Lys296 in red forms the Schiff’s base and Glu113 in orange forms the counterion. The cysteine residues at 110 and 188 that from a disulphide bridge are shown in blue and possible phosphorylation sites at the C-terminus are in green.
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Nucleotide

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<tr>
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<td>82%</td>
<td></td>
<td>88%</td>
</tr>
<tr>
<td>P. microdon</td>
<td>86%</td>
<td>80%</td>
<td></td>
</tr>
</tbody>
</table>

Amino acid

Table 3.2. Percentage nucleotide and amino acid identities found between the rod opsins identified from Aristostomias tittmanni, Pachystomias microdon and Malacosteus niger.

The amino acid residues found within the seven helical domains are shown aligned with rod opsins isolated from 28 species of deep-sea fish (figure 3.10) (Hope et al., 1997; Hunt et al., 2001). Three important substitutions are often found in short-wave shifted deep-sea rod opsins, these are Asp83Asn, Tyr261Phe and Ser292Ala (Hope et al., 1997; Hunt et al., 2002). Figure 3.10 highlights these residues across all species shown. The Tyr261Phe and Ser292Ala substitutions are commonly found in short-wave shifted rod opsins, but absent in the three long-wave shifted pigments. This is further evidence that sites 261 and 292 are important tuning sites in deep-sea rod opsins. The Asp83Asn substitution is present in the majority of species, including Malacosteus, Aristostomias and Pachystomias even though these rod opsins are long-wave rather than short-wave shifted. This substitution may be important for efficient regeneration under high pressure or low temperatures, and not key for spectral tuning. The absence of short-
Chapter 3 – Aristostomias and Pachystomias

wave substitutions at sites 261 and 292 would shift the $\lambda_{\text{max}}$ of the stomiid pigments to at or above 500 nm.
<table>
<thead>
<tr>
<th>Species</th>
<th>Helix 1</th>
<th>Helix 2</th>
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<tr>
<td>Aristostomias tittmanni</td>
<td>MNGTEGPDFYVPSMNAGIVRSYPYFQYYLVSVPVAYM</td>
<td>YLSNYILLNLAVNLFMVF CGFTTIYTCMN</td>
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<tr>
<td>Pachystomias microdon</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
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<td>I...RT...V...S..D.A.SL</td>
<td>I...M.N.</td>
</tr>
<tr>
<td>Ichthyococcus ovatus</td>
<td>Y...I...L.TT.M.</td>
<td>Y...I...L.I.F.S.I.</td>
</tr>
<tr>
<td>Stomias boa</td>
<td>T...K.V...F...E.WFSL</td>
<td>A...L.I.F.S.I.</td>
</tr>
<tr>
<td>Chauliodus sloani</td>
<td>Y...I...L.TT.V...T.R...RT.IV</td>
<td>A...L.S.I.</td>
</tr>
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<td>A...S...V...I.</td>
</tr>
<tr>
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<td>Y...I...L.TT.V...G</td>
<td>A...I.V...I.</td>
</tr>
<tr>
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<td>V...I...V...L.M.S.</td>
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<td>V...I...I.M.S.S.</td>
</tr>
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<td>Gonostoma bathyphilum</td>
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<td>A...V...I.M.S.S.</td>
</tr>
<tr>
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<td>A...L.I.F.A.I.</td>
</tr>
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<td>A...V...V...L.S.H</td>
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<td>A...G.L...I.S.H</td>
</tr>
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<td>A...G.L...I.S.H</td>
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<td>A...RG.I.</td>
</tr>
<tr>
<td>Cataetys laticeps</td>
<td>A...A.A...A...I.LG.I</td>
<td>VQ...I...M.S.H</td>
</tr>
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<td>A...I.LG.I.</td>
</tr>
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<td>A...I.LG.I.</td>
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<td>F...I...M...A</td>
<td>VQ...I...M.S.H</td>
</tr>
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<td>L.VG.I...H</td>
</tr>
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<td>L.VG.I...H</td>
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<td>Ichthyococcus ovatus</td>
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<td>Conocara salmonea</td>
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<td>Alepocephalus bairdi</td>
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<td>Anoplogaster cornuta</td>
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<tr>
<td>Bathysaurus ferox</td>
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</tr>
<tr>
<td>Bathysaurus mollis</td>
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Aristostomias tittmanni
Pachystomias microdon
Malacosteus niger
Ichthyococcus ovatus
Stomias boa
Chauliodus sloani
Idiacanthus fasciola
Chauliodus danai
Photostomias guernei
Gonostoma elongatum
Gonostoma bathypilum
Vincicorpus mimoraria
Arghyopelecus aculeatus
Arghyopelecus gigas
Diaphus rafinesquii
Bolinichthys indicus
Ceratocapella warmingii
Gonostoma subouritale
Bathysaurus elongatum
Bathysaurus mazzonii
Argyrosema mazzonii
Lampanyctus australis
Phyloceratichthys cystophorus
Coryphaenoides guntheri
Coryphaenoides leptolepis
Bassosetus compressus
Cataleyx laticeps
Conocara salmonea
Alepocephalus bairdii
Anoplogaster cornuta
Hoplostethus mediterraneus
Bathysaurus ferox
Bathysaurus mazzonii
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Pachystomias microdon
Malacosteus niger
Ichthyococcus ovatus
Stomias boa
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Idiacanthus fasciola
Chauliodus danae
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Gonostoma bathyphilum
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Ceratocopeius warmingii
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Phycis biennoides
Coryphaenoides guntheri
Coryphaenoides leptolepis
Bassoreus compressus
Cataetyx laticeps
Conocara salmona
Aleopocephalus bairdii
Anoplogaster cornuta
Hoplostethus mediterraneus
Bathysaurus ferox
Bathysaurus mollis

Fig 3.10 Deduced amino acid sequences within the seven helix domains for twenty eight species of deep-sea fish. The previously identified short-wave shifting substitutions at Positions 83, 261 and 292 are shown in blue. Residues identified in this study that may long-wave shift the rod pigments of *Malacosteus, Aristostomias* and *Pachystomias* are highlighted in red. The sequences from all species except *Pachystomias* and *Aristostomias* were isolated by Hunt *et al.*, 2002.
3.3.2 Identification of Potential Long-Wave Shifting Substitutions

To identify potential tuning sites that might further long-wave shift the $\lambda_{\text{max}}$ to around 590 nm, the amino acid sequences from *Malacosteus*, *Aristostomias* and *Pachystomias* rod opsins were directly compared. No substitutions were identified that affected a change in polarity in a residue that either faces the chromophore pocket or is in close proximity to the chromophore, and that was common to the *Aristostomias* and *Pachystomias* sequences. However, there is an alanine to serine substitution at position 42 (bovine numbering). The amino acid found here is not in direct association with the binding pocket or chromophore but is adjacent to the tyrosine and methionine residues at positions 43 and 44 respectively, which both lie very close to the Schiff’s base (Palczewski *et al.*, 2000; Teller *et al.*, 2003). All short-wave shifted deep-sea sequences contain alanine at this site, the only exception being *Photostomias guernei* and the lizardfishes *Bathysaurus ferox* and *Bathysaurus mollis*. *P. guernei* is a stomiid and a member of the Malacostinae (loosejaw) subfamily as are *Aristostomias*, *Pachystomias* and *Malacosteus* (Nelson, 1994). The lizardfishes are members of the Synodontidae family of the Aulopiformes order and are therefore not closely related to the stomiids (Nelson, 1994). It may be that serine at position 42 long-wave shifts the pigment, and a compensating short-wave shifting substitution is present in the *P. guernei*, *B. ferox* and *B. mollis* pigment that is as yet unidentified. Though it seems unlikely that the putative substitution would have been maintained in species that do not require long-wave sensitivity. A further possible tuning substitution is present in the sequence of *Malacosteus niger*, though it is absent from *Aristostomias* and *Pachystomias*. At position 208 there is a phenylalanine to tyrosine change. These are both aromatic residues though phenylalanine is non-polar whereas tyrosine is polar due to an extra OH
side chain. Residue 208 is within helix V and does potentially face into the binding pocket as demonstrated by Baldwin's model (Baldwin et al., 1993), though the crystallized structure of bovine rod opsin does not identify this residue as being near to the Schiff's base or chromophore binding pocket (Palczewski et al., 2000, Teller et al., 2003). In summary, there are no strong candidates for a potential long-wave shift to 590 nm in the rod opsins identified from Aristostomias and Pachystomias.
3.3.3 In Vitro Expression and Regeneration with 11-cis Retinal of Aristostomias Rod Opsin

Once the complete coding sequence for *Aristostomias tittmanni* had been identified from cDNA, primers were designed to amplify the whole opsin from the start and stop codons. The primers AristoRodF (3'-GCGCGAATTCCACCATGAACGGCACGGAGS') and AristoRodR (3'-CGGCGTCGACGCCGCCGGTGACACGGAG-5') contained EcoRl (AristoRodF) and SalI (AristoRodR) restriction enzyme sites, as shown in red. These sites were cut with the appropriate restriction enzyme to create complimentary ‘sticky ends’ to enable cloning into the expression vector pMT4. The ligated vector was then transfected into the 293T human embryonic kidney (HEK) cell line. Protein extraction, regeneration and bleaching proceeded as described in the methods section 2.2.6.

The experiment was carried out on five separate occasions, each time alongside a bovine rod control. The *Aristostomias* pigment failed to regenerate in each instance, whereas the bovine rod pigment generated a normal light sensitive response (figure 3.11). The *Aristostomias* rod opsin appears to be extremely unstable in this regeneration system. There is an increase in absorbance difference as wavelength increases, though the values are negative due to a baseline shift between the dark and bleached scans. A small peak at around 520 nm was present in the difference spectra when regenerated at pH 7 in PBS, though this could be artifactual because of the high level of noise. The experiment was also performed at pH 6.6 in an alternative buffer containing 50 mM HEPES (N-[2-Hydroxyethyl]piperazine-N’[2-ethanesulfonic acid]), 140 mM NaCl, 3 mM MgCl₂ and 20% (w/v) glycerol, but the pigment still did not show a light response.
It was proposed that the reason for the difficulty in regenerating the pigment was linked to its deep-sea function and that greater than atmospheric pressure is required to form a functional pigment.
Figure 3.11 Regeneration of bovine and *Aristostomias* recombinant rod opsins with 11-cis retinal. The bovine response displays the typical absorbance curve, with a $\lambda_{\text{max}}$ at 499 nm when fitted to a Govardovski template. The *Aristostomias* response is almost flat, very noisy and rises as wavelength increases. Graphs are on different scales. Absorbance Difference (Ab. Diff.).
3.3.4 Regeneration of Aristostomias and Bovine Rod Opsins Under Pressure

After many attempts to regenerate *Aristostomias* rod opsin, it was concluded that a reliable response was not possible in the system currently employed. It was speculated that the problem may be linked to regeneration at atmospheric pressure, 1000 times less than normally encountered. Perhaps the opsin protein will only form the correct 3D structure when subjected to these pressures, i.e. is 'squeezed' into a functional configuration. *In vivo* the whole process from translation of the protein, to bleaching of the pigment is under the same constant pressure of around 100 bar. This is impossible to recreate *in vitro*, as manipulation of the cells and extracted protein can only be performed at atmospheric pressure. However, a high pressure chamber could be utilised to administer high pressure as the opsin was actually being regenerated with 11-cis retinal.

Cells were transfected with pMT4 constructs containing either bovine or *Aristostomias* rod opsin (as described previously). The pressure chamber was filled with water to remove any air bubbles. The cells were thawed on ice, resuspended in 3 ml PBS pH 7, and 11-cis retinal was added to a final concentration of 40 μM. The bovine or *Aristostomias* sample was loaded into a glass cuvette, sealed with parafilm and placed into the pressure chamber. The chamber was sealed and the pressure gradually increased to 100 bar (atm). The sample was maintained at pressure for 1 hour. A pressure of 100 bar was created because *Aristostomias* Sp. is found at depths close to 1000 m, where the pressure will be 100 times atmospheric. It was impossible to carry out the remainder of the experiment under pressure because of the inaccessibility of the samples whilst in the...
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... chamber. It was therefore hoped that the initial insertion of the chromophore was the critical step, and that once the rhodopsin pigment was formed it would remain functional. After the opsin had been isolated and washed on a 1D4 antibody column (see methods section 2.2.6), the final pigment was eluted in 400 μl. Half of this volume was stored away from light at -20°C. The remainder for both Aristostomias and bovine were placed in a spectrophotometer and a dark scan recorded from 200 to 800 nm, under normal atmospheric pressure. Bovine rhodopsin gave a clear light response curve, with a λ<sub>max</sub> close to 500 nm. The sample was slowly bleached through a series of filters; 1 min at 625 nm, 1 min at 585 nm, 1 min at 495 nm, 2 min at 495 nm, 2 min at 495 nm, 4 min at 485 nm, 10 min at 495 nm and finally 10 mins with sunlight (figure 3.12). Regenerating under pressure had not affected the ability of bovine opsin to form a functional pigment. Aristostomias rhodopsin produced no initial dark response and therefore careful bleaching was not necessary (figure 3.13). The Aristostomias pigment stored at -20°C was thawed on ice and scanned under pressure (100 bar). This still elicited no light sensitive response. The limitations of this experiment may have been too great to ascertain whether high pressure is necessary for the Aristostomias rod opsin to function.
Figure 3.12 Dark and partial bleach spectra from recombinant bovine rod opsin, regenerated with 11-cis retinal at 100 bar. Difference spectrum is shown inset fitted to a Gavordovski template with a $\lambda_{\text{max}}$ of 500 nm.
Figure 3.13. (A) Dark and bleached spectra for recombinant *Aristostomias tittmanni* rod opsin regenerated with 11-*cis* retinal at 100 bar. (B) Difference spectrum shows no evidence of a light sensitive response, though the upward trend in absorbance is still evident.
3.3.5 Search for a Second Aristostomias Pigment

As only a single opsin had been identified in both *Aristostomias* and *Pachystomias*, it was necessary to undertake a comprehensive search for the proposed second visual pigment. MSP and whole-mount experiments had identified this pigment to have a $\lambda_{\text{max}}$ close to 590 nm. This sensitivity is more likely to be due to a LWS cone pigment than from a rod, but no cone-like cells had been identified in the retina of these species. Therefore a number of primers were designed to conserved regions in either the RH1 or LWS/MWS opsins from teleost species that were deposited on the NCBI database (http://www.ncbi.nlm.nih.gov). A list of all the forward and reverse primers can be found in table 3.3. These were used in RT-PCR and degenerate RACE on retinal mRNA and PCR and genomic walking on genomic DNA, under a whole range of conditions. On every occasion the rod primers amplified the rod opsin previously identified in this work. This was ascertained by cloning all amplified fragments into pGEM-T-Easy, followed by sequencing between 15-25 of these clones. In every instance only the known rod sequence was generated. The MWS/LWS primers did not amplify any opsin-like products, again determined by cloning and sequencing PCR amplified products. Degenerate primers designed to conserved regions of all opsin classes were also used in PCR. These again only amplified the identified rod opsin and no other. The primers were designed so that a vast number of different combinations of forward and reverse primers were possible, so that the number of different reactions performed was well over 100. The template and MgCl$_2$ concentrations, and length and temperature of cycling parameters were also varied, but even so no second pigment was identified.
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<th>LWS Reverse</th>
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<td>MAP4R-5'-TTATGCRGGAGCCACAGAGG-3'</td>
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<td>LW980R-5'-WGGRTGGAGGCATAKCC-3'</td>
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<td>MAP9R-5'-TCGTTGTGTAACCTTCCG-3'</td>
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<td>Rod607-5'-GCAGAAGACATGAGCTACATAG-3'</td>
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<td>RodATG-5’-ATGAACGGGCACRGAGGG-3’</td>
<td>Rod545-5’-CGGTCKGGGATGTAACG-3’</td>
</tr>
<tr>
<td>Rod1+-5’-ATGAACGGGCACRGARGGA-3’</td>
<td>Rod737-5’-GCCCTCTGSGTGTTCTC-3’</td>
</tr>
<tr>
<td>Rod52+-5’-GGCRTKGTBGGAGCCCC-3’</td>
<td>Rod932-5’-TGTTGATGCGARATGATG-3’</td>
</tr>
<tr>
<td>Rod186+-5’-CATCGACACAACAAGAGC-3’</td>
<td></td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>All Opsin Forward</th>
<th>All Opsin Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>796F-5’-ACCACCCAGAAGGCAGAGAAG-3’</td>
<td>993R-5’-GACATAGATGATGAGGGTTGTA-3’</td>
</tr>
</tbody>
</table>

Table 3.3. List of forward and reverse primers used to try to isolate a second opsin sequence from *Aristostomias* mRNA and gDNA.
A different approach was to perform a Southern hybridisation on digested genomic DNA and to probe with different opsin classes to determine their presence or absence in the genome. This gave no positive results and further investigation identified the problem as rapid degradation of the genomic DNA. All genomic DNA that had been stored at the recommended +4°C had completely degraded although aliquots stored frozen at -20°C had maintained their integrity (figure 3.14). However, when this gDNA was used in a restriction enzyme digestion experiment, control samples containing no enzyme showed rapid digestion at 37°C (figure 3.15). It was therefore not possible to carry out a Southern hybridisation. The most likely explanation for this is that a contaminant was extracted with the DNA that acted as a DNase. Degradation occurred at 4°C, but if the temperature was raised then the speed of degradation increased. Heating to 65°C can often denature DNases. This was attempted, but only speeded up the degradation reaction even further (figure 3.15).
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Figure 3.14. *Aristostomias* genomic DNA separated on a 0.8% agarose gel by electrophoresis. The first two lanes show DNA stored at +4°C. DNA stored at −20°C is shown in the lane on the right. High molecular weight DNA is marked with an arrow.

Figure 3.15. *Aristostomias* genomic DNA separated on a 0.8% agarose gel by gel electrophoresis. The first lane contains DNA thawed on ice and then held at 4°C for two hours. The next two lanes contain DNA held at 65 or 35°C with the restriction enzymes AccIII or EcoRI respectively and the appropriate buffer. The final two lanes were treated in the same way but contained dH₂O instead of enzyme.
3.3.6 Histology of the Pachystomias Retina

All eye specimens available from *Aristostomias* and *Pachystomias* had been stored at -80°C without prior cryoprotection. Therefore the tissue was thawed slowly to reduce cell disruption. A single *Pachystomias* eye was placed in cold Carnoys solution (60% (v/v) absolute ethanol, 30% (v/v) chloroform, 10% (v/v) glacial acetic acid) and kept at -20°C for five days, to promote penetration of the solution to replace water. After this period the eye was placed at 4°C for two hours and then held at room temperature. The eye was dehydrated and fixed for histology as described in the methods. The retina was orientated when embedded to lie transverse to the front of the block. Sections of 1-2.5 μm thickness were cut with a steel blade on a microtome, and dried onto glass slides. These were stained and viewed under a light microscope. Photographs taken by an attached digital camera are shown in figure 3.16. The different layers of the retina, especially the inner nuclear and plexiform layers, have become distorted probably due to the initial preparation of the tissue. The RPE layer can be distinguished and is rather thin, which is characteristic of deep-sea retinas. The rod outer segments have become intertwined, but it can still be seen that they are longer and thicker than those found in typical teleosts. Across all sections studied no regional variation was found, there being only a single distinct class of rod photoreceptor. There was no evidence for any cone-like cells in the retina of *Pachystomias*.
Figure 3.15. Photographs of transverse sections through the *Pachystomias microdon* retina. There is some cell disruption caused by freeze/thawing un-cryoprotected tissue. The long and thick outer segments (ROS) as commonly seen in deep-sea fish are clearly visible. The RPE layer is rather thin and sparse. The other retinal layers are hard to distinguish though the layer separated from the receptors and on the opposing side to the RPE appears to contain ganglion like cells (GCL). Scale bars = 100 μm.
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3.4 Discussion

The three genera of stomiid, Malacosteus, Aristostomias and Pachystomias are interesting because they emit long-wave light from sub-orbital photophores. The wavelength composition of this bioluminescence reaches into the far-red. Since the majority of deep-sea fish possess only a single visual pigment with a $\lambda_{\text{max}}$ close to 480 nm, this far-red bioluminescence would be undetectable. Malacosteus, Aristostomias and Pachystomias are closely related, all members of the sub family Malacosteinae, but Malacosteus appears to have evolved quite a different mechanism to become long-wave sensitive to that found in Aristostomias and Pachystomias. As previously mentioned Malacosteus expresses a single visual opsin within its photoreceptors, with $\lambda_{\text{max}}$ values at $\approx 517$ nm (when bound to 11-cis retinal) and $\approx 542$ nm (when bound to 3,4 dehydroretinal) (Bowmaker et al., 1988; Partridge et al., 1989; Douglas et al., 1999).

Sensitivity to longer wavelengths is achieved by bacteriochlorophylls c and d, which act as photosensitizers (Bowmaker et al., 1988; Douglas et al., 1998). A photosensitizer has not been identified in Aristostomias and Pachystomias but rather a second visual pigment has been discovered that has a $\lambda_{\text{max}}$ at 580-590 nm (Partridge and Douglas, 1995; Douglas et al., 1998), though Denton et al. (1970) identified a single pigment with a $\lambda_{\text{max}}$ at around 575 nm. Interestingly digitonin extraction, whole-mounts on frozen tissue or MSP on individual outer segments has never isolated this pigment from either Aristostomias or Pachystomias (O'Day and Fernandez, 1974; Knowles and Dartnall, 1977b; Bowmaker et al., 1988; Partridge et al., 1989; Crescitelli, 1991; Partridge and Douglas et al., 1998). The long-wave pigment has been found in both species by fresh whole-mount recording or by MSP on patches of cells (figure 3.16).
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(Partridge and Douglas, 1995; Douglas et al., 1998 showing unpublished data). The fresh Aristostomias retinal whole-mounts were sequentially bleached in the following manner: a). initial dark scan 10 min after the addition of hydroxylamine; b). 10 min exposure to 699 nm light; c). 60 min 699 nm; d). 10 min 666 nm; e). 30 min 666 nm; f). 60 min 666 nm; g). 60 min 666 nm; h). 60 min 666 nm; i). 77 s 609 nm; j). 77 s 609 nm and k). 6 min 521 nm. From these measurements, three separate difference spectra were extracted using scans a-c, c-e and h-j. To aid understanding of this method it would be helpful if the authors had included spectra taken from Malacosteus niger and showed that separating the partial bleaches into three groups in a similar manner identifies two pigments and not three. However, this may be impossible because of the presence of a red tapetum and retinal photosensitizers. The normalized difference spectra show three visual pigments with $\lambda_{\text{max}}$ (after best-fit templates have been fitted) at 531 nm, 550 nm and 586 nm. The 531 nm and 586 nm pigments were both fitted to rhodopsin templates while the 550 nm pigment is identified as a porphyropsin because of the breadth of its absorbance difference curve. All three pigments have been normalized to have a maximum absorbance of 1.0. This prevents any comparison of the relative proportions of the three pigments in the retinal whole-mount and no reference is given to the raw difference spectra data, which would provide this information. The experiment was repeated on Pachystomias fresh whole-mount retina and three pigments at 520, 563 and 595 nm were identified, though the data for the long-wave pigment is extremely noisy and does not fit well to the rhodopsin template curve. The published MSP data taken from Aristostomias shows the normalized absorbance recordings and identifies three visual pigments. Out of the 60 individual outer segments measured, 42 contained a 550 nm pigment, 18 contained a 518 nm pigment and none contained a more long-wave pigment. Further MSP on patches of several cells produced two absorbance scans,
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which were averaged. There is no indication of whether the 518 and 550 nm pigments were also present in the tissue patch MSP, in fact the data suggests that the patch only contains the 586 nm pigment. The absorbance curve is abnormal with a large short-wave absorbance (twice than at the reported $\lambda_{\text{max}}$) at around 419 nm. This is explained in the paper by three possibilities: the pigment may have bleached before measurement, is unstable when aldehyde treated or is due to a short-wave absorbing photostable pigment. The first explanation seems unlikely, as a scan from a bleached pigment would be the same as that for all-trans retinal, which has a $\lambda_{\text{max}}$ at 380 nm. This value is rather removed from the $\lambda_{\text{max}}$ reading of 419 nm. The pigment may have become denatured on aldehyde treatment, but there is no evidence to support or oppose this argument. Perhaps the most logical explanation is that the reading is due to a photostable pigment. Could the $\lambda_{\text{max}}$ at 581 nm also be attributed to the same pigment? It is unclear from the paper whether the 581 nm ‘pigment’ was photosensitive as no bleaching data is shown. As the long-wave pigment has only been identified using methods that record from whole retina, it may be that the long-wave absorbances recorded are from a photostable pigment or were not from outer segments. It is still unclear therefore whether Aristostomias and Pachystomias have two or three visual pigments, and this study aimed to address this.

A rod opsin gene was cloned from Aristostomias tittmanni by Hunt et al. (2001). The presence of this expressed sequence has been confirmed in this study and has also been identified in Pachystomias. Even though the two identified sequences are not 100% identical the variances can be explained by problems with species classification, or perhaps by sequencing errors In the previous study genomic DNA was isolated from a single species. However, in this work mRNA was extracted from two separate eyes and
gDNA from two livers and one body. A classification error is unlikely here as the RH1 opsin from all samples was identical, though is possible in the study by Hunt et al. (2001). The likelihood of sequencing errors has been reduced in this study as many clones have been sequenced from a large number of PCRs. The published Aristostomias tittmanni RH1 opsin sequence was obtained from a limited number of clones from a single PCR. In an attempt to isolate the cDNA gene sequence for a second opsin an exhaustive PCR has been carried out using a large number of primer pairs in different combinations. However, there is no evidence for two opsins, either expressed or in the genome of Aristostomias or Pachystomias. The quality of the histological sections produced from Pachystomias retina was not high, due to problems with tissue preservation but it is clear that the rod outer segments are extremely long when compared to shallow living teleosts and no cone cells or variation in rod cell morphology is present in any sections, throughout the retina.

The opsin sequences isolated from Aristostomias and Pachystomias were compared to other deep-sea rod opsins to identify potential long-wave shifting substitutions. Two candidates, Phe261Tyr and Ser292Ala are present in both sequences. A previous study inserted the Phe261Tyr mutation into the Astyanax rod opsin by site directed mutagenesis and the resulting pigment regenerated with a $\lambda_{\text{max}}$ 8 nm long-wave shifted compared to wild type (Yokoyama et al., 1995). The reverse substitution has been identified in the primate red and green sensitive LWS genes (Neitz et al., 1991; Merbs and Nathans, 1991b and Williams et al., 1992). Mutational studies on the human red/green visual pigments have also shown that this substitution has a spectral tuning effect (Asenjo et al., 1994). Further examination of the translated sequences does not identify many more potential long-wave tuning substitutions, the only two being
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Ala42Ser and Phe208Tyr. These were identified because both involve a polar change but neither is actually close to the retinal binding pocket or Schiff’s base in the crystallised model of bovine rhodopsin (Palczewski et al., 2000; Teller et al., 2003). The Ala42Ser change is common to all three long-wave sensitive stomiid species but also to Photostomias guerrei (λ_max 483 nm) and the lizardfishes Bathysaurus ferox (λ_max 481 nm) and Bathysaurus mollis (λ_max 479 nm). The Phe208Tyr change is found at site 208 in Malacosteus only. Sensitivity to long wavelengths is achieved in the LWS opsin class by possession of a chloride binding site close to the Schiff’s base. When Cl− ions are present the LW pigment from zebrafish has a λ_max of 572 nm, however when the solution is anion free the λ_max is at 542 nm (Kleinschmidt and Harosi, 1992). The chloride binding sites have been identified as His197 and Lys200 in the LW pigments (Wang et al., 1993). The sequences isolated from Aristostomias and Pachystomias contain the typical Glu181 and Gln184 residues found at the corresponding sites in bovine rod opsin. The long-wave shift of these rod opsin pigments must be by a different mechanism to that found in LWS opsins.

Unfortunately the Aristostomias rod opsin failed to regenerate properly in vitro, under normal or deep-sea pressures, and the λ_max could not be confirmed. The regeneration protocol is described in the methods section and produces good results for all other rod opsins tested. So why is the Aristostomias pigment so different? It may be that the opsin needs to be synthesised under pressure to attain the correct structure and shape, hence the lack of regeneration success when the protein is formed at atmospheric pressure in vitro. Another factor may be the lipid membrane environment that surrounds the protein. The fluidity of the membrane under high pressure would be much reduced to that encountered at atmospheric pressure. The composition of the cell membrane of the
HEK cell line may not mimic closely enough the true environment. A problem may also have occurred earlier in the protocol, the opsin may not have translated and/or been modified correctly, which may have led to problems in inserting the protein into the membrane. Producing a protein extract from transfected HEK 293T cells could be used to help confirm expression of *Aristostomias* rod opsin. A Western blot could be performed by separating the extracted proteins on an SDS-PAGE gel. This could then be blotted and probed with the 1D4 antibody. If the protein is expressed then the antibody will bind, and it can be detected by binding a secondary antibody that is FITC (fluorescein-5-isothiocyanate) labelled. To identify whether the protein is being inserted into the cell membrane the 1D4 antibody can be probed against whole cells. These can then be viewed by confocal microscopy, and the fluorescence tag identified. If the protein is inserted into the membrane the label should be localised to the outer rim of the cell. Both the Western blot and the immunohistochemistry would be worthwhile experiments for the future. All three explanations for the lack of *in vitro* regeneration success are in accordance with positive results achieved by MSP, whole-mount and extract recording. As in all these cases the protein was synthesised, modified and localised *in vivo*.

On finding no molecular genetic evidence for a second long-wave visual pigment, there are two possible explanations. The first is that its presence was overlooked and the second is that *Aristostomias* only uses a single opsin. *Aristostomias* and *Pachystomias* fish emit blue/green light like other deep-sea fish, with maximum emittance at around 480-490 nm, as well as the long-wave light > 600 nm and require visual sensitivity to both ends of the spectrum. Without sensitivity to short wavelengths, *Aristostomias* and *Pachystomias* would be blind to communication from nearly all other bioluminescent
species and to their own short-wave light emissions. However, sensitivity is also required for long wavelengths so that the far-red bioluminescence is useful in finding prey and for communication. Previous evidence suggested that these fish express two opsins, one forming pigments maximally sensitive to \( \approx 517 \) nm and to \( \approx 542 \) nm, and the second to \( \approx 590 \) nm and potentially \( > 600 \) nm. Why have both pigments been long-wave shifted since it would seem more advantageous to have a long-wave and a short-wave pigment, which would confer more sensitivity to both bioluminescent emissions. Evidence taken from the sensitivity of the human LW cone suggests that at 700 nm the pigment is approximately 2.4 log units less sensitive than at its \( \lambda_{\text{max}} \) of 560 nm. (Estevez, 1979, cited from Bowmaker et al., 1988) However this is a rhodopsin pigment, a porphyropsin pigment would be 0.5 to 0.8 log units more sensitive (Bowmaker et al., 1988). The 542 porphyropsin and the 590 rhodopsin of Aristostomias and Pachystomias will be sufficient to long-wave light over 600 nm to detect their own long-wave bioluminescence (Bowmaker et al., 1988). With one pigment very long-wave shifted, why is the other not more short-wave shifted to confer greater sensitivity to blue bioluminescence? This pigment is neither optimally sensitive to short or long wavelengths, but is somewhat in the middle. This can be explained if at one time during evolution or perhaps still to this day a single opsin is being expressed. It is necessary that the opsin provides sufficient sensitivity to both short and long wavelengths. This can be achieved by forming rhodopsin and porphyropsin pigments and by balancing their \( \lambda_{\text{max}} \) values. There needs to be a compromise. If the rhodopsin pigment was more short-wave sensitive, then sensitivity to blue/green bioluminescence would be greater, but the porphyropsin pigment would also be short-wave shifted and therefore less sensitive to far-red light. The same pay off would occur if the porphyropsin pigment was long-wave shifted. To be sensitive to both ends of the
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spectrum requires a rhodopsin/porphyropsin pair and a carefully tuned opsin. The expression of a second more long-wave sensitive pigment would be a relatively recent event, as it must have occurred after the speciation of Malacosteus, which followed the separation of Pachystomias (Nelson, 1994). This would suggest that the long-wave pigment evolved independently in Aristostomias and Pachystomias, which seems particularly doubtful. A $\lambda_{\text{max}}$ value of around 590 nm, would suggest a MWS/LWS opsin had either been retained or sporadically acquired. However, it is also possible that it is an RH1 or RH2 like pigment that has been substantially long-wave shifted. The 590 pigment may not be more long-wave shifted to prevent increased susceptibility to spontaneous thermal isomerisations. Studies on levels of dark noise in salamander show that rod and SWS2 pigments are $10^6$ fold more stable than MWS/LWS pigments because of the thermal noise problem (Rieke and Baylor, 2000).

The Asp83Asn substitution, previously thought to short-wave shift the rod pigments 10 nm (Hope et al., 1997; Hunt et al., 2001) is present in the stomiid sequences. There are two possible explanations for the substitution to be present in the long-wave shifted pigments. Firstly, the Asp83Asn change may have occurred very early during the evolution of deep-sea opsins. Ancestors of Aristostomias, Pachystomias and Malacosteus may have possessed short-wave shifted pigments as in other deep-sea fish. Over time the pigments were long-wave shifted, but the Asp83Asn change was maintained. A second explanation is that the Asp83Asn substitution is not an important tuning candidate, but is present in deep-sea fish for an alternative purpose. Evidence to support this comes from a study that identified key sites for rhodopsin activation (Weitz and Nathans, 1993). The equilibrium of metarhodopsin I-metarhodopsin II was affected when Asp was substituted for Asn at position 83, as found in deep-sea pigments (Weitz
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and Nathans, 1993). Metarhodopsin I was stabilised when aspartic acid was present, however asparagine at 83 tipped the equilibrium in favour of metarhodopsin II formation. This work shows that site 83 may have a kinetic role, as well as a potential tuning function. The question that needs to be addressed is why favouring metarhodopsin II formation is important for deep-sea pigments? The answer is found in earlier work that showed the Asp83Asn mutation in bovine rod opsin increased its ability to activate transducin (Nakayama and Khorana, 1991). This can be explained by the equilibrium skew towards metarhodopsin II shown by Weitz and Nathans (1993), which is the step proceeding transducin activation. The low light conditions encountered by deep-sea fish is a sensitivity problem. These fish need to elicit a visual response when the photon count is low. The Asp83Asn mutation allows a single regeneration event to be amplified, increasing the number of transducin molecules activated, and hence providing a greater phototransduction cascade response. At the C-terminus of the Aristostomias and Pachystomias rod opsins is a three residue deletion. It is the loss of Ser338, which is interesting as it is one of the residues important for rhodopsin phosphorylation by rhodopsin kinase as discussed in chapter 4. The deletion would be expected to reduce the rate of deactivation of metarhodopsin II. A longer activation period will increase the number of transducin molecules that are activated. The outcome would be a greater amplification of the phototransduction cascade, a similar effect as that shown by the Asp83Asn substitution.

3.5. Conclusions

Further work is needed to determine if one or two opsins are expressed in Pachystomias and Aristostomias. This study identified a single opsin gene in both species. The
pigment from \textit{Aristostomias} was refractory to regeneration \textit{in vitro}. This suggests that the pigment is unlike the typical rod opsins that have been isolated and regenerated. The problem may be linked to its adaptation to a high pressure environment, but attempts to regenerate under pressure also failed. Histology provides evidence for a single morphological class of rod photoreceptor in the retina of \textit{Pachystomias microdon}.

### 3.6. Future Work

A retinal cDNA library would be a very valuable tool in identifying conclusively which opsins are expressed in the retina of \textit{Aristostomias} and \textit{Pachystomias}. If more samples could be obtained than this would be an extremely interesting and worthwhile experiment. Fresh genomic DNA free from DNase contamination, could be used for Southern blotting. This would show which opsin classes are present in the genome. If sufficient eyes could be obtained, RNA could be extracted for Northern blot analysis. This would identify which opsin genes are expressed. However, information could be obtained from a single eye, if as soon as the fish is caught, the eye is removed and fixed for \textit{in situ} hybridisation. The retina could be probed with rod and cone opsin DIG-labelled RNA and detected with anti-DIG alkaline phosphatase antibody conjugate. A positive result would be expected with the rod opsin probe, but could interestingly determine the expression of a LW or an RH2 opsin. A negative result would suggest it is either a second rod pigment or only a single opsin is expressed. A request for more tissue was recently made to researchers embarking on a sample collecting cruise, but no species were caught, a reflection on the sparcity of these species of stomiid and the difficulties faced in projects of this type.
4.1 Introduction

The Scopelarchidae are a family of teleosts that possess asymmetrical or tubular eyes (figure 4.1). Most of the previous work on this family has been on the bigfin pearleye, *Scopelarchus michaelsarsi* and the smallfin pearleye, *Scopelarchus analis*. Both of these fish are mesopelagic predators and possess a large horizontal mouth with long needle-like teeth (Nelson, 1994; Collin *et al.*, 1998).

![Figure 3.1](image.jpg)
Chapter 4 - Scopelarchus analis

Young fish have been found as shallow as 200 m though the adults appear to inhabit depths between 500 and 1000 m. This suggests that once mature the fish descend to inhabit deeper water. The tubular eyes develop from normal lateral eyes. At maturity they are immobile but allow for a large binocular visual field. The position of tubular eyes within the head can vary between species, but are angled dorsally in Scopelarchus to catch sunlight falling down the water column. Tubular eyes are cylindrical in shape, with the base of the cylinder comprising a main retina (figure 4.2). An accessory retina is located on the medial wall of the eye (Locket, 1977). The main retina receives binocular information from the dorsal visual field, with the accessory retina subserving monocular lateral visual space. In S. michaelsarsi, the main and accessory retinae are separated by a choroidal fissure (Collin et al., 1998). The reason why some teleost fish have evolved dorsally directed eyes is still not fully understood. It is often suggested that this angle of vision allows for the silhouette of organisms swimming above to be viewed against the down-welling space light. Tubular eyed species may scan the water above and wait for passing prey (Collin et al., 1997).

As often found in fish the lens is large and spherical and fills a large oblique pupil. The lens of S. analis is yellow and allows for very little light transmission below 450 nm, an adaptation thought to enhance the contrast of bioluminescence against a background of residual sunlight (Douglas and Thorpe, 1992). The retractor lentis muscle is well developed, which suggests that accommodation of the lens may occur (Munk, 1966). The iris is covered by a silver argentea, apart from on the medial wall in front of the accessory retina. Matthiessen’s ratio (Matthiessen, 1880) confirms that the main retina will receive a focussed image; the focal length for the accessory retina however is too short, which suggests that its role is not for fine visual acuity but more for gross light
Chapter 4 – *Scopelarchus analis*

perception. The main retinae of both the *Scopelarchus* species examined by Collin et al. (1998) show a blue reflection, which suggests the presence of a retinal tapetum. The accessory retina from *S. analis* elicited a white eye shine. This suggests that the accessory retina also overlies a tapetum but that its composition differs from that behind the main retina.

**Figure 4.2.** (A) Photograph of the tubular eye of *S. analis*, courtesy of Dr. Shaun Collin. (B) Diagram of a vertical section through the eye; AR, accessory retina; L, lens; LP, lens pad; MR, main retina; ON, optic nerve. Redrawn from Partridge et al., 1992.

The presence of a lens pad (sometimes termed pearl organ) allows extension of the visual field. The lens pad is an invagination in a region of the lateral iris, formed from a collection of regularly spaced and orientated corneal lamellae (Munk, 1966, Locket, 1977). Locket proposed that this extends the ventrolateral monocular field of view by wave guiding light onto the accessory retina (Locket, 1977). The fields of view capable from the *Scopelarchus* tubular eye are shown in figure 4.3.
As well as extending the visual field, the *Scopelarchid* eye has evolved a number of retinal adaptations to improve sensitivity in a low light environment. Collin *et al.* (1998) have reported seven retinal specializations at the level of the photoreceptor or ganglion cell in *Scopelarchus michaelsarsi* and *Scopelarchus analis.*
4.1.1 Retinal Specializations in Scopelarchus analis

Ungrouped Rod-like Photoreceptors in the Main Retina

A single bank of elongated rod photoreceptors is found in the nasal two thirds of the main retina (Collin et al., 1998). The photoreceptor thickness in this region is 475 μm in *S. analis*, with rod cells reaching lengths of 340 μm. This is exceptionally long even for deep-sea receptors, especially when compared to the rods of *S. michaelsarsi*, which reach lengths of 87 μm. The elongation of the rod photoreceptor cells is likely to be an adaptation to increase photon capture in low light conditions by ensuring a longer light path. There is also a region of shorter rods (around 40 μm in length) caudal to the rods with very long outer segments (Locket, 1971a). The junction between these two regions is abrupt as shown in figure 4.4. All ungrouped rod receptors in the nasal region of the main retina are packed into a regular hexagonal array, which ensures optimal utilisation of retinal area for photon capture. The peak density reaches $65.0 \times 10^3$ receptors per mm$^2$ in an area located centrolaterally in the retina of *S. michaelsarsi*. In *S. guntheri* an interesting observation has been made regarding the receptor pedicles. In this species five or six synaptic ribbons are present, which rise to basal filaments from their lateral borders (Locket, 1977). This complexity is more often associated with cones even though the outer segments of these receptors are typically rod-like. The RPE underlying the nasal two thirds of the main retina is thick and contains a high number of spherical- and rod-shaped pigment granules, though it is not certain if these act as a tapetum as no reflecting crystals were identified (Collin et al., 1998).
Figure 4.4. Light photomicrograph demonstrating the junction of short and long rod regions in the main retina of *Scopelarchus analis*. The junction is abrupt and gives rise to a marked increase in retinal thickness. There are no obvious differences in the inner retina related to the short and long rod regions. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer, OPL, outer plexiform layer; OS, outer segments. Redrawn from Locket, 1971b.
Chapter 4 – Scopelarchus analis

Grouped Rod-like Photoreceptors in the Main Retina

In the remaining caudal third of the main retina, the rod photoreceptors form groups of approximately 35-36 receptive cells (Collin et al., 1998). Each group is isolated from its neighbours by the outer membranes of RPE cells, and hence form large macroreceptors. The groups of receptors are arranged in an array that is similar to the grouping of single photoreceptors in the nasal main retina. The synaptic pedicles of these photoreceptors are more like typical teleost rod synapses and unlike those found in the non-grouped main retina (Locket, 1977). The use of macroreceptors is another adaptation to limiting light conditions. The surrounding RPE cells contain reflecting crystals, probably guanine, which reflect light back into the receptive cup of rod photoreceptors if not absorbed on its first entry (Collin et al., 1998). Although greatly increasing the likelihood of light capture, spatial acuity would be much reduced. The macroreceptors are likely to be both optically and electrically isolated from each other, with rods within the cups joined by gap junctions. The fact that the photoreceptors in the nasal two thirds of the main retina remain ungrouped suggests that the visual field that this area subtends is visually important, such as for prey identification.

Grouped Rod-like Photoreceptors in the Accessory Retina

An accessory retina lies on the medial wall of both eyes. This appears less differentiated than the main retina with a sparsely populated ganglion cell layer (Collin et al., 1998). The rod photoreceptor cells are grouped as in the caudal main retina, but with only 20 receptors per group instead of 35 (Collin et al., 1998). The rods are smaller than in the main retina and are less regularly arranged, though rod cell density is actually higher. In S. analis, the RPE behind the accessory retina is thick and contains reflecting crystals, whereas in S. michaelsarsi, the RPE is thin and has no reflecting crystals. This may
reflect differing visual roles being played by the accessory retina in the two species, perhaps arising from differences in the ecological niches inhabited.

**Cone-like Photoreceptors**

Cone-like photoreceptors have been described in the retina of both *S. michaelsarsi* and *S. analis*. These have been identified by their small, short tapering outer segments and thick inner segments. Also, the inner segments of these cells contain high numbers of mitochondria and large, lightly staining granular nuclei, which are located vitread of the rod nuclei (Collins et al., 1998). In *S. michaelsarsi*, cone-like receptors were only identified in a band at the ventral base of the accessory retina. The number of cone-like receptors was small, only 2 - 4 per group. In *S. analis* however, none were found in the accessory retina, but were present in the temporal main retina (Collins et al., 1998). In most deep-sea fish studied the retina has lost the expression of cone opsin visual pigments and relies totally on rod opsin. However, in the retinae of *Scopelarchus* sps. there is a population of cone-like cells. Is a cone-like or a rod-like visual pigment expressed in these receptors? Or are these cells defunct? It seems unlikely that a species with such a highly adapted eye to its environment would retain cone cells if they were not playing a visual role. The increased efficiency of cone transduction may confer some advantage, although in what situation this would be useful is unknown, especially as behavioural data is extremely difficult if not impossible to obtain at the present time.

**Area Centralis of the Main Retina**

Shallow-water teleosts often have an area of retina that contains a high density of photoreceptors (Ahlbert, 1976) or ganglion cells (Collin and Pettigrew, 1988a,b). This region is termed an area centralis and is linked to acute visual tasks. Studies on the
density of ganglion cells across the main and accessory retinas of *Scopelarchus michaelsarsi* shows that the distribution is uneven. The peak ganglion cell density of $56.1 \times 10^3$ cells/mm$^2$ is located in the centrolateral region of the main retina (Collin *et al.*, 1998). This is substantially higher than in the shallow living goldfish ($5.96 \times 10^3$) (Mednick and Springer, 1988), and closer to the densities found in the highly visual coral reef fish *Parapercis cylindrical* ($40 \times 10^3$) and *Balistoides conspicillum* ($51 \times 10^3$) (Collin and Pettigrew, 1988). When compared to the peak ganglion cell density of the accessory retina ($1.7 - 7.8 \times 10^3$), the centrolateral main retina appears to be wired to receive a high input of visual information. This is supported by the presence of single rod photoreceptors rather than receptive groups and by the comparable densities of photoreceptors to ganglion cells in the *area centralis* of *S. michaelsarsi*, maintaining a low summation ratio. On average the number of ganglion cells in the main retina compared to the accessory is 33:1 with a sharp transition between the two. The presence of an area centralis would suggest that the region of the visual field that it receives visual information from is important, for example in prey capture.

**Alpha-Like Ganglion Cells in the Temporal Main Retina**

A subset of large ganglion cells was discovered in the temporal retina of *S. michaelsarsi*, with areas ranging from 26.2 to 32.2 $\mu$m$^2$, and $\approx 45$ $\mu$m in length. The soma of these cells were statistically larger in size than the soma of the ganglion cells found in the rest of the retina. In fact the temporal ganglion cell soma were approximately four times greater in area than those in the nasal retina. This population makes up approximately 1% of the total ganglion cells in the retina, a situation also present in the cichlid *Oreochromis spilurus*, the channel catfish, *Ictalurus punctatus*, and the Florida garfish, *Lepisosteus platyrhincus* (Cook and Becker, 1991; Collin and
Collin et al. (1998) described these ganglion cells as alpha-like because of their soma size, thick primary dendrites and distribution in a regular mosaic. This pattern has previously been seen in the temporal retinæ of procellariiform seabirds and was termed an *area giganto cellularis* (Hayes et al., 1991). These cells may be motion sensitive and have transient receptive fields like the alpha or Y class cells of cats (Wassle et al., 1981; Collin et al., 1998). In Scopelarchid fish, there seems to be a correlation between the number of alpha-like ganglion cells and the proportion of large, myelinated axons in the optic nerve. In the retina of *S. analis* there is an increase in the number of large axons in the optic nerve, which correlates to an increase in the area covered by alpha-like ganglion cells in the temporoventral main retina (Collin et al., 1998). This would provide fast conduction for visual information received by the macroreceptors, transmitted to the alpha-like ganglion cells and then relayed along the large myelinated axons to the visual centres of the brain. The α ganglion cells receive information from rostro-dorsal visual space and may provide increased temporal sensitivity to enable prey capture.

The Retinal Diverticulum

The retina of *Scopelarchus michaelsarsi* contains a third area of differentiated retina. As well as the main and accessory retinæ, there is an outpocketing of retina or retinal diverticulum located in the temporal region of the eye, below the optic nerve head (Collin et al., 1998). This area was originally believed to record hydrostatic pressure and indicate depth (Pütter, 1902 cited from Collin et al., 1998). More recently it has been proposed that it may be receiving light stimulation from below the eye or from within the mouth (Bertelsen et al., 1965). The light reaching the retinal diverticulum would not have passed through the lens and is therefore not forming a focussed image.
The retinal diverticulum is backed by an unpigmented retinal epithelium. Collin et al. (1998) describe the retinal diverticulum as transitional in structure between the main and accessory retinae. In *S. michaelsarsi* a mixture of rod and cone photoreceptors were found in the retinal diverticulum.

### 4.1.2 Visual Pigments of *Scopelarchus analis*

MSP preparations were made for both the main and accessory retinae of *Scopelarchus analis* (Partridge et al., 1992). The results of this technique demonstrate the presence of three visual pigments, with $\lambda_{\text{max}}$ at 444, 479 and 505 nm, all fitting a rhodopsin template (Partridge et al., 1992). As most of the deep-sea fish studied to date contain rod-only retinae, it is interesting to determine if these are all spectrally tuned rod opsins or if one or more cone opsins have been retained. The outer segments were subjected to partial bleaching experiments to determine if they contained mixed pigments. However, all bleached in a normal way suggesting that they were all homogeneous. The main retina contained the 505 and 444 nm pigments only, with both present in the same outer segment. The accessory retina contained all three pigments, with the 505 and 444 nm pigments again found together in the same outer segments. Sequential scans at 8 μm intervals along the outer segment demonstrated that the 505 nm pigment was always located at the distal end and the 444 nm pigment at the proximal end. There were no regions where a mixture of both pigments was present. The transition between the two was consistently abrupt and found 40–50 μm from the inner segment in the main retina. The change over was also abrupt in the accessory retina although the position varied from 30 to 180 μm from the inner segments. The accessory retina also contained receptors that solely expressed the 444 or 479 nm pigments, a situation not present in
the main retina. This suggests that the main and accessory retinae have different visual roles. The observations were taken from a single fish and Partridge et al. (1992) suggest that it was in the process of maturing from a shallow-living juvenile to a deep-sea adult and that the 505 nm pigment was being actively replaced by the 444 nm pigment, hence dual localisation in the same photoreceptor. A change over from a $\lambda_{\text{max}}$ at 505 nm to 444 nm confers greater short-wave sensitivity, which suggests that the visual system is adapting to perform in bluer, deeper waters. Opsin substitution has not been previously identified in a deep-sea species. Replacement of an opsin with another more blue-shifted has previously been reported in the eel, *Anguilla anguilla* (Wood & Partridge, 1993). Coexpression of opsins in cone photoreceptors has been shown in the guppy (Archer and Lythgoe, 1990) and in a number of small nocturnal mammals (Rölich et al., 1994, Szél et al., 2000) including the mouse (Applebury et al., 2000). A polymorphic trait found in the guppy can lead to two LWS opsins with different $\lambda_{\text{max}}$ to be expressed in the same cone photoreceptor, with the mixture of pigments having a $\lambda_{\text{max}}$ intermediate between the two pure LWS pigments (Archer and Lythgoe, 1990).

Whether the 505 nm pigment is present in the adult or not, it is possible for *Scopelarchus* to have basic dichromatic vision. This may enable the spectral composition of different light sources to be compared. Sensitivity to differences in hue between bioluminescence and residual sunlight would confer an ability to detect prey that use counterillumination camouflage.
4.2 Aims

The main aims of this chapter are:

1. to identify the visual pigment genes that are expressed in the main and accessory retinas of *Scopelarchus analis*,

2. to obtain the $\lambda_{\text{max}}$ of the opsin proteins coded by these genes by regeneration of the recombinant opsin with 11-cis retinal,

3. to locate the mRNA transcripts of these genes in the retina.
4.3 Results

4.3.1 Identification of Two Rod Opsin Sequences from *S. analis* Genomic DNA and mRNA

Genomic DNA was extracted using phenol/chloroform from a single liver sample (0.05 g) but the quality of DNA was low due to the limited amount of tissue. The extraction was therefore repeated using the whole body minus the head (1.50 g). This gDNA served as the template for a PCR with the degenerate rod opsin primers FRHOB (5'−CTTCCCYRTCAACTTCCTCAC-3') and FRHOB− (5'−TGCCCTTGTCAWGCAGATGTAG-3'). The PCR conditions were standard as described in the methods section, apart from increased MgCl₂ (4 mM) and primer (12.5 μM) concentrations. Gel electrophoresis identified a product of the expected 650 bp size (figure 4.5). The DNA was extracted from the agarose gel and cloned into the pGEM-T-Easy vector (Promega). A large number of clones (30) were sequenced so that the presence of more than one amplified product could be detected. Two distinct rod opsin sequences were present in an approximate 50:50 ratio (13:17). Neither contained introns, which is typical of teleost rod opsin genes (Fitzgibbon *et al*., 1995). The two opsins have been termed Rod A and Rod B.
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Figure 4.5. Gel image of PCR on Scopelarchus analis gDNA with FRHOB+ and FRHOB- primers. The faint band of approximately 650 bp was cloned and sequenced.

Messenger RNA was isolated from two eyes taken from an individual caught at 300 m (combined mass of eyes = 0.042 g) and separately from two eyes from a fish caught at 950 m (mass = 0.096 g) using the QuickPrep® Micro mRNA Purification Kit (refer to methods section 2.2.1). Double stranded DNA was synthesised from these templates with the SuperScript™ First-Strand Synthesis System for RT-PCR (again refer to section 2.2.1.1) using a poly(T) primer, which anchors to the poly(A) tail of mRNA. This produced two cDNA templates, one from a smaller, shallow dwelling fish (juvenile) and the other from a larger, deeper dwelling fish (adult).
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The FRHOB/B- primers were first used on 'juvenile' cDNA, under standard PCR conditions with 4 mM MgCl₂ and 12.5 μM primer concentrations. The amplified fragment viewed by gel electrophoresis (figure 4.6, A) was cloned into pGem-T Easy and 12 clones were sequenced. Rod A sequence was generated from all clones. Specific primers, which amplified either Rod A or Rod B, were designed. Primers Scop 295+ (5'-CATGGCTACTTTGTCTTAGG-3') and Scop 850- (5'-GGATGGTCATGAAGACGG-3') are Rod A specific, whereas primers ScopB 170+ (5'-CTGGTTGTAACGTCGAGG-3') and ScopB 714- (5'-CGAAGAAGGACGGCACC-3') are Rod B specific. Rod B specific PCRs on 'juvenile' cDNA and gDNA only amplified a product from the gDNA template (Figure 4.6, B). PCR reactions were performed on 'adult' cDNA amplifying from either the Rod A or Rod B specific primers (Figures 4.6, C and D). Both rod opsins were present in the 'adult' cDNA population. Therefore, both rod opsins are expressed in the *S. analis* individual collected from 950 m ('adult') but only Rod A is expressed in the fish collected at 300 m ('juvenile'). Rod B expression would appear to be temporally controlled.
Figure 4.6. Gel Images of RT-PCRs amplifying opins Rod A and Rod B from *S. analis* retinal mRNA. (A) B/B- degenerate primers amplified a product from 'juvenile' cDNA, which when cloned and sequenced generated only Rod A sequence. (B) Rod B specific primers amplified Rod B opsin from gDNA but not from 'juvenile' cDNA. (C) However, Rod B specific primers did amplify Rod B from 'adult' cDNA. (D) Rod A specific primers amplified Rod A from adult cDNA.
4.3.2 Completion of Rod A Sequence

The 5' end of the gene was completed by a genomic walk (refer to methods section 2.2.2) with the outer primers Scop WK396- (5'-GGTCCTCAACTCTTTGGCTCG-3') and UNI33 (5'-TTTTTTTTTTTTTGGTTGTTGCTGGGGT-3'), followed by a nested PCR with the inner primers Scop Wk364- (5'-GAGTGAGGAAGTTGACGG-3') and UNI17 (5'-TTTTGTTTGTGGCTGGG-3'). The primary walk reactions contained either 1.5 or 3.0 mM MgCl₂. The secondary walk products were visualised by gel electrophoresis. A fragment of \(\approx 400\) bp in size was strongly amplified though there were two other fainter bands of \(\approx 250\) bp and \(850\) bp (figure 4.7, A). All three fragments were excised, cloned and sequenced from at least two different PCRs. Only the 400 bp fragment sequenced as opsin, producing the 5' end of the Rod A gene up to a potential TAATA box site. The sequence was confirmed by amplifying a 300 bp fragment from gDNA with a forward primer designed to the proposed Rod A TAATA box (ScopBoxF 5'- TGGAACTAATAATCCAGG-3') and the Scop Wk396- reverse primer (5'-GGTCCTCAACTCTTTGGCTCG-3') (figure 4.7, B). The product was sequenced and confirmed the sequence generated by genomic walking.

The 3' end of Rod A was completed using the 3' RACE amplification system (see methods section 2.2.2.4). The outer 3' gene specific primer Scop 3'O (5'-GTCTGAGACCACCAAGG-3') and the kit supplied 3' RACE Outer Primer (5'-GCGAGACAGAATTAATACGACT-3') were used in a standard RACE PCR on 'juvenile' cDNA at a range of annealing temperatures (56-60.5°C). The inner nested primers Scop 3'I (5'-TGGTACATCTTCACACACC-3') and the kit supplied 3' RACE Inner Primer (5'-CGCGGATCCGAAACTGCGTTTGTGGCTTGTGATG-3')
amplified a fragment of \(\approx 650\) bp in size from each outer reaction as determined by gel electrophoresis (figure 4.7, C). These were excised, cloned and sequenced. The sequence generated read up to and included the poly(A) tail of the mRNA. To check the coding region sequence a reverse primer was designed to the stop codon (Scop Stop R 5' - TTATGCAGGAGCCACACTGG-3'), which with the Scop 3'O forward primer amplified a 350 bp product from ‘juvenile’ cDNA (figure 4.7, D). This was direct sequenced and confirmed the identified 3' sequence. A contiguous Rod A cDNA sequence was then produced by combining all sequence information together (see figure 4.8 for how the contig was produced and figure 4.9 for the completed cDNA sequence). The 5' upstream sequence identified by genomic walking is shown in figure 4.10.
Figure 4.7. Gel images of PCRs used to complete the *S. analis* rod A sequence. (A) Secondary genomic walk on primary reactions at 1.5 or 3.0 mM MgCl₂ concentration. The outer (NCO) and inner (NC1) negative controls are also shown. (B) PCR with primers ScopBoxF and ScopWk396- on gDNA. (C) 3’ inner RACE reaction on outer reactions run at 56°C –60.5°C. All amplified a bright product which when cloned and sequenced identified the 3’ end of rod A cDNA. This was confirmed with a PCR with primers 3’ Outer and ScopStopR (D).
Figure 4.8. Amplified sequences from *Scopelarchus analis* Rod A used to create a cDNA contiguous sequence. PCRs are shown by double-headed arrows, identifying the position of the forward and reverse primers. (A) FRHOB+/B-; (B) UNI17/ScopWk364-; (C) ScopBoxF/ScopWk396-; (D) Scop3'I/RACE 3' Inner and (E) Scop3'O/ScopStopR.
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ATGAAACGGCA CAGAGGGACC AGATTTTTAT GTCCCTATGG TAAATACACC 50
TGGTGTTGTC CGGAGCCCTCT TTGAAATACC TCAGTACTAC CTTGTGACGCC 100
CGGCGGCTTA CCTCCTGGCT GATTGCTTATCG ATCTCGACCC CTTCTGCTGC 150
GGCTTCCTGG GCACCTGCTAT GTTACCATCG AGCACAAAGA 200
GTTAGAGAGC CGCTAAACTAC ACAAATGCTCT CAAACTGGCT GTTCTAACC 250
TCTGGATGGT GTGGAGGAGA TTCAAACCAAA CAAATGACAG GCTTCTGCTC 300
GGCATTTTGG TCTTACGGCG ACCGCTGCTG AATTTGAAGAG GCTTCTTGGC 350
TACCTTTTGGC GCTGAAATAG CTCCTCTGCTG CTTGCTGATC TGGCTATGG 400
AGAGATGTGGT GTGCCCTGAG GACGTTGTGA CCACTTCGCC ATTTGGGAG 450
AAACCATGCTA TTCCTCCTCT GTGTTTCTCT GTGATACTGG CCGCCGCTTG 500
CGCCTGATCCC CCTCTTTTGG GCTCCTCTGG TTTACATCCCA GAGGCATGC 550
AGTGGTGATGG TTTGAACTTC TTCTACACAC GTGCCCCTTG TATCAAAAT 600
GAACTTTTGG TACCTTCATG TTGCTTCTGG CACTTCACCT TTCCACCTGT 650
CGTCACTACGC TTCTGTATGG CGCCTCTGGT CGTCTCAGTC AAGGGAGCT 700
CTGCCGCCCA GACAGGATCT GACACACACC GAAAGGGCGA GAGGGAGTCC 750
AGCGGCTAGG TTATATAGCT GCTCATGCGC TTCTTGATGT GTTGGCTGCC 800
CTACGCACAG GTGCCCCTGT ACACTTTTAC ACACAGGGGC AGTGATTTTG 850
GACCATGACG TATGACATGC CTTCTCTGCT TTTCAAGAGT CTCCTCACTC 900
TACACACCTT CTTACATCG CGGCTCTTGG CGTCTCAGTC AAGGGAGCT 950
GATTACCACC CTGGCTCCTG GGAGAAATCC CTTCGAAGAG GAGGAAGGAG 1000
GCTGTCCTCCG CTCCTGCAAG AACGAGAAAT GCTCTCAAGAT TTCCTCACTC 1050
GTGGCTCTTG CATAAAGGC TCTCACAAC AGGCTCTAAG ATCCATATGC 1100
CAGCAAGAAG AATCTCTACG TCTCCGCAAT GCTACAAGCA ACAACTCTCT 1150
TTTATTAATTT TTACACCAAC AGTTGTTCTA ACCAAGAAC ATTGAGCCAG 1200
AAGGGCGGCC CACTACATGG TTGGTCTGGT ATGTACAGAC ATGTAACGCC 1250
AACAATACGT GAGATAAAT ATTTTTCTCG AGTTGAAGG AAGGAAATCT 1300
TTTAAATCTTT TACAGTTGGA TCTATACTAG TACTGGAATTA TTTGTAATG 1350
TAGAGGCGATG TAATCAACGC AAGCTAAATA AAGACGCTACT TGCCAATAG 1400
AAAAAAAAAA 1409

**Figure 4.9.** Complete cDNA sequence for *Scopelarchus analis* Rod A opsin. The coding sequence is 1062 bp in length. The start and stop translation codons are highlighted in blue and the poly(A) tail is shown in red.

TTTTGTGTGT TGTTGGGGGG TTAGATATTA ACTGTTGCCCT AAATCCTATT 50
GTGGTGACGG ATTTGGAAAC TAATAATCCA AGGATGCCTT TAAATAGCAG 100
GATTGCGCC ACTCGGCTCC ATTTGTAGCA GCTCCTCTCC CATCCTCTC 150
CCTTACAGCT AGAGAAACAA GCACCGCGCA AGGGTGCTAT CGCAACCAGA 200
AACCGCAACC ATG 210

**Figure 4.10.** 5’ upstream sequence of *S. analis* Rod A opsin reading up to the start codon (blue). A proposed TAATA box is highlighted in green, approximately 140 bp upstream of the start codon.
4.3.3 Completion of Rod B Sequence

To complete the 5' end of the Rod B gene, genomic walking was again utilised alongside a degenerate PCR technique. The genomic walk required a number of attempts to obtain primers that were specific enough to Rod B, as Rod A was preferentially amplified on a number of occasions. Primers ScopBWKO (5'-TCGACGTTACAACCAGACGGTC-3') and ScopBSTnner (5'-TGACTACGAAGAGGTTGGC-3') however worked well, in conjunction with the UNI walking primers. The nested PCR produced fragments of two sizes approximately 1,300 and 650 bp in size as determined by gel electrophoresis (Figure 4.11, A). Both of these were excised, cloned and sequenced. Both fragments were identified as Rod B, with the size difference due to the UNI primer clamping down 600 bp further upstream in the larger fragment. A degenerate PCR technique was used to confirm the sequence information. A forward primer was designed to the start codon of the Rod B sequence (5'-GCGCGAATTCCACCATGAACAAGCACCGCCGA-3') and was used in conjunction with the ScopBST reverse primer (5'-TGACTACGAAGAGGTTGGC-3') on 'adult' cDNA. The PCR was performed at four annealing temperatures (53-56°C), with each amplifying two fragments of 300 and 500 bp in size (Figure 4.11, B). Both fragments from two separate PCRs were excised, cloned and sequenced and the 300 bp fragment confirmed the 5' end of the Rod B sequence.

3' RACE was used to obtain the 3' end of the Rod B cDNA sequence. The gene specific primers were Scop B 3'O (5'-CCTGGTGACCTCCGCTAC-3') and Scop B 3'I (5'-GACCTATTCCGTCATGACGG-3'). The products amplified in the inner PCR were viewed by gel electrophoresis, which showed a rather smeary result (figure 4.11, C).
However, a band was discernable of \( \approx 500 \text{ bp} \) in size and this was excised, cloned and sequenced. The resulting sequence read up to the poly(A) tail. The complete cDNA sequence for \textit{S. analis} rod B was made by summating the sequence information obtained (figure 4.12) and the cDNA sequence is shown in figure 4.13, with \( \approx 400 \text{ bp} \) of 5' upstream sequence in figure 4.14.
Figure 4.11. Gel images of PCRs used to complete the *S. analis* Rod B sequence. (A) Primary and secondary genomic walks on *S. analis* gDNA. The first round PCR was run at 1.5 or 3.0 mM MgCl₂ concentration, these were then used as templates for the secondary walk, amplifying two products at ≈ 1.3 kb and 650 bp. (B) Degenerate PCR to confirm the identified 5' sequence with Scop start B and ScopB5'1 primers amplified two fragments at ≈ 300 and 500 bp which were cloned and sequenced. (C) Inner 3' RACE at a range of annealing temperatures (57.5 - 64°C) amplified a 500 bp product.
Figure 4.12. Amplified sequences from *Scopelarchus analis* Rod B used to create a cDNA contiguous sequence. PCRs are shown by double headed arrows, identifying the position of the forward and reverse primers. (A) FRHOB+/FRHOB-; (B) UNI17/ScopBWkO; (C) ScopStartB/ScopBWkO; (D) ScopB3'I/RACE 3'Inner.
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ATGAACGGCA CAGAAGGACC GGAACCTTCAC GTCCCTTTTT CCAACTTAC  50
AGGCCGTGGTG CGAGGCCCCCT TTTGAAATAC ACAGATTATAC CTGGGCTCCAC  100
CAGAGGGCAT CTGGGCCCTTG GCTGCTTACATG CATTGCCGGT  150
GGTGCTCCCCA TCACTCTCCT ACTCTCTACGG ATGCCACATGG AGCACAAAGAA  200
GCCTCGGAGT CCCCCCCTGC GCTCCTTCCCT TAACTTGGAC CTCGCCAAC  250
TCTTGTGAGT CATGCGGAGG GCTTACACGA CGTGATACAC GGGGGTGAC  300
GGATACCTTTGG TCCTGGGACC GTCTGTTGGT AAGCTGAGGG GCTTCTTGGC  350
CACCCACGCA GGAGAGATGT CCCTGGTGTCT TTTAGTGGTG CTCGCCATCG  400
AGAGGGGTGT GTTGGTGGTG AAGCCCCGGA GCCACTTCCA CTCGGGGAGG  450
AAACACCGCA TTGGGCGGCT GGCTTCACCC TGGATGATGG GTGGGCTCTG  500
CTGGTGCCGC CCCCTCTTGG GCTGCTTACAG TTACATCCCC GAGGGCGATG  550
AGTGGTCCTTG CGGGTGCAG TACTACACCC TGAACCGGGA ATCCCACAC  600
GAGTCTCTTTTG TGGCTCATAT GTCTCTTGGTC CACTTGGCCG TCCCTGTTGC  650
CACAATCTCC TTCTGTTCAG GCGCTCTTGTC TGGCCGCTTAC AAGGGCGGCC  700
CAGCCGCCCC AGCAGGAGTC GAGACCCACCC AGAGGGCGGA ACAGGAGGTC  750
ACCCGCATGG TGGCTGACAG TGGCACTACG TTCTTGCTTG GCTGCTGCTC  800
CTACGCGGAG TGGGCTGCTG AACCTTTCC ACACCGGGGC AGCACTTCCG  850
GACCTATCTG CAGTGAATCT GCCCTCTTCT TCGCCAGAAG TCTACGCCCTG  900
TCAACTCCCAAT CTCTCATCAT CTGCGTAAAC AGCAAGTCCCC GCGGCTCGAT  950
GCTCAGGCCCT GTGTCCTGGC GGAAGACCAC GCTGGAGGAG GACACCCCTGC 1000
TGCTCCGCTC TCCGGCTGCC TCCAGCTTGC TCTTTCTTGC ATGAGACTGA 1050
TGTAATAAGG CTTGCTGCCC CTTTATTGAAG CGAGTTTAC ACGAATAACC 1100
TCAGATTGCC ACTCTGTCCAT CTGAAATTTA ACAAGACAAA AAGGACAAAA 1150
TCATGACCTA TGGCCTTCAA GGAACATATA TATATATATA TATATATATA 1200
TGTAACCTAT TTGAAAAAT GAACCTTAAA ACATCCTTCT TCCTACTCTTT 1250
TTTTTTGATG TGTGTCGCCAC ATTTCCACAA GGAATGGCTA ATACAAATTG 1300
AAAGACCAAC AAAGTGTTGG ATTCAGTTCT CATGAGTGT CAAGGATTTG 1350
CCCTAAAATA ATTTTAAAAA GATTTTAAAA AAAAAAAA  1398

Figure 4.13. The complete cDNA sequence for S. analis Rod B opsin. The coding sequence is 1041bp in length, 21 bp shorter than Rod A. The start and stop codons are highlighted in blue and the poly (A) tail is shown in red.

TATTAAGGTCG ACGTATGTCAA ACTAAAAAG AAAGCGGTTGA GACGGAGACC  50
TTTAAAGTCTG CGAAGCGGATG ACAGACCTCC TCAAGCTTTA CGGCTGGATG  100
TACACTCAATA TCAATCTAAA TCATAGCTCC AGATCATGCT AGAGTTTTTT  150
AGACTAATAG GATGGGGAGC TGCCACATGCT AGTAAACGCT GTGGGATAGG  200
CATACAGGCC AGAAGGTCTTA CTCCCATCAT GTGAGTGGATT ATCCCTGATT  250
TGCTAAATCCA TTCTCTCTGC ATACACACGC ACAATAACTC ATAGTCTCAG  300
TAAGATCCTCT TATTAAGGCT CCAACTGAGC TGCTCTACAG ATAAACACAC  350
ACACCTTCTC TGGGCGGCCC TTGAGGGTCA CACATACAAA TATAGTGGGG  400
CCATG  405

Figure 4.14. 400 bp of 5’ upstream sequence of S. analis Rod B opsin. A proposed TAATA box is shown in green, about 240 bp 5’ of the start codon (blue).
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The coding regions of Rod A and Rod B were translated and the alignment is shown in figure 4.15. The amino acid identity is 80%, a little higher than the nucleotide identity of 73%. Both show highest identity to RH1 opsins, as determined by nucleotide and protein BLAST (BLAST database at http://www.ncbi.nlm.nih.gov/BLAST). The most striking difference between the two protein sequences is the absence of seven amino acids at sites 336 to 343 in Rod B opsin. The 5’ upstream sequences for Rod A and Rod B share only 42% identity (not shown), which suggests a relative high degree of divergence if the genes arose by duplication.

| Scop rod B | MNGTEGPDVF VPFSNLTQV YSPFEYPOQV LAPPERSAL RAYNFLLIV 5E |
| Scop rod A | MNGTEGPDVF VPYNTNQG RRSPFEYQOU LVSFASLYQ RAYNFLLIV 5E |
| Scop rod B | GFPVNLTYLF VTEHKLKLL PLNYILLNLRA YANLFVVYG FTTATTAVH 10E |
| Scop rod A | GFPVNLTYLF VTEHKLKLL PLNYILLNLRA YANLVAVVVG FTTMVVSMH 10E |
| Scop rod B | GVFVLQPSGR NVEFFFAHGG GEILWLSLVA LAIERAWVVC KPVSQHAGSE 15E |
| Scop rod A | GVFVLQPSGR NIEFFDAFSG GEIALWLSLVA LAIERAWVVC KPVSQHAGSE 15E |
| Scop rod B | KHAIGVATI WMTASACSVV PLGLGSRVTP EGMQSCDDV VYTLKPDHIN 20E |
| Scop rod A | NHAIMGVSS FIMARACAVP PLLGGSRYTP EGMQAGID YVTREPQDDN 20E |
| Scop rod B | ESFVVYMLSY HYFAPLTEIS FCVRGLLCV KQARARRAES ETORRENGV 25E |
| Scop rod A | ESFVVHMLSC HYFIPLIVIS FCVRGLLCV KQARARRAES ETORRENGV 25E |
| Scop rod B | TRAMVMVIA FLYCIRPWAS YAVVYFTFTHG STFGPVTMT PSFASRSSAL 30E |
| Scop rod A | SRMVIMMVIA FLYCIPWAS WAVVYFTHOG SEFGPVTMT PSFSSSBSAL 30E |
| Scop rod B | VNPLVICHNN ROFRACLLTT VFCOQNLNE DEGLS----- --RBSYSSSS 34E |
| Scop rod A | VNPLVICHNN ROFRACLLTT LCCOQNPEE EERAASISTEKE RBSYSSSS 35E |
| Scop rod B | VSPA 34E |
| Scop rod A | VAPA 35E |

**Figure 4.15** Amino acid alignment of *S. analis* Rod A and Rod B translated sequences. Conserved regions are highlighted in grey. Lys(296) which binds to retinal and forms the Schiff’s base is shown in red. Glu(113) provides the counterion to the Schiff’s base and is shown in orange. The two cysteine residues at 110 and 187 (in blue) form a disulphide bridge and multiple serine and threonine residues (green) at the C-terminal of the protein are important for rhodopsin kinase phosphorylation.
4.3.4 Quantitative Expression of Rod A and Rod B

Forward and reverse primers were designed to regions of the identified Rod A and Rod B sequences that were 100% identical, these were ScopRodsF (5'-CCGTCAACTTCCTCACTC-3') and ScopRodsR (5'-CGCTGGCGTAGGG-3'). As the primers are specific to both rod sequences there should be no bias in annealing and amplification during the PCR. The reaction was performed on cDNA synthesised from ‘juvenile’ or ‘adult’ mRNA with standard reagents and cycling parameters. The amplified products were viewed by gel electrophoresis, and the ~700 bp fragments were excised. Within the region amplified, Rod B contains a SalI restriction enzyme site, whereas Rod A does not (see figure 4.16). This site is almost exactly half way along the fragment, and therefore digestion of the site would produce two products of the same size.

15 µl of the products amplified from both 'juvenile' and 'adult' cDNA were digested with 2U SalI and 10X buffer in a 30 µl reaction for 2 hours at 37°C. After this time the 30 µl reaction containing 15 µl digested DNA was run on a 1.6% agarose gel alongside
15 μl undigested DNA (diluted to 30 μl with dH2O). The results are shown in figure 4.17. The ‘adult’ digest clearly shows two products. The larger is the uncut fragment, and is therefore Rod A. The smaller of approximately 350 bp is the two halves of the digested fragment, Rod B. A DNA hyperladder (Bioline) has also been run to aid size and concentration quantification. The uncut band appears to be close to 30 ng, which in 30 μl is a concentration of 1 ng/μl. The digested fragment is closer to 20 ng, and therefore at a concentration of 0.67 ng/μl. As this is composed of the two halves of the digestion reaction, the original fragment would have been at a 0.33 ng/μl concentration. Therefore in the final PCR, Rod A was amplified to a final concentration of 1 ng/μl and Rod B to 0.33 ng/μl. This suggests that in the individual ‘adult’ fish retina Rod A expression is 3 fold that of Rod B, though Rod B is still at a detectable level. However, in two separate ‘juvenile’ reactions, one being on freshly synthesised cDNA, there was a single undigested product. There may be a very faint digested fragment in the ‘juvenile’ reactions but there is no real detectable expression. This shows that Rod A is expressed in the ‘juvenile’, whereas Rod B appears to be absent at this time point.

Figure 4.17. Gel images of cDNA products amplified from ‘adult’ and ‘juvenile’ cDNA. (A) SalI cut and uncut ‘adult’ PCR product. The two fragments following digestion are indicated with white arrows. (B) SalI cut and uncut ‘juvenile’ PCR product. There is only a single fragment present after digestion, again indicated by a white arrow.
4.3.5 In Vitro Expression and Regeneration of S. analis Rod Opsins with 11-cis Retinal

The full length coding regions for Rod A and Rod B were amplified from ‘adult’ cDNA using the primers Scop AF (5’-GCGCGAATTCACCATGAAACAGCACCACCGCA-3’) and Scop AR (5’- CCGCGTGGAGGAGGACACACTGG-3’) and Scop BF (5’- GCGCGAATTCACCATGAAACAGCACCACCGCA-3’), and Scop BR (5’- CCGCGAATTCACCATGAAACAGCACCACCGCA-3’), respectively. The amplified fragments were digested with restriction enzymes, EcoR1 and SalI for Rod A and EcoR1 and XhoI for Rod B (sites shown in red in primers). This created complementary ends that were used to ligate into the EcoR1/SalI digested pMT4 expression vector. The Rod B fragment was digested with XhoI because a SalI site is present in the coding sequence. XhoI will not cut at a SalI site but will leave a three base overhang that can ligate to the digested SalI site of pMT4. The inserts were sequenced to check for PCR incorporated errors.

A single clone was selected for each gene and was transfected into HEK 293T cells. Isolation and regeneration of the protein proceeded as described in the methods section (see section 2.2.6). Dark spectra between 250-700 nm were obtained for the regenerated pigments by spectrophotometry. The pigments were then bleached. The dark and bleached spectra for Rod A are shown in figure 4.18, and the difference spectrum fitted to a Govardovskii template is shown in figure 4.19, the same for Rod B are shown in figures 4.20 and 4.21. As the absorbance readings were low three separate scans were measured and summed for both the dark and bleached spectra.
Figure 4.18. Dark (A) and bleached (B) spectra for *Scopelarchus analis* Rod A. Both show summed measurements from three separate scans.
**Scopelarchus analis Rod A**

Figure 4.19. *Scopelarchus analis* Rod A difference spectrum (open squares) fitted to a Govardovskii template (black line) with a $\lambda_{\text{max}}$ at 490 nm and a bleached retinal curve subtracted.
Figure 4.20. Dark (A) and bleached (B) spectra for *S. analis* Rod B opsin. Both show summed measurements from three separate scans.
Figure 4.21. *S. analis* Rod B difference spectrum (open squares) fitted to a Govardovskii template (black line) with a $\lambda_{\text{max}}$ at 480 nm and a bleached retinal curve subtracted.
The overall level of absorbance was low for both Rod A and Rod B pigments, though Rod B consistently regenerated with a higher absorbance reading than Rod A. Both difference spectra were fitted to a Govardovskii template that gave \( \lambda_{\text{max}} \) values at 490 nm for Rod A and 480 nm for Rod B. The recording for Rod B correlates well to the MSP data measurement of 479 nm. Rod A is approximately 10 nm long-wave shifted compared to Rod B. MSP data identified three retinal visual pigments with \( \lambda_{\text{max}} \) at 444, 479 and 505 nm. None of these values closely match the \textit{in vitro} \( \lambda_{\text{max}} \) of 490 nm found for Rod A, the closest being the 479 nm measurement.
4.3.6 Spectral Tuning Analysis of S. analis Rod Opsins

The identified rod opsins have $\lambda_{\text{max}}$ values that are short-wave shifted to 480 and 490 nm, as often found in deep-sea fish. Previous studies have identified potential substitutions responsible for this shift in other deep-sea fish at positions 83, 122, 124, 132, 168, 208, 261, 292, 299, 300 (Hope et al., 1997; Hunt et al., 2001). Table 4.1 shows the residues found at these sites in Rod A and Rod B opsins. Potential short-wave tuning amino acids are highlighted in blue.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Substitution</th>
<th>Rod A</th>
<th>Rod B</th>
</tr>
</thead>
<tbody>
<tr>
<td>83</td>
<td>Asp $\rightarrow$ Asn</td>
<td>Asn</td>
<td>Asn</td>
</tr>
<tr>
<td>122</td>
<td>Glu $\rightarrow$ Gln/Val</td>
<td>Glu</td>
<td>Glu</td>
</tr>
<tr>
<td>124</td>
<td>Ala $\rightarrow$ Ser</td>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>132</td>
<td>Ala $\rightarrow$ Ser</td>
<td>Ala</td>
<td>Ala</td>
</tr>
<tr>
<td>168</td>
<td>Ser $\rightarrow$ Ala</td>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>208</td>
<td>Phe $\rightarrow$ Tyr</td>
<td>Phe</td>
<td>Phe</td>
</tr>
<tr>
<td>261</td>
<td>Tyr $\rightarrow$ Phe</td>
<td>Phe</td>
<td>Phe</td>
</tr>
<tr>
<td>292</td>
<td>Ala $\rightarrow$ Ser</td>
<td>Ser</td>
<td>Ser</td>
</tr>
<tr>
<td>299</td>
<td>Ala $\rightarrow$ Ser/Thr</td>
<td>Ser/Thr</td>
<td>Ala</td>
</tr>
<tr>
<td>300</td>
<td>Ile $\rightarrow$ Thr/Leu</td>
<td>Ile</td>
<td>Leu</td>
</tr>
</tbody>
</table>

Table 4.1. List of amino acids from S. analis Rod A and Rod B, found at sites previously identified as potentially short-wave tuning in other deep-sea rod opsins. Where the short-wave shifting amino acid is present it has been highlighted in blue.
Chapter 4 – *Scopelarchus analis*

Three substitutions are commonly found in most deep-sea rod opsin sequences; these are Asp83Asn, Tyr261Phe and Ala292Ser. All three of these occur in both Rod A and Rod B. Four of the identified sites differ between the two opsins. At positions 168 and 299, Rod A has acquired the potential blue shifting residues whereas Rod B has these at positions 124 and 300. *In vitro* regeneration identified Rod B as approximately 10 nm further short-wave shifted than rod A. This difference may be explained by the combined effect of the differences found at positions 124, 168, 299 and 300.

### 4.3.7 Comparison of the Rod Opsin C-Terminus

The C-terminus of Rod B is missing seven amino acids when compared to Rod A. This region was compared between all 26 completed RH1 sequences deposited on the NCBI database (figure 4.22). These include thirteen mammalian, two avian (one not complete) and thirteen teleost species. Three serine residues at positions 334, 338 and 343 in bovine rod opsin have been identified as main phosphorylation sites (McDowell et al., 1993; Ohguro et al., 1993, 1994, 1996; Papac et al., 1993; Adams et al., 2003). These three sites are conserved in twelve of the thirteen mammalian species, the only change present at site 334 in the Caribbean manatee. Interestingly in the two avian species only serine at 343 is conserved, with glycine at 338 and aspartic acid at site 334. This suggests that the deactivation of rhodopsin by phosphorylation may occur differently in birds. Teleosts however have maintained all three serine residues in the majority of species, although a deletion of site 338 is present in sand goby, Atlantic halibut, the two stomiid species and the deep-water expressed rod opsin of conger eel. Rod A from *Scopelarchus analis* has conserved serines at the three sites. Rod B has a substitution to
leucine at site 334 and a seven amino acid deletion including site 338 but Site 343 is retained.
<table>
<thead>
<tr>
<th>Mammalian Sp.</th>
<th>334</th>
<th>338</th>
<th>343</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bos taurus</em> (bovine)</td>
<td>S T V S K T E T S</td>
<td>Q V A P A</td>
<td></td>
</tr>
<tr>
<td><em>Homo sapiens</em> (human)</td>
<td>A A * * * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Mus musculus</em> (mouse)</td>
<td>S A * * * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Rattus norvegicus</em> (rat)</td>
<td>A A * * * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Canis familiaris</em> (dog)</td>
<td>A S A * * * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Macaca fascicularis</em> (macaque)</td>
<td>A * * * * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Tursiops truncatus</em></td>
<td>* * A * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td>(bottle nosed dolphin)</td>
<td>A * * * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Delphinus delphis</em> (saddleback dolphin)</td>
<td>* * A * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Globicephala melas</em> (pilot whale)</td>
<td>* * A * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Mesoplodon bidens</em> (Sowerby’s beaked whale)</td>
<td>* * A * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Phoca vitulina</em> (harbor seal)</td>
<td>A S A * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Phoca groenlandica</em> (harp seal)</td>
<td>A S A * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
<tr>
<td><em>Trichechus manatus</em> (Caribbean manatee)</td>
<td>A * * * * * * * *</td>
<td>* * * * * * * *</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Avian Sp.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Melopsittacus undulates</em> (budgerigar)</td>
<td>D T S A G K T E S S S S V S T S Q V S P A</td>
</tr>
<tr>
<td><em>Serinus canaria</em> (canary)</td>
<td>D * * * * * * * T *</td>
</tr>
</tbody>
</table>
Table 4.2. Amino acid alignment of the C-terminal region of RH1 opsin. All sequences except those for *Scopelarchus analis* are from the NCBI database. The sequence from canary is not complete. Positions 334, 338 and 343 are indicated with arrows and have been identified as important phosphorylation sites in bovine rod opsin. Identity is shown by an asterisk. The accession numbers in the order shown in the table are AH001149, U49742, AY318865, U22180, Y09004, S76579, AF055456, AF055314, AF055315, AF055316, AF055317, AF055318, AF055319, AF021242, AJ277926, L11863, AB087811, AF201471, AF201470, Y14484, X62405, Z71999, AF156265, AB043818, AB043817, AJ249203, AJ249202 and U81514.

<table>
<thead>
<tr>
<th>Teleost Sp.</th>
<th>334</th>
<th>338</th>
<th>343</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Carassius auratus</em> (goldfish)</td>
<td>S</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td><em>Danio rerio</em> (zebrafish)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Takifugu rubripes</em> (pufferfish)</td>
<td>*</td>
<td>S</td>
<td>T</td>
</tr>
<tr>
<td><em>Salmo salar</em> (salmon)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Zeus faber</em> (John Dory)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Pomatoschistus minutus</em> (sand goby)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em> (carp)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Hippoglossus hippoglossus</em></td>
<td>S</td>
<td>*</td>
<td>A</td>
</tr>
<tr>
<td><em>(Atlantic halibut)</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Conger myriaster</em> Deep</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>(conger eel)</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Conger myriaster</em> Fresh</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>(conger eel)</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Anguilla japonica</em> Deep</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>(Japanese eel)</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Anguilla japonica</em> Fresh</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>(Japanese eel)</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Raja erinacea</em> (skate)</td>
<td>A</td>
<td>S</td>
<td>*</td>
</tr>
<tr>
<td><em>Scopelarchus analis</em> (Rod A)</td>
<td>S</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Scopelarchus analis</em> (Rod B)</td>
<td>L</td>
<td>S</td>
<td>*</td>
</tr>
<tr>
<td><em>Aristostomias tittmanni</em></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><em>Pachystomias microdon</em></td>
<td>G</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Figure 4.22. Amino acid alignment of the C-terminal region of RH1 opsin. All sequences except those for *Scopelarchus analis* are from the NCBI database. The sequence from canary is not complete. Positions 334, 338 and 343 are indicated with arrows and have been identified as important phosphorylation sites in bovine rod opsin. Identity is shown by an asterisk. The accession numbers in the order shown in the table are AH001149, U49742, AY318865, U22180, Y09004, S76579, AF055456, AF055314, AF055315, AF055316, AF055317, AF055318, AF055319, AF021242, AJ277926, L11863, AB087811, AF201471, AF201470, Y14484, X62405, Z71999, AF156265, AB043818, AB043817, AJ249203, AJ249202 and U81514.
4.3.8 Divergence Time of Rod A from Rod B

To determine the molecular divergence time of the two *Scopelarchus analis* rod opsins, a calculation was made using an equation generated by Hunt *et al.* (1997). A calibration of the molecular clock was made based on the divergence of the rod opsin sequences from goldfish, *Carassius auratus* (Johnson *et al.*, 1993), and sand goby, *Pomatoschistus minutes* (Archer *et al.*, 1992). The fossil record suggests that the Ostariophysi superorder separated from the goldfish lineage close to 140 million years ago (mya).

The rate of divergence was calculated by Hunt *et al.* (1997) using the formula:

\[
\text{Rate of divergence (} R \text{)} = \frac{\text{Substitutions/site (} S \text{)}}{[\text{time of divergence (} T \text{)}*2]}
\]

This gave a value of \(2.46 \times 10^{-9}\) substitutions/site/year. This was taken as the standard rate of divergence for fish rod opsin. The substitutions per site between Rod A and Rod B opsin nucleotide sequences is 27-29%, or for the calculation 0.27-0.29. If this and the value already identified as \( R \) are used back in the same equation, then:

\[2.46 \times 10^{-9} = 0.27/[T*2],\] which rearranged is \(2T = 0.27/2.46 \times 10^{-9}\). \(T = 54.9\) mya.

This is the lower limit, if 0.29 is put into the equation then \(T = 58.9\) mya. Therefore, assuming a standard rate of divergence, the two rod opsins of *Scopelarchus analis* separated between 54.9 and 58.9 mya.
4.3.9 Identification of an RH2 Opsin from *S. analis* Genomic and cDNA

Degenerate forward primers, Green224+ (5'-CAACTWTATKTTGGTCAACC-3') and Green599+ (5'-TACAACAATGAATCATATGTC-3') were designed to conserved regions of teleost RH2 opsins found on the NCBI database and used for 3' RACE on 'juvenile' cDNA. The inner RACE PCR amplified three products of $\approx 500$, 650 and 850 bp in size as determined by gel electrophoresis (Figure 4.23, A). All three were excised, cloned and sequenced, and all were identified as fragments of the same novel RH2 opsin. The gene sequence was completed by a combination of degenerate PCR and genomic walking. A specific reverse primer was designed to the novel sequence, SG596- (5'-CATATGATTCATTGTTGTAGCC-3') and was used with one of two degenerate primers designed to the first seven codons of teleost RH2 sequences on the database, Greenstart1 (5'-ATGAAYGGCACTGARGGMAA-3') and Greenstart2 (5'-ATGGYTTGGGAMGGMGG-3'). The PCR on 'juvenile' cDNA with Greenstart1 amplified a bright product of $\approx 650$ bp in size as detected by gel electrophoresis (Figure 4.23, B). This was directly sequenced from two different reactions and completed the 5' end of the *S. analis* RH2 sequence. At this point the sequence was virtually complete, except for the first 20 bp, which were derived from the Greenstart1 primer. Specific primers were designed for a genomic walk (ScopgreenWKO 5'-TCTGACTCCTGCTGCTGAGCTG-3' and ScopgreenWKI 5'-TGAAGAAGATGGTGAAGACG-3'). The resulting product was sequenced and extended information into the 5' upstream region of the gene (Figure 4.23, C). Interestingly, the walk identified an intron within the RH2 gene sequence, confirming that the gene is not RH1-like. The presence of the RH2 transcript in both 'adult' and 'juvenile' cDNA was examined by PCR with Green checkF (5'-
CAAACAGTTCCGTAACTGC-3') and Green checkR (5'-GGAAACTGAGGAGACTTCT-3'). The expected product was amplified from both templates (figure 4.22). The fragments were excised and sequenced to confirm that the same opsin had been amplified in each case. The RH2 opsin is expressed in both 'juvenile' and 'adult' cDNA. The complete cDNA sequence is shown in figure 4.25, and the 5' upstream sequence is shown in figure 4.26.

**Figure 4.23.** Gel images of PCRs used to amplify RH2 opsin from *S. analis* genomic and cDNA. (A) Inner 3' RACE PCR with degenerate RH2 forward primers at annealing temperatures 50.5 - 55°C. The 650 bp fragment sequenced as novel RH2 opsin. (B) Degenerate PCR to amplify the 5' end of the RH2 cDNA sequence. Two separate primer pairs were used, with Greenstart1 and SG596- amplifying a 650 bp product. (C) Primary and secondary genomic walks amplified a 650 bp product that provided 5' UTR sequence.
Figure 4.24. PCR identifying RH2 expression in both 'juvenile' and 'adult' cDNA. NC, negative control.

Figure 4.25 Complete cDNA sequence for Scopelarchus analis RH2 opsin. The coding sequence is 1053 bp in length. The start and stop translation codons are highlighted in blue and the poly(A) tail is shown in red.
Figure 4.26. Approximately 200 bp of *S. analis* RH2 opsin 5’ upstream sequence. The start codon is shown in blue and a proposed TAATA box is in green, about 110 bp upstream from the start site.

The translated sequence shares 86% identity to pufferfish (*Takifugu rubripes*), 84% to goldfish (*Carassius auratus*) and 80% to zebrafish (*Danio rerio*) RH2 opsin cDNA sequences. An amino acid alignment of these four RH2 opsins is shown in figure 4.27. The *S. analis* RH2 opsin has a number of extra serine residues at the C terminus. These may be involved in an alteration in the kinetics of rhodopsin kinase phosphorylation.
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Figure 4.27. RH2 amino acid sequences for RH2 opsin from goldfish (Carassius auratus), zebrafish (Danio rerio), pufferfish (Takifugu rubripes) and Scopelarchus analis. Conserved regions are highlighted in grey. Using bovine rod opsin numbering lys296, which forms the Schiff’s base with retinal, is shown in red and glu113 shown in orange forms the counterion. The two cysteine residues at 110 and 188 form a disulphide bridge and are shown in blue and potential rhodopsin kinase phosphorylation sites are shown in green.
4.3.10 In Vitro Expression and Regeneration of *S. analis* RH2 Opsin with 11-cis retinal

It seemed likely that the MSP measurement at 505 nm is from cells expressing the RH2 opsin. To try to confirm this the full coding sequence of *S. analis* RH2 opsin was amplified with the primers ScopGF (5'-CAAACAGTTCCGTAACTGC-3') and ScopGR (5'-GGAAACTGAGGAGACTTCT-3') from ‘adult’ cDNA and cloned into pMT4 as described in the methods section 2.2.6. The construct was then transfected into HEK 293T cells and the protein regenerated with 11-cis retinal. On four separate occasions the pigment failed to regenerate. During one of these regenerations phosphatidylcholine (0.8 mg/ml) was sonicated and added as the membrane prep was being made. Phosphatidylcholine is a lipid thought to stabilise RH2 opsin by mimicking the plasma membrane environment, however, it did not affect the inability of *S. analis* RH2 opsin to regenerate.

4.3.11 Regeneration of Rod B and RH2 Opsins Under Pressure

To try to provoke a light sensitive response from *S. analis* RH2 opsin the pigment was regenerated with 11-cis retinal in a high pressure chamber at 100 bar (as described in methods section 2.2.6.7). *S. analis* Rod B was also regenerated in this way to determine if high pressure increased its responsiveness. The RH2 pigment failed to produce a dark response and was bleached with light for 10 mins. Rod B elicited a dark response curve, and was bleached slowly using a series of filters; 1 min at 625 nm, 1 min at 585 nm, 1 min at 495 nm, 2 min at 495 nm, 4 min at 495 nm, 4 min at 495 nm, 10 min at 495 nm and finally 10 min with sunlight. Scans were taken after each bleach. The difference
spectra for both Rod B and RH2 pigments are shown in figure 4.28, a Govardovskii template with a $\lambda_{\text{max}}$ of 478 nm with a bleached retinal curve subtracted has been fitted to Rod B. This is slightly short-wave shifted compared to the 480 nm measured at atmospheric pressure though this is probably not significant.

**Rod B**

![Graph of Rod B spectrum with $\lambda_{\text{max}} = 478$ nm](image)

**RH2**

![Graph of RH2 spectrum](image)

**Figure 4.28.** Difference spectra for *S. analis* Rod B and RH2 pigments regenerated under pressure. The Rod B absorption curve (open squares) has been fitted to a Govardovskii template (dark line) with a $\lambda_{\text{max}}$ of 478 nm, with a bleached retinal curve subtracted.
4.3.12. Phylogenetic Analysis of *S. analis* Opsins

A phylogenetic tree was constructed by the neighbour joining method (Saitou and Nei, 1986) with bootstrapping analysis, to confirm the classification of the three identified opsins (figure 4.29). Rod A and Rod B clade with goldfish (*Carassius auratus*) rod opsin and not with zebrafish (*Danio rerio*) exo-rod opsin with a high level of confidence. It seems unlikely therefore that either Rod A or Rod B is a non-visual opsin. The *S. analis* RH2 opsin clades with the goldfish RH2 opsin. The outgroup is *C. auratus* SWS2 opsin.

![Phylogenetic Tree](image)

**Figure 4.29** A phylogenetic tree showing the three *S. analis* opsins with the rod (L11863), RH2 (L11866) and SWS2 (L11864) opsins of *Carassius auratus* and the exo-rod opsin (NM131212) of *Danio rerio*. Neither Rod A or Rod B clades with exo-rod opsin. Accession numbers are shown in brackets.
4.3.13 In Situ Hybridisation on *S. analis* Retinal Sections

The opsin responsible for the 444 nm MSP recording had still not been identified. The $\lambda_{\text{max}}$ value suggests that the opsin responsible is a member of the SWS2 class. One approach to test this hypothesis was to perform in situ hybridisation experiments on retinal cryosections probing with an SWS2 opsin anti-sense RNA. Additionally, the sections were also probed with rod and RH2 anti-sense RNA to establish the pattern of expression of these genes. The eye used was from a sample caught at 1,200 m that had been frozen on board ship, without prior cryoprotection. The tissue required dehydration and therefore had to be thawed. This was done gradually over seven days, in Carnoys solution containing 60% (v/v) ethanol, 30% (v/v) chloroform and 10% (v/v) glacial acetic acid. Once at room temperature the cornea was removed and the eye dehydrated in increasing concentrations of ethanol (70% (v/v) - 100%). The retina and RPE were then dissected away from the choroid and other superfluous tissue, cryoprotected in 30% (w/v) sucrose and fixed in OCT compound medium. Only the main retina could be identified, the accessory retina could not be discerned due to tissue distortion during thawing. The main retina was orientated to lie transverse to the cryostat blade. Sections were cut 10 μm thick and dried onto glass slides. These were then stored at −20°C. Single stranded DIG-labelled RNA probes were synthesised from Rod A and RH2 opsin fragments in pBluescript and sea bream, *Acanthopagrus butcheri*, SWS2 opsin in pGem-T Easy (kindly donated by Dr. J. A. Cowing). See methods section 2.2.7 for full details on probe production and hybridisation. Both anti-sense and sense probes were generated. As mRNA reads in the sense direction, the anti-sense sequence will bind specifically. The sense probes provided an experimental control. Hybridisation and washing steps were performed at 60°C. The results for RH1
and SWS2 probes are shown in figure 4.30, the RH2 results are in figure 4.31. Cross hybridisation was found to be low as shown in chapter 5, figure 5.26.
Figure 4.30. *In situ* hybridisation on transverse cryosections from *S. analis* main retina. (A, B) Positive labelling with Rod A DIG-labelled anti-sense RNA, with a double photoreceptor layer visible in A. (C) No labelling when probed with Rod A sense RNA. (D, E) Positive labelling with SWS2 DIG-labelled anti-sense RNA, again a double layer is present in some sections (D). (F) Negative control probed with SWS2 sense RNA. Scale bar = 500 μm.
RH2

Figure 4.31. In situ hybridisation on transverse cryosections from *S. analis* main retina demonstrating RH2 expression. (A) Retina probed with anti-sense RH2 RNA shows no positive labelling. (B) Negative control probed with sense RH2 RNA. Scale bar = 500 μm.

Both rod and SWS2 mRNA is expressed in the main retina of *Scopelarchus analis*. RH2 opsin expression was not detected, and therefore expression is likely to be either in the accessory retina only, or limited to more juvenile fish. The SWS2 and rod probes have bound to produce a single or double layer of labelling. The two layers occur more often in sections from peripheral retina. Tissue distortion makes identification of the specifically labelled cells difficult, though there is strict localization, which is likely to be within photoreceptor inner segments.
4.3.14 Search for an SWS2 Cone Opsin

A number of degenerate primers were designed to teleost SWS2 cone opsin sequences deposited on the NCBI database (see table 4.2). These were used in many different combinations in a number of PCR and RACE experiments but no SWS2 opsin has been amplified from 'juvenile' or 'adult' cDNA, or from genomic DNA. A Southern Blot was attempted many times but was unsuccessful because of degradation of the gDNA, as also found with *Aristostomias* and *Pachystomias* (see chapter 3, section 3.3.5).
<table>
<thead>
<tr>
<th>SWS2 Forward Primers</th>
<th>SWS2 Reverse Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFblue+ 5'- CCATTTTTGTCCCGTATC-3'</td>
<td>GFblue- 5'- AGAGAAAAAGCTAGCATATGGC3'</td>
</tr>
<tr>
<td>GFB373+ 5'- CAACGCTCGAGAATGG3'</td>
<td>GFB818- 5'- CCAGCATACCAAGAGCC3'</td>
</tr>
<tr>
<td>BlueF 5'-AAGAARCTMMGNTNMMCTNACTAYATGCTGTAAN</td>
<td>BlueR 5'- CCGAACTGYTTGCTATKARGAYRTANATKAYRGG</td>
</tr>
<tr>
<td>Bull blue1+ 5'- TCTCATAGAATGTGTTCTC-3'</td>
<td>Bull blue2- 5'- CATAGCATGGTCGGGCTT-3'</td>
</tr>
<tr>
<td>Bull blue2+ 5'- GTCTTCTCTTGTGGGATG3'</td>
<td>Bull blue3- 5'- TGCAGCCCTCTGGGATG3'</td>
</tr>
<tr>
<td>Blue 3' outer 5'- CTNAACTAYATYCTKSTGAAC3'</td>
<td>ZF blueR 5'- CGGCAGTTCTTATGTATACCAGTCAGG</td>
</tr>
<tr>
<td>Blue 3' inner1 5'- GYYTGTGTCTKTGTGC-3'</td>
<td>Blue800- 5'- CACCAANAYCACCACCATC-3'</td>
</tr>
<tr>
<td>Blue 3' inner2 5'- TCTGCTTYTGCTTTGCBGT-3'</td>
<td>Blue650- 5'- GCAAAGCARAAGCAGAA-3'</td>
</tr>
<tr>
<td>ZF blueF 5'- GCGCCTCGAGAGAGAACTGTTCGAAGACCC</td>
<td>GFB2- 5'- CGTCACCTCCCTCTCTGCT-3'</td>
</tr>
<tr>
<td>Blue400+ 5'- YYTGTGTCTKTGTGCTGT-3'</td>
<td>Blue744- 5'- GCCTTCTGGTGNGRGC-3'</td>
</tr>
<tr>
<td>Blue600+ 5'- TGTGMMCCDGACTGTGAYAC-3'</td>
<td></td>
</tr>
<tr>
<td>GFB1+ 5'- ACAACCTTCAGCCTACAGC-3'</td>
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</tr>
<tr>
<td>GFB2+ 5'- TGGCTGGTACTTGGCAAAACC-3'</td>
<td></td>
</tr>
<tr>
<td>Blue619+ 5'- CAATGAAWCCCTACGTSAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. List of SWS2 forward and reverse primers used to try to amplify an SWS2 sequence from *S. analis* mRNA and gDNA.
Chapter 4 – Scopelarchus analis

4.4 Discussion

Three visual pigment genes have been identified and sequenced from Scopelarchus analis mRNA and gDNA templates. These are classified as two rod opsins (Rod A and Rod B), and an RH2 opsin by phylogenetic analysis and by BLAST alignment. PCR demonstrated that both Rod A and RH2 genes are expressed in retinal mRNA from a smaller, shallow dwelling ‘juvenile’ and a larger, deeper dwelling ‘adult’. However, Rod B is only expressed in the ‘adult’ fish. In situ hybridisation experiments on the main retina from an ‘adult’ fish identified expression of rod and SWS2, but not RH2 opsin.

MSP has identified three visual pigments with $\lambda_{\text{max}}$ values at 444, 479 and 505 nm (Partridge et al., 1992). The opsins identified in this study need to be correlated to the MSP values. Rod A and Rod B have both been expressed and regenerated in vitro with 11-cis retinal to give $\lambda_{\text{max}}$ values of 490 and 480 nm respectively. Rod B is likely to have formed the pigment responsible for the 479 nm MSP value. The $\lambda_{\text{max}}$ obtained for Rod A does not closely match any of the MSP findings. There are two main possible explanations for this. The first is that the individual sample examined by Partridge et al. (1992) was at a different stage of development as the fish used in this study. It may be that the expression of Rod A is restricted during a particular developmental time point, which was observed by Partridge et al. (1992). An alternative explanation is that the MSP readings were not from pure pigments, but that there was a degree of co-expression. Rod A may have been expressed but was masked by Rod B or the 505 or 444 nm pigments. The RH2 pigment will not regenerate with or without
phosphatidylcholine, which suggests that it is particularly unstable. Attempts to regenerate under pressure also failed. It maybe that the protein needs to be synthesised under pressure for it to attain the putative structure and shape, or that it requires post translational modification, which is not possible in the cell line used. As the spectral range of RH2 rhodopsin sensitivity typically lies between 460 and 520 nm, it seems unlikely that this pigment is responsible for the 444 nm reading, but that it is rather the 505 nm pigment. This is not atypical for a teleost RH2 pigment. Goldfish express two RH2 opsins, one of which regenerates with 11-cis retinal to give a $\lambda_{\text{max}}$ of 505 nm (Johnson et al., 1993), and one of the four RH2 opsins expressed in zebrafish also has a $\lambda_{\text{max}}$ of 505 nm (Chinen et al., 2003). Attempts have been made to identify potential tuning sites by comparing the $S. \text{analis}$ sequence to the goldfish and zebrafish RH2 opsins. However, it is difficult to draw conclusions because the assumption that the tuning mechanism is the same in these three species cannot be made. The SWS2 opsin identified by in situ hybridisation is most likely to be the 444 nm pigment. Partridge et al (1992) identified the 505 nm pigment at the apical tip of rod outer segments, and it was believed that the expression of this gene was being switched off and replaced with that of the 444 nm pigment. This is supported by the in situ result reported in this study, which shows SWS2 but no RH2 expression in an adult main retina, providing further evidence that the mature $S. \text{analis}$ expresses an SWS2 opsin, perhaps with a $\lambda_{\text{max}}$ of 444 nm. PCR shows that RH2 opsin is still expressed in ‘adult’ mRNA and is therefore not replaced completely. It is therefore likely that some cells, perhaps in the accessory retina, retain RH2 expression. A switch in opsin expression to coincide with sexual maturity has been reported in the rod opsins of Atlantic eel, Anguilla anguilla (Archer et al., 1996) and Japanese eel, Anguilla japonica (Zhang et al., 2000) and the SWS2 opsins of the pollack, Pollachius pollachius (Shand et al., 1988). However, if this
interpretation is correct, the retina of *S. analis* is the first example where an RH2 pigment is replaced by an SWS2 pigment within the same photoreceptor. The switch in rod opsin expression that occurs in *A. anguilla* can be induced experimentally by administering gonadotropin hormone (Wood and Partridge, 1993; Hope *et al.*, 1998) the increase in thyroid hormones circulating in the blood stream is thought to induce the opsin switch. Hormone levels may also underlie switches in cone pigments since when the gene encoding thyroid hormone receptor β2 (Trβ2) was knocked out of the mouse it led to a decrease in M-cones, and an increase in S-cones (Ng *et al.*, 2001). The result suggests that in mice, cones develop along an S-cone default pathway, and the thyroid hormone receptor is essential for commitment to the M-cone pathway. The data relates to MWS/LWS and SWS1 gene expression in mammals, but the principle may be similar for RH2 and SWS2 genes in fish.

This study has identified four retinally expressed visual pigments from *Scopelarchus analis*, one more than the three identified by MSP. The extra pigment is a second rod opsin. Rod B is expressed in ‘adult’ but not ‘juvenile’ mRNA (figure 4.6) and when both rod opsin genes are expressed, the level of Rod A mRNA is roughly 3 fold that of Rod B (figure 4.17). The translated sequences from Rod A and Rod B share only 80% identity. This is particularly low for two pigments of the same class, especially as they are from the same species with one presumably arising from a duplication of the other. A calculation of the separation time based on the assumption of a standard rate of divergence is around 55 mya, and is therefore not a recent duplication event. The $\lambda_{\text{max}}$ of 480 nm for Rod B is slightly short-wave shifted compared to the 490 nm of Rod A. This is likely to be explained by only a couple of amino acid differences. Table 4.1 shows the amino acid residues found at ten candidate tuning sites identified by Hope *et
al. (1997) and Hunt et al. (2001) in previous work on deep-sea opsins. The Asp83Asn substitution is common and appears to short-wave shift the $\lambda_{\text{max}}$ by about 10 nm (Hope et al., 1997). This is found in both the *Scopelarchus* rod opsin sequences. The Tyr261Phe and Ala292Ser substitutions are also present in both sequences. Ala124Ser is an important site, which has been identified in aulopiformes and osphidiiformes (Hunt et al., 2001). *Scopelarchus* is a member of the aulopiformes and at position 124 there is an alanine to serine substitution in Rod B opsin and is a likely candidate for the difference in $\lambda_{\text{max}}$ found between the two rod opsins.

The C-terminus of Rod B has seven amino acids absent, which are present in Rod A and common in most previously identified rod sequences (figure 4.22). A number of mutational studies have identified three important phosphorylation sites at 334, 338 and 343 in bovine and mouse rod pigments (McDowell et al., 1993; Ohguro et al., 1993, 1994, 1996; Papac et al., 1993; Adams et al., 2003). The kinetics of phosphorylation and dephosphorylation at Ser$^{338}$ and Ser$^{343}$ are faster than those at Ser$^{334}$ (Ohguro et al., 1995, 1996), which may indicate differing roles in phototransduction. Ser$^{338}$ is phosphorylated after a light flash, whereas Ser$^{334}$ becomes phosphorylated after a continued light response (Ohguro et al., 1995). Phosphorylation at 338 and 343 precedes that of 334 in mice (Kennedy et al., 2001). The C-terminus of avian and teleost rod opsins contains five extra amino acids between residues 343 and 344 of bovine rod. Of these three in avian and four in teleost species, are serine residues. Comparing to the mammalian model, site 343 is conserved in all rod sequences identified from birds and fish. The two avian rod opsins on the NCBI database have not retained serine at sites 334 and 338. The deactivation of these rod opsins must occur differently to that in mammals as two of the identified phosphorylation sites are absent.
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The three extra serine residues in the budgerigar may replace the role of sites 334 and 338. In most species of teleost, 334, 338 and 343 are conserved. However, three rod opsins from the NCBI database, and Aristostomias tittmanni, Pachystomias microdon and Scopelarchus analis rod B have a deletion that includes site 338. Atlantic halibut (50-2000 m), deep-living conger eel (as low as 4000 m), the two stomiid species (1000-2000 m) and Scopelarchus analis (1000 m) all inhabit deep waters. The sand goby lives in shallow, muddy waters where visibility can be low because of the high density of suspended particles. It may therefore be common for fish that inhabit water where light levels are reduced to acquire a deletion mutation at this site. How could this be beneficial? Activated rhodopsin is deactivated by phosphorylation at multiple sites by rhodopsin kinase (Xu et al., 1997). Mendez et al. (2000) studied the effect of a Ser loss at site 334 and/or 338 by expressing rhodopsin mutants in transgenic mice. When either one or both of these sites were mutated, the rod response was greatly prolonged. Rhodopsin is maintained in its active state of metarhodopsin II for a longer period of time. This could lead to an amplification of the photoresponse. In a low light environment where photons are limited, sensitivity to light needs to be increased. A greater amplification of the phototransduction cascade would lead to a stronger signal being relayed to the bipolar and horizontal cells even though photon capture remains the same. The deletion of Ser$^{338}$ and the substitution of Ser$^{334}$ in S. analis rod opsin B may well be an adaptation to low light conditions and could be a common feature of deep-sea opsins.

4.5. Conclusions

The visual system of S. analis has become highly adapted to increase sensitivity. This is also true of its visual pigments. This study has identified four expressed visual opsins.
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Rod A is expressed in both ‘juvenile’ and ‘adult’ whole eye mRNA. Rod B is only expressed in ‘adult’ mRNA. An RH2 opsin is expressed in both ‘juvenile’ and ‘adult’ mRNA, but in situ hybridisation did not identify expression in the main retina from an ‘adult’ eye. Adult RH2 expression may therefore be limited to the accessory retina, be localised to a region of the main retina that has not been examined, or has been turned off. Expression of an SWS2 opsin was identified in the ‘adult’ main retina by in situ hybridisation. Rod A and Rod B have \( \lambda_{\text{max}} \) values at 490 and 480 nm respectively. The RH2 opsin is most likely responsible for the 505 nm MSP recording, and the SWS2 opsin for the 444 nm pigment. The major functional difference between Rod A and Rod B pigments may be linked to differing rates of deactivation by rhodopsin kinase. The deep-water Rod B may have a longer activation time, which promotes amplification of the phototransduction cascade to elicit a greater photoresponse.

4.6. Future Work

The most important aspect that requires completion is the identification of an SWS2 opsin sequence from mRNA or gDNA templates. In vitro regeneration of this and the RH2 pigment may prove to be very difficult and perhaps impossible because of problems in recreating typical ‘deep-sea’ conditions. Recently some fresh samples have been caught and properly fixed for in situ hybridisation work. These could provide some very interesting data on the expression pattern of rod, RH2 and SWS2 opsins in the main and accessory retinæ from ‘juvenile’ and ‘adult’ fish.
Chapter 5 - The Visual Pigments of the Notothenioid Family of Ice Fish

5.1 Introduction

The waters of the Antarctic are a hostile environment. Not many species are able to flourish in such extreme conditions. One suborder of telesosts, the Notothenioidei have been extremely successful in adapting to life at sub-zero temperatures (figure 5.1). In fact the Notothenioidei or icefishes includes five families and represents greater than 90% of the Antarctic fish fauna in terms of both biomass and number of species (Eastman, 1993).

Figure 5.1 Photograph of Dissostichus mawsoni, a member of the notothenioidei. The lack of colour is due to the reduction of haemoglobin and provides good camouflage for life under pack ice. D. mawsoni can grow up to 2 metres in length. Photograph courtesy of Prof. Jim Bowmaker.
The majority of notothenioids are benthic, though some have moved into the pelagic realm and a few species live just below the pack ice, these are classified as cryopelagic (Nelson, 1994). As the notothenioids lack a swim bladder it has been suggested that they may have evolved from a benthic ancestor (Andersen, 1984).

Ice fish are extremely interesting to evolutionary biologists because of the numerous adaptations to life in freezing waters. The most extensively examined adaptation is the presence of antifreeze proteins in the blood plasma. These were first described by DeVries and colleagues (1969, 1970) and have been found to be present in a variety of organisms including bacteria, plants, insects and vertebrates (for reviews see Fletcher et al., 2001; Cheng, 1998). Work on the winter flounder has demonstrated that there are two antifreeze gene families. The protein expressed from the first family is found in the liver and is then distributed throughout the body via the blood stream. The second is expressed in gill and skin epithelia and protects tissues that come into direct contact with ice (Gong et al., 1996). The antifreeze proteins work by binding to and preventing embryonic ice crystals from forming (Raymond and DeVries, 1977).

A second adaptation to life at low temperatures is the reduction of erythrocytes and haemoglobin in the blood (Tamburrini et al., 1996; Bargelloni et al., 1998). This situation is viable because the cold temperature allows for a lower metabolic demand and higher oxygen solubility. The amount of haemoglobin found in different species of notothenioid fish varies. The most extreme case is seen in the Channichthyidae family (crocodile icefishes) where all haemoglobin has been lost (Ruud, 1965).
Chapter 5 - Notothenioids

There is limited information at present on the ice fish visual system. The eyes of notothenioid species can be orientated laterally as in the Gymnodraconinae and Bathydraconini, or dorsolaterally as in the Bathydraconini (Eastman and Lannoo, 2003). All members of the Bathydraconidae family have duplex retinae (rod and cone photoreceptors), with deeper living species exhibiting a higher proportion of rod receptors. Double and single cones are present in all species studied taken from various depths (Miyazaki et al., 2001; Eastman and Lannoo, 2003). Previous work on the retinae of three nototheniid fish that inhabit different depths showed an increase in rod density in the deepest living species examined, Dissostichus mawsoni (Meyer-Rochow and Klyne, 1982). The rods in this species were described as having unusually long inner and outer segments (Meyer-Rochow and Klyne, 1982). ERG recordings of dark- and light-adapted retina were taken from Pagothenia borchgrevinki. The scotopic response elicited a maximum at 490 nm and is considerably higher in amplitude than the photopic or cone response (Morita et al., 1997). Studies on the brain morphology of the Brachydraconidae have shown that these fish are not sensory specialists, a feature often found in deep-sea fish (Eastman and Lannoo, 2003). However, deeper living species do have reduced olfactory and visual centres and enlarged lateral line regions.

Visual scientists have been interested by the notothenioids because of their unique visual environment. During the Antarctic summer there is continuous light for several months. However, the thick layers of pack ice and snow limit the amount and spectral composition of the light reaching the water below. Light transmission is also influenced by the low sun angle and impurities present in the ice. A light intensity reading was taken at noon under 3 m solid sea ice covered by 5 cm of snow, which showed the average light transmission to be 0.25% (Littlepage, 1965). Pankhurst and Montgomery
(1989) plotted depth light-intensity profiles at different times of day and found highest light intensities at 15:00 h with penetration down to 40 m. UV and long-wave light are unable to penetrate thick sheets of ice and therefore there would be little advantage gained by vision in these regions of the spectrum. Have the ice fish that have adapted so neatly to their atypical environment also adapted their visual system? It may be expected that UV and LWS cones are absent or few in number. There may also be some variation in spectral sensitivity between cryopelagic, mesopelagic and benthic species. Fish that live deeper will encounter light that has been filtered through the water column. The visual pigments may have become spectrally tuned to match the down-welling light, as seen in fish from the deep-sea (for review see Douglas et al., 1998) and Lake Baikal (Bowmaker et al., 1994; Hunt et al., 1996). A group at the University of Illinois led by Dr. Chi-Heng Christina Cheng, collect notothenioid samples from the Antarctic to analyse antifreeze proteins. A collaborative project with Prof. David Hunt and Prof. Jim Bowmaker is aimed at isolating the expressed opsin sequences and identifying the spectral detecting capabilities of these fish that live in a reduced light environment.
5.2 Aims

1. Identification and isolation of the cone visual pigments present in *D. mawsoni* and other selected species.

2. Estimation of $\lambda_{\text{max}}$ values for these pigments with MSP and *in vitro* regeneration with 11-cis retinal.

3. Localisation of opsins in the retina.

4. Examination of the retinal cone mosaic of selected species using basic histological techniques.
Chapter 5 - Notothenioids

5.3 Results

The majority of the work in this chapter is on the Antarctic toothfish, *Dissostichus mawsoni*, though there is some histological data for *Trematomus hansonii* and molecular genetic data for several species including *Trematomus loenhergii*, *Pagothenia borchgrevinki*, *Gymnodraco acuticeps* and *Notothenia angustata*. Possible experiments were reliant on available tissue. *D. mawsoni* was the only species with RNA and genomic DNA readily available and was therefore studied most intensely. However, as both molecular genetic and MSP data gave highly similar results across all ice fish species, conclusions made for one can probably be extrapolated to all.

5.3.1 MSP Recordings from Ice Fish Photoreceptors

MSP analysis was carried out by Prof. J. K. Bowmaker and Dr. J. Parry on the retinæ of five nototheioid species; *T. loenhergii*, *P. borchgrevinki*, *T. hansonii*, *T. bernacchii* and *D. mawsoni*. These inhabit a range of depths under the ice, from cryopelagic to benthic habitats. The MSP study was aimed at identifying any differences in wavelength sensitivity with relation to depth inhabited. The results show the presence of one rod and three cone photoreceptor cell classes (personal communication, figure 5.2). The rod cells gave $\lambda_{\text{max}}$ values around 500 (499-503) nm. This is slightly short-wave shifted with respect to the more commonly studied goldfish and zebrafish. The cone composition of the retina consisted of single blue cones ($\lambda_{\text{max}}$ 414-426 nm), single green cones ($\lambda_{\text{max}}$ 489-491 nm) and double green cones ($\lambda_{\text{max}}$ 489-491 nm). It is likely that the same green cone visual pigment is expressed in the single and double cones. A
single species, *P. borchgrevinki*, appeared to be missing the single blue cones. That result aside, all five species showed very little difference in $\lambda_{\text{max}}$ for rods, green and blue sensitive cones. No UV or LW sensitive cones were found in any species.
Figure 5.2. MSP analysis on the retinas of five species of notothenioids. In all ice fish studied there was a population of rods (grey, 499-503 nm) and single and double green cones (light and dark green respectively, 489 – 491 nm). In four of the five there was also a population of blue sensitive cones (blue, 414 – 426 nm). No UV or red (LW) sensitive cones were detected. Bowmaker and Parry (personal communication).
5.3.2 Southern Hybridisation on D. mawsoni gDNA with Cone Opsin

**Probes**

To identify the cone opsin genes present in the genome of *D. mawsoni*, digested gDNA was probed with species specific SWS1 (see section 5.3.4) and RH2, SWS2 and LWS/MWS opsin from sea bream, which had been cloned for an earlier experiment by Dr. J. A. Cowing. 45μg of *D. mawsoni* genomic DNA was digested in each case. The first blot contained three lanes of gDNA digested with EcoR1, Bam H1 or HinD III and was probed solely with *D. mawsoni* SWS1 opsin. The blot was washed twice with [6X SSC, 0.5% SDS (w/v)], twice with [3X SSC, 0.5% SDS (w/v)] and twice in [1X SSC, 0.5% SDS (w/v)] for 10 mins at 65°C. The blot was exposed to film for 5 nights. The resulting radiograph was clean of unspecific labelling and shows single bands in the EcoR1 and HinD III lanes of comparable signal strength. In the Bam H1 lane there were three bands all of slightly less signal strength suggesting the presence of two BamH1 restriction sites in the SWS1 genomic sequence (Figure 5.3).

The experiment was repeated with sea bream SWS2, RH2 and LWS/MWS cone opsin probes, digested from maxiprep stocks, and labelled with αP32 dCTP as before. The genomic DNA digests and transfer conditions were also exactly as previously used. The temperature for hybridisation was lowered to 50°C to aid binding of non-species specific probes, but was not lowered any further to reduce the possibility of the probes cross-hybridising to non-specific opsin genes. The same washing reagents were used but at 55°C instead of 65°C to prevent removal of the non species-specific probe. The blot was exposed to film for 5 nights. The results are shown in figure 5.4. A signal was
present when probed with SWS2 and RH2 opsin. There was no detectable signal with the LWS/MWS opsin probe.

*Figure 5.3* (A) Southern hybridisation on *D. mawsoni* gDNA with the *D. mawsoni* SWS1 opsin specific probe. Lanes 1-3 show the results for the three restriction enzymes Bam H1, EcoR1 and HinD III respectfully. The ladder is taken from the original gel (B) and has been scaled appropriately.
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Figure 5.4. (A) Southern hybridisation of EcoRI digested D. mawsoni genomic DNA probed with sea bream SWS2, RH2 and LWS/MWS opsin fragments. A signal is present in the SWS2 and RH2 lanes. There is no signal with the LWS/MWS probe. The ladder is from the original gel (B) and has been scaled appropriately.

On figures 5.3 and 5.4 a ladder has been superimposed onto the side of the blot to aid band size determination. The ladder was taken from the original agarose gel on which the digested genomic DNA was separated overnight. Using this method it is clear that the SWS1, SWS2 and RH2 fragments in the Eco R1 digested lanes are all of different sizes. The SWS1 band is at 3.8 kb, the SWS2 band is at 8 kb and RH2 is at 7.3 kb as determined by plotting size (bp) against \( \log_{10} \) (distance run) and using the generated graph to identify the size of the labelled fragments. This provides evidence that the
result is not due to cross-hybridisation but that each of the three positive probes have labelled different genes. Probes that cross-hybridise with opsins of a different class would light up digested fragments of equal size in different lanes, i.e. if the SWS1 gene hybridised to both the SWS1 and SWS2 probes, the visualised bands would be of the same size because the same gene is labelled in both experiments. Here all probes hybridised to fragments of different size. The Southern hybridisation blots strongly suggest that SWS1, SWS2 and RH2 genes are present in the genome of *D. mawsoni*. The MWS/LWS gene appears to be absent. The presence of both SWS1 and SWS2 genes is interesting as the MSP only provided evidence for a single short-wave pigment, which at a $\lambda_{\text{max}}$ of 410 nm is likely to be an SWS2 opsin.
MSP identified two cone classes, whereas the Southern hybridisation experiments show that there are three different genes. This led us to suspect that perhaps one of the genes is not being expressed. Northern blots were performed using retinal RNA to ascertain which of the identified cone classes are retinally expressed. Northern hybridisation is a technique, which probes extracted RNA for expressed genes. A collaborative group led by Dr. Chi-Hing Christina Cheng at the University of Illinois probed *D. mawsoni*, *P. borchgrevinki*, *T. hansonii*, *G. acuticeps*, *P. antarcticus* and *C. gunneri* retinal RNA with *P. borchgrevinki* SWS1 opsin and sea bream SWS2, RH2 and MWS/LWS opsin fragments. The results are shown in figure 5.5. The RH2 probe hybridised very strongly to the retinal RNA for all six species. The results for the other probes are not so conclusive. The SWS1 probe only hybridised to *P. borchgrevinki* RNA, the other five lanes remaining blank. There is faint hybridisation with the SWS2 probe and very little with the LWS/MWS probe. Unfortunately, all four probes were hybridising against RNA fragments of approximately the same size (1.7 kb) and the gel has not run straight. This makes it very difficult to determine the exact size and whether the SWS2 probe is specifically binding to an SWS2 RNA sequence or if it is cross-hybridising to another opsin sequence, i.e. that of the highly expressed RH2. It seems likely that cross-hybridisation is the reason for the very faint binding with the LWS probe. The lack of SWS1 hybridisation to the RNA of five of the species may suggest that the SWS1 transcript is rare or absent in these fish, but is strongly expressed in *P. borchgrevinki*. Interestingly the MSP for *P. borchgrevinki*, could not measure any single cone specific pigments that were either blue or UV sensitive.
Figure 5.5. Northern blots of Notothenioid RNA. Total retinal RNA from six species was run on formamide gels (row A) and then transferred to a membrane. The two bands present on the RNA gel are those for 28S and 18S ribosomal RNA. The images in row B show Northern blots hybridised with (from left to right) SWS1, RH2 and SWS2 probes. In row C the same membranes were stripped and then hybridised with LWS, RH2 and SWS2 probes. A strong signal was present in the RH2 probed blots for all species. There is a faint signal for all species except *P. antarcticus* when probed with SWS2 opsin. The SWS1 probe elicited a signal from *P. borchgrevinki* only. None of the species gave a signal when probed with LWS/MWS opsin. The gel has not run straight, which makes exact size determination difficult.
5.3.4 *Isolation of the Ice Fish SWS1 Opsin*

Dr. Cheng and colleagues used a molecular genetic approach to isolate the cone visual pigments from retinal RNA. A complete sequence for an SWS1 (UV/violet) sensitive opsin gene was obtained, but there was no success in obtaining SWS2 (blue), RH2 (green) or LWS (red) opsin sequence data (personal communication). The SWS1 sequence has been identified in retinal RNA from six different ice fish species: *Pagothenia borchgrevinki, Trematomus loenbergii, Gymnodraco acuticeps, Notothenia angustata, Pagetopsis macropterus* and *Dissostichus mawsoni*. The cDNA and amino acid sequences for all species are shown in figures 5.6 and 5.7 respectively. Table 5.1 show the nucleotide and amino acid percentage identity values. Amino acid identity is approximately 90% with the nucleotide values slightly higher at around 94%. The percentage identities are roughly similar between all species, except for the slightly lower amino acid identity of the *N. angustata* opsin to *D. mawsoni, P. borchgrevinki* and *T. loenbergii*. The SWS1 sequences have remained highly conserved after speciation, this plus the fact expression is retained suggests that SWS1 vision has an important role in these fish. Isolation of an SWS1 pigment suggests that UV/violet sensitivity is present in the ice fish retina.
Chapter 5 - Notothenioids

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Chapter 5 - Notothenioids

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**Pagothenia borchgrevinki**

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278
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**Figure 5.6.** Complete SWS1 cDNA sequences from six notothenioid species. Cheng et al. Personal communication.
Figure 5.7. SWS1 translated sequences from six notothenioid species. Lys (296) (red) forms the Schiff’s base, Glu (113) (orange) is the counterion. The disulphide bridge forming cysteines at positions 110 and 188 (blue) and the residues important for phosphorylation (green) are also shown. The phenylalanine at position 86 (purple and underlined) is commonly found in UV sensitive pigments. Numbering used for highlighted sites is that for bovine rod opsin.
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### Amino acid

Table 5.1. Amino acid and nucleotide percentage identities for the SWS1 opsins isolated from six species of nototheniid.
5.3.5 In Vitro Expression and Regeneration of Recombinant D. mawsoni SWS1 Opsin with 11-cis Retinal

The full coding sequence for *D. mawsoni* SWS1 opsin was amplified from a pGEM-T Easy clone that had been donated by Dr. Cheng and colleagues. The modified primers D.mawsUVF (5’-GCGCGAATTCC ACC ATGGGGAAGG ACTTCC AC-3’) and D.mawsUVR (5’- CGGCGTCGACGCAGACACTGAGGACACCTC-3’) and the proof reading DNA polymerase *pfu* were used with the usual PCR reagents. The SWS1 fragment and pMT4 vector were both digested with the restriction enzymes EcoRI and SalI to create complimentary sticky ends allowing ligation of the SWS1 opsin into the expression vector. The opsin gene was placed next to a strong promoter within the vector, which runs transcription of the opsin plus the 1D4 epitope tagged at the 3’ end. The construct was then used to transfect 293T HEK cells. The opsin protein was isolated from the cell membranes, bound to 11-cis retinal, captured on a column using the 1D4 epitope and after several washes eluted in solution (see methods section 2.2.6). Dark and acid denatured spectra were taken by spectrophotometry and are shown in figure 5.8. The difference spectrum fitted to a Govardovskii template is shown in figure 5.9. A $\lambda_{\text{max}}$ value of 369 nm is calculated from the template, which does not correspond to any MSP measurement. It seems unlikely that the SWS1 opsin is responsible for the $\lambda_{\text{max}}$ found at 414 nm, but that this reading is from an SWS2 pigment. A $\lambda_{\text{max}}$ value at 369 nm for an SWS1 pigment is quite typical when compared with other teleost species. Even though there is evidence for UV absorption by the overlying pack ice, six species of notothenioid that inhabit a range of depths all express a UV sensitive SWS1 opsin.
Figure 5.8. Dark (A) and acid denatured (B) spectra for *D. mawsoni* SWS1 opsin.
**Figure 5.9.** *D. mawsoni* SWS1 difference spectrum (open squares) fitted to a Govardovski template (black line) with a $\lambda_{\text{max}}$ at 369 nm and with a bleached retinal curve subtracted.
5.3.6 Isolation of Ice Fish RH2 Opsins

cDNA was synthesized from 4μg of *D. mawsoni* retinal total RNA (kindly donated by Dr. C. Cheng). A number of degenerate teleost RH2 opsin primers were designed and a complete RH2 opsin cDNA sequence was isolated from cDNA in four steps. All the PCR reactions were under standard conditions unless otherwise stated.

1. Two degenerate primers were designed to amplify a 600 bp fragment from the start codon, Greenstart1 (5’-ATGAAYGGCCTGARGGMAA-3’) and Greenstart2 (5’-ATGGYTTGGGAMGGMGG-3’). Greenstart1 and SG596-amplified a fragment of the expected size from cDNA, Greenstart2 was not effective (Figure 5.10 A). The band was excised, cloned and sequenced. The resulting cDNA sequence showed highest similarity to teleost RH2 opsins deposited on the NCBI database.

2. The isolated sequence began at the start codon but as the first 20 bp were derived from the forward primer, the 5’ end of the sequence required completion. *D. mawsoni* RH2 specific walking primers (WK Outer 5’-CTAGAAGCTTGAACATGATGGG-3’, WK Inner 5’-TAAGGACTTCTAACAATCCC-3’) were designed and a standard genomic walk (see methods 2.2.2.3) performed on ≈ 0.35 μg genomic DNA (kindly donated by Dr. C. Cheng) at 1.5 or 3.0 mM MgCl₂ concentration. This amplified a 1 kb fragment which when sequenced confirmed the presence of the RH2 opsin gene in the genome of *D. mawsoni*, and extended the sequence into the 5’ untranslated region (Figure 5.10, B).
3. To complete the cDNA sequence a series of 3’ RACE reactions with different RH2 forward primers, Ice 3’ Inner (5’- GCTGGAATTGGTGTGGGA-3’), GreenCheckF (5’- CAAACAGTTCCGTAACTGC-3’), SG 615+ (5’- ATGTCACTACATGTTCACC-3’),  SG 908+ (5’- ACCAACCAGTCATCTACG-3’), and Green 599+(5’- TACAACAATGAATCATATGTC-3’) and a standard polyA reverse primer were performed over a range of three annealing temperatures (55-60°C). This amplified a number of products, which were all excised and directly sequenced (Figure 5.10 C and D). The result was 450 bp of further RH2 opsin sequence completing the very 3’ end, but this did not overlap with the previously isolated 5’ sequence.

4. To bridge the gap in the cDNA sequence, combinations of primers with one on either side of the missing section were used. Three different standard reactions were performed and primers Ice Green 3’O (5’- TCCAGATACTTCTGAGGG-3’) and D. maws GR (5’- CGGGCGTCGACGCAGACACAG and D. maws GF (5’- CGCGAATTCCACCATGGAGACCAATGGGCACAG-3’) and SG 596- (5’- CATATGATCTGGTGAGGCCAG-3’) amplified appropriately sized fragments (figure 5.10, E). These were direct sequenced and when all the information was put together the completed RH2 cDNA sequence was obtained as shown in figure 5.11 and 500 bp of 5’ upstream sequence isolated by genomic walking is shown in figure 5.12. The translated sequence is found in figure 5.13.
Figure 5.10. Gel images of degenerate PCR, 3’ RACE and genomic walking on *D. mawsoni* genomic and cDNA to amplify an RH2 opsin. (A) Degenerate PCR on *D. mawsoni* retinal cDNA amplified a 600 bp product. (B) Genomic walking amplified a 1 kb fragment to complete the 5’ end. (C, D) Degenerate 3’ RACE obtained the final 400 bp of coding sequence. (E) The RH2 cDNA sequence was completed with PCRs bridging the missing middle region.
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Figure 5.11. Complete *D. mawsoni* RH2 opsin cDNA sequence. Start and stop codons are highlighted in green, the poly(A) tail is shown in red.

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Figure 5.12. Approximately 500 bp of *D. mawsoni* RH2 5' upstream sequence, reading up to the start codon shown in green.
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Figure 5.13. *D. mawsoni* RH2 translated sequence. The protein consists of 346 amino acids. Key functional residues have been highlighted. Lys(296) is shown in red and forms the Schiff’s base. Glu(113) is in orange and is the counterion. The two cysteine residues (110, 188) that form a disulphide bridge are shown in blue and phosphorylation sites necessary for binding of rhodopsin kinase are shown in green. Numbering for key residues is for bovine rod opsin.

Rh2 sequences were amplified from *P. borchgrevinki, G. acuticeps* and *N. angustata* retinal cDNA using the *D. mawsoni* specific primers *D.mawsF* (5’-GCGCGAATTCACCACCATGGAGACCAATGGCACAG-3’) and *D.mawsR* (5’-CGGCGTCGACGCAGACACAGAGGACACTTCTG-3’). These primers bind from the start and stop codons and amplify the full coding sequence. Products from all three species (figure 5.14) were excised, cloned and then sequenced.

The four isolated notothenioid RH2 sequences show 93% identity, excluding the conserved forward and reverse primer sequences. The cDNA sequences from *G. acuticeps, N. angustata* and *P. borchgrevinki* are shown in figure 5.15. The translated sequences are shown aligned in figure 5.16. Table 5.2 shows the nucleotide and amino
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acid percentage identities between the sequences, identifying high similarity between all four.

Gymnodraco acuticeps

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**Pagothenia borchgrevinki**

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Figure 5.15. Complete RH2 cDNA sequences from three species of nototheniod. The forward and reverse primer sequences are shown in green, as these are not species specific.

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### Amino acid

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**Figure 5.16.** Amino acid alignment of *D. mawsoni*, *P. borchgrevinki*, *G. acuticeps* and *N. angustata* RH2 (green) opsins. The residues shown in blue are translated from the forward and reverse primers and are therefore not species specific. Conserved regions in all four sequences are highlighted in grey.
5.3.7 Regeneration of Recombinant D. mawsoni RH2 Opsin with 11-cis Retinal

Once the RH2 sequences had been completed, the entire coding region was isolated from *D. mawsoni* retinal cDNA using the modified primers D.mawsF (5'-GCGCGAATTCCACCATGGAGACCAATGGCACAG -3') and D.mawsR (5'-CGGCGTCGACGCAGACACAGAGGACACTTCTG -3'). The resulting fragment was cloned into the expression vector pMT4 and transfected into HEK 293T cells as described for SWS1 opsin. The cells were harvested, the opsin isolated and then regenerated with 11-cis retinal before bleaching with light for 15 mins. The resulting dark and bleached spectra are shown in figure 5.17. The difference spectrum is fitted to a Govardovski template with a $\lambda_{max}$ of 488 nm and with a bleached retinal curve subtracted (figure 5.18). The $\lambda_{max}$ is close to the MSP value of 491 nm.
Figure 5.17. Dark (A) and bleached (B) spectra for *D. mawsoni* RH2 opsin.
Figure 5.18. Difference spectrum for *D. mawsoni* RH2 opsin (open squares), fitted to a Govardovski template (black line) with a $\lambda_{\text{max}}$ of 488 nm with a bleached retinal curve subtracted.
5.3.8 Isolation of Ice Fish SWS2 Opsins

The search for an SWS2 cone opsin involved the same techniques that proved successful for the RH2 cone opsin. A number of SWS2 primers were designed from teleost SWS2 opsin sequences deposited on the NCBI database (shown previously in table 4.2). The preferred location of primers was in conserved regions of sequence, normally found in the areas coding for the transmembrane domains. PCRs were performed on retinal cDNA and genomic DNA and degenerate RACE reactions were performed on retinal cDNA. Any amplified products were excised, cloned and sequenced. None of the fragments contained an SWS2 opsin sequence. A possible explanation for the lack of SWS2 RT-PCR success was that the area of retina used for extraction showed low SWS2 expression. To prevent this being a factor all further PCRs were performed using genomic DNA. More degenerate SWS2 primers were designed and further reactions run altering the reagent concentrations, the annealing temperature, and length of annealing and extension steps. Still no SWS2 sequence was isolated and unfortunately all the *D. mawsoni* genomic DNA had been used. Further reactions were performed with *Trematomus loenbergii* genomic DNA as the template. Eventually successful amplification conditions were found. The gradient PCR conditions used were standard except the annealing temperatures were low, 48-54°C. Forward primer blue400+ (5′- YYTGTGGTCTCTKCTGT-3′) and reverse primer GFB818- (5′-CCAGCATACCAAGAAGCC-3′) amplified two products of approximately 800 bp and 1 Kb in size (figure 5.18 A). When these were excised, cloned and sequenced the 1 kb fragment contained novel SWS2 sequence encompassing exons 3-5 including the introns between these exons. With degenerate PCR it is common to amplify non-specific genes, especially if both the forward and reverse
primers contain unspecified bases. This was one of the problems in isolating the SWS2 sequence as other opsin-like genes were preferentially amplified. However, primers too specific to other teleost SWS2 genes were unsuccessful because of nucleotide differences present in the ice fish sequence. With primers blue400+ and GFB818-, only the forward primer was degenerate, the reverse primer being goldfish specific. Having one primer specific for SWS2 increased the likelihood of specific binding and amplification of the SWS2 opsin. The low annealing temperature enabled binding of the non-ice fish specific GFB818- primer. The isolated SWS2 genomic sequence was extended in the 3' and 5' directions with specific PCR and genomic walking respectively.

A genomic walk was performed using gene specific primers T.loen WKO (5'-GGACGGAAGCAATCAGTGCAA-3') and T.loen WKI (5'-CCCAGGTTAATGCACAGC-3') for outer and inner reactions respectively. The reactions were set up in a standard manner as described in the methods at two MgCl₂ concentrations, 1.5 and 3.0 mM. Gel analysis identified a 500 bp product (figure 5.18 B), which was excised from two different PCRs and directly sequenced. This produced roughly 380 bp further sequence but did not complete the 5' end, as the primer had bound within the first intron. Further genomic walks were performed using new primers but none of these extended the sequence into the first exon. A reason for this difficulty could be that the first intron contains a sequence that binds tightly to the universal primer, and therefore it is difficult to optimise the PCR to remove this binding while promoting binding in the 5' untranslated region. Degenerate primers designed to exon 1 were used in PCR reactions with gene specific reverse primers but no products were
amplified. The 5’ end of the *T. loen* SWS2 sequence ends approximately 170 bp before the start codon.

To extend sequence information in the 3’ end of the gene PCR reactions were performed on gDNA with *T. loenbergii* specific forward primers (T.loen 3O (5’-CATTACACTGAAAGCAGTGA-3’), T.loen 3I (5’-GCAGAGTCTGCCTCCACC-3’)) and degenerate reverse primers (GF blue stop (5’-TTATTTCTCTGGYGCAACRG-3’, MZ blue stop (5’-CTAWGCAGGYSCCYACTTTRG-3’)). The two reverse primers were designed after aligning a number of teleost SWS2 sequences. It was noted that the final six codons showed two different basic forms. Two separate degenerate primers were designed, one to each basic variant. One was termed GF blue stop, as one of the species with this stop sequence was goldfish and the second MZ blue stop, after the cichlid Metraclima zebra. The two forward and two reverse primers were used in their four permutations in PCR reactions. T.loen 3I and MZ blue stop amplified a 700 bp fragment (figure 5.18 C), which when cloned and sequenced extended the *T. loenbergii* SWS2 sequence up to the final codon. The final 20 bp were derived from the primer and not from *T. loenbergii* opsin. Therefore, the isolated *T. loenbergii* SWS2 sequence includes all exons except for the first 170 bp of the first exon and the last six codons of the fifth. The contiguous nucleotide sequence with introns removed is shown in figure 5.19. The translated sequence is shown in figure 5.20.
Figure 5.19. Gel images of the products amplified from T. loenbergtii genomic DNA using SWS2 primers. (A) Degenerate PCR with blue 400+ and GFB818primers amplified two bands at standard conditions with annealing temperatures increasing from left to right at 47.9°C, 50°C, 52°C and 53.9°C. The 1 kb band generated novel SWS2 sequence. (B) Inner genomic walk with a specific T. loen SWS2 reverse primer and the universal primer UNl 17. Inner and outer reactions were performed at both 1.5 and 3.0 mM MgCl₂. (C) Degenerate PCRs on the 3’ end of the gene with T. loen 3’O and 1 forward primers and GFbluestop and MZbluestop reverse primers. The ≈ 700 bp amplified fragment in the I/MZ lane (arrow) was excised and sequenced as the 3’ end of the SWS2 gene.
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**Figure 5.20.** *T. loenbergii* SWS2 partial genomic sequence. Exons 1, 3 and 5 are highlighted in grey, exons 2 and 4 are in between these blocks. Introns have been removed from the sequence. The final seven codons are that of the degenerate primer MZbluestop.

**Figure 5.21.** *T. loenbergii* SWS2 amino acid sequence missing the first 60 amino acids. Key conserved residues are highlighted. Lys(296) forms the Schiff’s base and is shown in red and Glu(113) forms its counterion and is in orange. The two cysteine residues (C110 and C188) that form a disulphide bridge are shown in blue.

Only gDNA from *T. loenbergii* was available, but sequence needed to be isolated from retinal cDNA to establish gene expression. A primer pair designed to the isolated *T. loenbergii* SWS2 sequence, *T.loen+* (5’- CGGATCGAGCTAC-3’) and *T.loen-* (5’- GGTGAGGCAGACTTCGC-3’) was used on *D. mawsoni, P. borchgrevinki, N. angustata* and *G. acuticeps* cDNA (figure 5.21). Products amplified from all four species were cloned. A number of colonies were miniprepped and sequenced.
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Approximately 200 bp of novel SWS2 sequence was isolated from *G. acuticeps* and *P. borchgrevinki*. This confirms the expression of SWS2 opsin in the retina of these two species. Only SWS1 sequence was obtained from the *D. mawsoni* and *N. angustata* clones. The lack of PCR success may suggest that the level of SWS2 expression is lower in these species, and may help to explain the difficulties in isolating SWS2 sequence from *D. mawsoni* cDNA as mentioned earlier. The SWS2 cDNA sequences are shown in figure 5.22. The two species show 98% identity over this small region.

**Figure 5.22.** Gel image of the 250 bp fragments amplified with *T. loenbergii* specific SWS2 primers from *D. mawsoni*, *G. acuticeps*, *N. angustata* and *P. borchgrevinki* retinal cDNA. NC is the negative control.

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**Figure 5.23.** 170 bp of SWS2 sequence isolated from *P. borchgrevinki* and *G. acuticeps* retinal cDNA.
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It was not possible to express *T. loenbergii* SWS2 opsin and to regenerate *in vitro* because the complete coding sequence was not identified. However, the $\lambda_{\text{max}}$ value of 416 nm found by MSP is likely to be from cells expressing the SWS2 opsin. This $\lambda_{\text{max}}$ is particularly short-wave shifted compared to most members of this class, which have values close to 440 nm, but matches the values obtained for the SWS2 opsin of zebrafish, *Danio rerio*, by MSP and *in vitro* expression of 410-416 nm (Nawrocki *et al.*, 1985; Chinen *et al.*, 2003). The SWS2 amino acid sequences from seven teleost species and for *T. loenbergii* are shown aligned in figure 5.24. The $\lambda_{\text{max}}$ for these pigments are: *Metriaclima zebra*, 488 nm (Carleton and Kocher, 2001); *Cottus gobio*, 470 nm (Cowing *et al.*, 2002b); *Astyanax fasciatus*, 453 nm (Yokoyama and Yokoyama, 1993); *Cottus kessleri*, 450 nm (Cowing *et al.*, 2002b); *Carassius auratus*, 441 nm (Johnson *et al.*, 1993); *Batrachocottus nicolskii*, 428 nm (Cowing *et al.*, 2002b), and *Danio rerio*, 416 nm (Chinen *et al.*, 2003). Potential short-wave shifting substitutions have been previously identified in the SWS2 pigments of cottoid fish (Cowing *et al.*, 2002b). Two key sites were highlighted in this work, the Thr269Ala and the Thr118Ala/Gly substitutions. The former is present in the translated sequence of *T. loenbergii* SWS2 opsin. There is no change at position 118. Both of these sites remain conserved in the non-cottoid species and are therefore not involved in the tuning of the non-cottoid pigments (see figure 5.24). The amino acid residues found near to the chromophore binding pocket have been analysed to identify candidate substitutions for the shift down to 416 nm. There are three substitutions that involve a change in polarity. The Phe133Lys substitution is present in both zebrafish and *T. loenbergii*. This residue is not particularly close to the binding pocket or the Schiff’s base but it has been identified that the Phe133Lys substitution is present between RH2 and SWS1 opsins with lysine being present in the more blue-shifted pigment (Teller *et al.*, 2003). A good candidate
for the short-wave shift of the zebrafish pigment is an alanine to serine substitution at position 117. This residue faces the retinal binding pocket, and is in a position that may well interact with the chromophore (Palczewski et al., 2000). However, this substitution is not present in the ice fish sequence. A threonine to alanine change can be found at position 299 in the T. loenbergtt sequence. This residue interacts with the amino acid at position 83 (Palczewski et al., 2000), which is a site previously identified as having a potential tuning effect (see introduction, sections 1.4.4 and 1.4.6).
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**Figure 5.24.** Amino acid alignment of teleost SWS2 opsins, helices II to VII. The tuning sites identified by Cowing *et al.* (2002b) at 118 and 296 are highlighted in red. Candidate short-wave shifting tuning sites for *D. rerio* (Ala117Ser) and *T. loenbergii* (The299Ala) are shown in blue.
5.3.9 In Situ Hybridisation on D. mawsoni Retinal Whole-mounts

In situ hybridisation experiments were performed to determine which of the identified cone opsins are expressed in the retina of D. mawsoni, and to try to localise this expression to morphologically distinct cone receptors. Single stranded RNA probes were synthesised from double stranded DNA in both sense and anti-sense directions. The mRNA coding for protein in the cell is in the sense direction, and therefore will bind to a homologous anti-sense probe. The sense probes were used as an experimental control. Specific SWS1 and RH2 D. mawsoni RNA probes were synthesised from cDNA and cloned into the pBluescript vector (refer to methods section 2.2.7). The SWS2 probe was synthesised from Sea Bream SWS2 cDNA in pGem-T-Easy. The DIG-labelled RNA probes were generated as described previously. The probes were detected on an RNA blot to check that the labelling reaction had been successful (figure 5.25) using an anti-DIG-AP (alkaline phosphatase) conjugated antibody, which in the presence of 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) produces an insoluble blue precipitate. After 45 mins colour development all probes were detectable, even at the lowest concentration of 3 pg/µl. A DNA/RNA blot was then performed to determine the degree of probe cross-hybridisation. Approximately 10 ng of the original DNA plasmids, from which the probes were synthesised, were loaded onto a membrane and fixed by baking at 120°C for 30 mins. As well as the plasmids containing SWS1, SWS2 and RH2 opsins, a pGem-T Easy clone of Aristostomias tittmanni rod opsin was also fixed onto the membrane. Three separate membranes were then probed with SWS1, SWS2 or RH2 anti-sense DIG-labelled RNA and detected using the anti-DIG-AP conjugated antibody (figure 5.26). There was very little cross-hybridisation with any of the three probes, when hybridised...
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at 60°C. Fixed eyecups from *D. mawsoni* were donated by Dr. Cheng, and the retinae were dissected away from the RPE before mounting onto slides, photoreceptor layer face up. These sections were dried flat and then probed with the sense or anti-sense DIG-labelled SWS1, SWS2 or RH2 RNA probes at 60°C and detected with anti-DIG-AP. The results are shown in figure 5.27. A positive result was achieved with the RH2 anti-sense probe (photos A-D). The labelling is localised to the inner segment of double cone receptors, and is present in both partners of a pair, in just one side or in neither side. The absence of labelling in some inner segments is unlikely to be caused by incomplete probe penetration because the unlabelled cones are randomly located across the retinal section. To obtain a rough guide to the proportion of equally labelled pairs, unequally labelled pairs (single cone labelled) and unlabelled pairs, 100 differently-labelled cones were randomly selected from the probed region of retina (Table 5.4). Almost half of the double cone receptors showed labelling in one of the pair only. This is an interesting result as it is contradictory to the MSP result, which found all double cones contained the same opsin protein in both partners, as there were no real differences in $\lambda_{\text{max}}$ between any two halves studied.
**Figure 5.25.** RNA blot to check DIG-labelling and probe synthesis. The concentrations are rough guidelines given by the manufacturers, for the four different dilutions made.

**Figure 5.26.** DNA blots probed with DIG-labelled anti-sense RNA. Each blot contains 10 ng of SWS2, RH2, SWS1 or rod opsin fragment digested from a pGem-T Easy plasmid. These were then probed with SWS2, RH2, SWS1 and rod DIG-labelled RNA at 60°C. Hybridisation was detected with the DIG-AP conjugated antibody with the colour reaction proceeding for 45 minutes.
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Figure 5.27. *In situ* hybridisation on *D. mawsoni* flat mounted retina. (A-D) Positive labelling with the RH2 anti-sense probe (blue arrows). (E) Negative control with the RH2 sense probe. (F, G) Negative results for the SWS2 and SWS1 anti-sense probes respectively. Scale bars = 100 μm.
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<td>Single Side Positive</td>
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Table 5.4. Table showing the approximate percentage of three types of double cone labelling with the RH2 anti-sense probe. The sample size was 100 double cone receptors.

The SWS1 and SWS2 antisense probes did not label any of the cone photoreceptors. The most likely explanations are either that the probe did not penetrate to the layer of the single cone inner segments, or that the much lower levels of mRNA in single cones is difficult to detect.

5.3.10 In Situ Hybridisation on D. mawsoni Retinal Sections

In order to obtain labelling in the single cone photoreceptors, the tissue was cut into sections on a cryostat, either transverse to the retinal layers or horizontally through the photoreceptor layer. These sections were probed with the same SWS1, SWS2 and RH2 anti-sense and sense RNA probes that were used for the whole-mount sections.

Transverse Sections

Transverse cryostat sections were taken from central and peripheral retina, mounted on slides and hybridised against SWS1, SWS2 and RH2 anti-sense DIG-labelled RNA
probes. The results are shown in figures 5.28 and 5.29. Positive labelling occurred with the SWS2 and RH2 probes, and is clearly visible as a purple band localised to the outer nuclear layer. RH2 labelling was present in both peripheral and central retina, whereas the SWS2 probe only labelled receptors found in the peripheral retina. The SWS1 probe did not produce any positive labelling on either peripheral or central sections. Negative controls were performed using sense SWS1, SWS2 and RH2 opsin probes and all were devoid of non-specific labelling.

*En Face Sections*

Cryostat sections were cut enface to the photoreceptor layer again from central and peripheral retina. These were probed with sense and anti-sense SWS1, SWS2 and RH2 DIG-labelled RNA. A positive result was achieved with the RH2 anti-sense for both the central and peripheral retina. The outer segments of double cones can be clearly seen in rows across the sections. As the sections were cut at an angle, there are also photoreceptor inner segments on view and it is these that have stained dark purple (figure 5.30). Neither the SWS1 nor SWS2 probes hybridised to any sections (figure 5.31). This may be due to difficulties with probe penetration, or because of low mRNA transcript levels.
Figure 5.28. *In situ* hybridisation on transverse cryosections of retina taken from *D. mawsoni*. (A) Positive labelling with the RH2 antisense probe, restricted mainly to the outer nuclear layer in the central retina. (B) Positive RH2 labelling of the outer and inner segments of cone photoreceptors in the peripheral retina. D, labelling of both partners of a double cone. S, labelling of a single partner of a double cone. (C) Negative control probed with an RH2 sense probe. INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer; OS, outer segment; RPE, retinal pigment epithelium. Scale bars = 100 μm.
Figure 5.29. *In situ* hybridisation on transverse cryosections from *D. mawsoni* retina. (A) Positive labelling of the peripheral retina with the SWS2 anti-sense probe mainly localised to the outer nuclear layer. (B) No SWS2 labelling of the central retina. (C) Negative control with SWS2 sense probe. SWS1 probed peripheral (D) and central (E) sections show no positive labelling. (F) Negative control with sense SWS1 probe. INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments; RPE, retinal pigment epithelium. Scale bars = 100 μm.
Figure 5.30. *In situ* hybridisation of enface cryosections from *D. mawsoni* retina probing with RH2 opsin anti-sense RNA. There is positive labelling of double cones with the RH2 anti-sense probe in both peripheral (A) and central (B) retina, indicated with a black arrow. (C) Negative control probed with RH2 sense RNA. Scale bar = 100 μm.
Figure 5.31. *In situ* hybridisation of enface cryosections taken from *D. mawsoni* central retina probing with SWS2 and SWS1 anti-sense RNA. There was no positive labelling with SWS2 (A) or SWS1 (C) anti-sense probes. (B, D) Negative controls probed with sense SWS2 (B) and SWS1 (D) RNA respectively. Scale bar = 100 μm.
Teleosts have highly organised retinae containing distinct photoreceptor classes. It is common to find double cones, as well as large and small single cone cells. The different morphology of the cone cells can be an indication to which visual pigment is being expressed. The double cones generally express RH2 and LWS/MWS genes, with the single cones more usually expressing the SWS1 or SWS2 genes. RH2 expression of *D. mawsoni* has been localised to double cones and so fits the typical teleost pattern.

5.3.11 Histology of *D. mawsoni* and *T. hansoni* Retinae

As attempts to localise SWS1 and SWS2 to a particular cell type was not achieved with *in situ* hybridisation, it was interesting to determine the arrangement of single cones in the ice fish retina. To investigate the organisation of cone cells, basic histology was performed on flat whole-mounted and transverse sections from two species of ice fish, the midpelagic *Dissosticus mawsoni* found at depths as low as 1,600 m and the demersal *Trematomus hansoni*, which lives in shallower waters and found between 6-549 m from the surface (www.fishbase.org). *D. mawsoni* eyes are large, with a diameter of approximately 5 cm. This made manipulation of the whole retina impossible. Sections of retina were dissected from the eyecup, with the RPE intact and flat mounted onto slides. The retina of *T. hansoni* is considerably smaller (1.5 cm diameter) and therefore the whole retina with RPE attached could be dissected and flat mounted. The retinae were dehydrated and then embedded in technovik historesin. Horizontal sections enface to the plane of the photoreceptor layer were taken, varying in thickness between 1 and 2.5 μm. Slides were produced as described in methods section 2.2.8. The slides were then viewed under a light microscope and photographs taken via an attached digital camera. The results for *D. mawsoni* are shown in figure 5.32 and those for *T. hansoni*...
hansoni are in figure 5.33. The retina of D. mawsoni is organised in a row mosaic array. There are rows of large double cone photoreceptors, with each pair orientated in the same direction. This is typical of a row mosaic rather than a square pattern, in which adjacent double cones lie at right angles to each other. In all sections the double cones were orientated in the same plane across a single row. Though this arrangement was consistent it may be that a square mosaic is present in a region not examined here, missed because of difficulties in examining an eye of such size. Lying in between the doubles are rows of very small single cone photoreceptors. These appear to be of roughly the same size throughout the sections. There are some gaps in the mosaic caused by missing single cones. This is not uncommon in teleost retinæ though in general the D. mawsoni mosaic is highly maintained across all the sections analysed.

The results for T. hansoni show a different cone arrangement, the retina is organised as a square mosaic. The adjacent double cones lie at right angles to each other. Sections taken encompass the whole retina, with no regions showing a shift towards a row array. The single cones again all appear to be of comparable size, though there does seem to be a higher number of gaps in the array due to missing single cones than found in the retina of D. mawsoni. This arises from the absence of central single cones in the square mosaic of T. hansoni, which leads to an alternating pattern of single cones being present or absent across the retina. The outer segments of the single cones from T. hansoni (0.82 μm) have a diameter near to twice that of single cones from D. mawsoni (0.44 μm). However, the double cones from D. mawsoni are larger in diameter (3.08 μm) than those from T. loenbergii (2.46 μm). The histology shows evidence for different retinal arrangements between different species, even though the same visual pigments are expressed.
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A

B
Figure 5.32 Flat whole-mounted retinal sections from *D. mawsoni*. A to D show the rows of double (D) and single (S) cones clearly. The two partners of the double cones are orientated in roughly the same direction in each row, as can be seen by the angle of the central dividing line. Photographs E and F show the highly organised mosaic across a wide retinal region. There are few empty spaces in the rows of single cones. Scale bars = 100 μm.
Figure 5.33. Flat whole-mounted retinal sections from *T. hansoni*. A, B and C show the square mosaic array at different magnifications. The central dividing line between two halves of a double cone lies at roughly 90° to the dividing lines of the adjacent double cones. Single cones are present in the square corners but are absent from the centre. D is a section through the tip of the double cone outer segments, displaying the close packing of receptors in the retina of *T. hansoni*. Scale bars = 100 μm.
To gain an insight into the different layers of the ice fish retina, transverse sections were cut perpendicular to the plane of the retinal layers. The procedure was exactly the same as that for the enface sections, except the retina was embedded to lie side onto the plane of the microtome blade. Sections were cut 2 \( \mu \text{m} \) thick and mounted onto slides. The sections were incubated in osmium solution for 10 mins to highlight cell membranes before staining in 1% (w/v) toluidine blue. Slides were coverslipped with DepX and then viewed under a light microscope. Results are shown in figure 5.34.
Figure 5.34. Transverse retinal sections from the ice fish *Trematomus hansoni*. A and B show the retinal layers, C shows the optic nerve head (ON). COS, cone outer segments; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; ROS, rod outer segments; RPE, retinal pigment epithelium. Scale bars = 100 μm.
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5.4 Discussion

The Southern hybridization experiments show quite clearly the existence of SWS1, SWS2 and RH2 genes in *Dissostichus mawsoni* genomic DNA. This is the first evidence to show three distinct cone classes present in an ice fish species, and to highlight the loss of the MWS/LWS gene. The absence of LWS cones is also demonstrated by MSP and Northern hybridization experiments. This loss must have occurred a significant time ago as recently acquired pseudogenes can elicit a positive Southern hybridisation result. Absence of long-wave sensitivity may have proved advantageous in species that live in an environment much reduced in red light. Water, snow and thick sheets of ice limit penetration of long wavelengths. The red sensitive cones would be inactive and occupying retinal space that could be used more efficiently. Expression of an opsin gene, which can function in ice and water filtered light would be much more effective. Loss of long-wave sensitivity is not unusual in fish, as found in the coelacanth (Yokoyama et al., 1999; Yokoyama and Tada, 2000), many deep-sea species (Munk, 1966; Bowmaker et al., 1994; Douglas et al., 2003) and some midwater and shallow dwelling species (Levine and MacNichol, 1979). This is probably achieved by accumulating mutations in the MWS/LWS gene, which in turn leads to a loss of expression. The same process has been identified in aquatic mammals that have lost SWS1 expression (Levenson and Dizon, 2003). Good chromatic sensitivity in the red end of the spectrum is useful for fish that live within the top few metres of the water column. Any deeper and the need for chromatic sensitivity begins to be replaced with that for overall light sensitivity.
RH2 expression was first identified by MSP and Northern hybridization and then PCR, with the whole gene amplified and sequenced from four notothenioid species; *D. mawsoni, G. acuticeps, N. angustata* and *P. borchgrevinki*. A $\lambda_{\text{max}}$ of approximately 490 nm for the *D. mawsoni* RH2 pigment was determined first by MSP and then confirmed by *in vitro* expression and regeneration with 11- cis retinal. This value is not unusual for a teleost RH2 pigment.

PCR's on *T. loenbergii* genomic DNA amplified the majority of an SWS2 gene, missing only the first 170 bp of the first exon. Expression in retinal RNA has been confirmed in two species, *P. borchgrevinki* and *G. acuticeps*, by PCR and in a number of others by Northern hybridization and MSP. Isolating sequence by PCR has been very problematic and may be explained by the low level of expression highlighted by the Northern blots. The $\lambda_{\text{max}}$ value for this pigment obtained by MSP is at 414- 426 nm, though this has not been confirmed by *in vitro* regeneration. However, it remains highly likely that the SWS2 pigment isolated is the pigment identified by MSP.

SWS1 opsin was amplified from retinal mRNA from all species of ice fish studied. The potential for SWS1 vision is therefore certainly present in many ice fish, even though MSP provided no evidence. The lack of ultraviolet/violet sensitive cones could be due to a sampling problem. If ultraviolet cones are very few in number, or localized to a small region of the retina they could easily be missed. A spectrum could not be measured from the majority of single cones of *P. borchgrevinki*. Only green sensitive singles could be found, the rest appearing empty. It may well be that these were UV sensitive but fixation had damaged the pigment. The Northern blot probed with SWS1 opsin showed a positive result for *P. borchgrevinki* but not for the remaining five
species. Why then may SWS1 expression be so much greater in this species? P. borchgrevinki is cryopelagic, and adequate levels of UV light may reach just below the ice to be visually useful. Looking at all the evidence, the most likely conclusion is that the SWS1 gene is expressed, but at a much lower level to the RH2 and SWS2 genes, and that the level of expression may vary between species. The number of SWS1 cones in the retina is therefore probably very low in most ice fish species, but even so ultraviolet/violet sensitivity is possible. The SWS1 opsin from D. mawsoni has been expressed in COS cells and regenerated with 11-cis retinal. The $\lambda_{\text{max}}$ resulting from the difference spectra is at 369 nm. This confirms the pigment as ultraviolet rather than violet sensitive. Ultraviolet sensitivity must therefore have a functional role, so why do ice fish require it? Many fish possess UV sensitivity while immature, which is then lost upon maturity, one example being the brown trout (Bowmaker and Kunz, 1987). UV sensitivity at early life stages is thought to aid foraging for plankton (Loew et al., 1993). It may be that ice fish also show this pattern, with the isolation by PCR due to a leaky shut-off mechanism in the adult. Though the fact that the SWS1 sequence was easily isolated from RNA from a number of species, may suggest that its expression is valid.

The $\lambda_{\text{max}}$ measurement of $\approx 414$ nm from single cones must be from SWS2 expressing cells as the RH2 and SWS1 pigments do not regenerate near this value. This value is rather short-wave shifted, as the majority of SWS2 pigments have $\lambda_{\text{max}}$ close to 440 nm, except zebrafish, Danio rerio, which has a similar $\lambda_{\text{max}}$ at 410-416 nm (Nawrocki et al., 1985; Chinen et al., 2003). There are not many potential tuning substitutions in the T. loenbergi sequence. The short-wave shifting substitution Thr269Ala was identified in the cottoid fish of Lake Baikal (Cowing et al., 2002b) and is present in the ice fish sequence, but is also present in the teleost SWS2 pigments with $\lambda_{\text{max}}$ close to 440 nm. A
potential candidate for short-wave tuning the \textit{T. loenbergii} pigment is a Thr299Ala change. This substitution has previously been identified in the short-wave shifted rod opsins of Sowerby's beaked whale, \textit{Mesoplodon bidens}, and harp seal, \textit{Phoca groenlandicus} (Fasick and Robinson, 2000). Site directed mutagenesis of the bovine and harp seal opsins showed a 4 nm blue shift when there is a Ser299Ala change. The crystal structure of bovine rod opsin has identified the amino acid at position 299 as interacting with the residues at positions 55 and 83, shown in figure 5.34 (Palczewski et al., 2000). Substitutions at site 83 are well characterized as affecting both the spectral (Hope et al., 1997; Hunt et al., 2002) and kinetic (Nakayama and Khorana, 1991; Weitz and Nathans, 1993) response of rhodopsin. A change from a hydrophilic to a hydrophobic residue at site 299, must affect the hydrogen bonding between sites 83 and 299. A hypothesis is that this change in polarity may lead to a tuning effect via an alteration of charge in the environment surrounding site 83.
MSP identified all double cones as green sensitive, and single cones as either blue or green sensitive. However, the in situ hybridization experiments show distinctly that not all double cones contain RH2 RNA. In fact around 50% of the double cones studied displayed expression in only one half of the pair. Why is there a discrepancy between these two sets of results? One possible explanation is that probe penetration was incomplete, and therefore RNA was present but not labelled in some cones. This seems unlikely because both partners of a pair will have been treated in exactly the same manner throughout the experiment. A more likely explanation is that the MSP sample size was too small, missing variation across the retina. The eyes from D. mawsoni are very large, the median diameter being around 6 cm. Sampling the whole retina from an eye this big is a mammoth task. Recordings were taken from five species with 8-17 double cones studied from each. If all recordings were taken from the same region of the retina, such as all from the centre, then differences that occur elsewhere would be
missed. The in situ experiment has identified a population of double cones that are not expressing the RH2 gene, so which gene are they expressing? Further in situ experiments did not produce much positive labelling. No single cones were labelled from en face sections, identifying a problem with the lability of RNA from single cones, or low levels of expression for both SWS2 and SWS1 opsin. However, there was a significant signal when transverse sections of peripheral retina were probed with SWS2 DIG-labeled RNA. Here there must be enough stable RNA present to create a signal. As there was no evidence for single cone labelling, this may be due to expression in double cones. Interestingly the whole-mount in situ, which show clear double cone labelling was on very peripheral retina. The empty doubles were found here, which agrees with the SWS2 in situ result, where positive labelling was found in the peripheral but not central retinal sections. It may be that the majority of double cones in the retina of D. mawsoni express RH2 in both sides. However, in the most peripheral doubles there is also expression of SWS2, in either both partners or in just one half. This hypothesis requires further in situ experiments to provide more substantial evidence.

The organizations of the cone receptor mosaics are different between D. mawsoni and T. hansoni retinas. The deeper dwelling D. mawsoni has double and single cones positioned in a row mosaic. All double cones in a single row lie in the same orientation as can be identified by the midline between the two outer segments of a pair. The single cones are very small and form single rows in between the doubles. This arrangement is commonly found in fish that have reduced visual requirements, i.e. deep dwelling or have a non-predatory lifestyle. This is corroborated by a study that demonstrates the retinomotor response as absent from D. mawsoni, explained as being a consequence of living at depth and not encountering fluctuations in light intensity (Meyer-Rochow and
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Klyne, 1982). The second species examined, *T. hansoni*, is demersal but lives in shallower water (down to 549 m) than *D. mawsoni* (down to 1,600 m). The retina of this species is organized into a typical square mosaic, as seen in goldfish and zebrafish. The double cones sit at the four corners of a square, each orientated 90° to its closest neighbours. Single cones are present in the centre but absent from the four corners. In *T. hansoni*, there appears to be only a single morphologically distinct class of single cone, with corner cones absent. The cones that are absent could be the UV sensitive cones. This may explain why MSP did not identify them as they have been lost from the mosaic. This study shows two distinct retinal arrangements in two phylogenetically close species of fish. The visual pigments of these fish have not been spectrally tuned in species inhabiting deeper water. The $\lambda_{\text{max}}$ values for rod, RH2, and SWS2 pigments match almost exactly across all ice fish species studied, whether deep or shallow dwelling. This agrees with Eastman and Lannoo (2003) and Miyazaki *et al.*, (2001), who demonstrated double and single cones in the ice fish irrespective of depth. So why is spectral tuning not apparent in these fish? How is optimal sensitivity achieved further down the water column? The light that reaches through the pack ice has already been substantially filtered. The spectrum is already narrowed because penetration of long and short wavelengths is restricted. Therefore, cryopelagic species must already express pigments tuned to this narrow bandwidth. Species living deeper will not encounter light much altered from that found above because the longer and UV wavelengths, which cannot penetrate far in water, are already absent. An hypothesis is that the spectrum of light available for the shallow and deeper species does not differ considerably, therefore evolutionary pressure to acquire tuning substitutions has been small. However, the overall level of light that reaches greater depths will be less, the number of photons of all wavelengths being limited. The deeper dwelling species therefore need to increase
overall sensitivity. This is demonstrated in *D. mawsoni* by Meyer-Rochow and Klyne (1982) as the proportion of rod photoreceptors is greater, and the outer and inner segments longer than in shallower species. This increases the likelihood of photon capture and is a common mechanism in deep-sea fish. It may well be that the deeper living ice fish rely much more on their scotopic visual system than shallow species. The differences identified in retinal arrangement agree with this idea. The shallow species organize the cone receptors in a square mosaic to ensure optimal packing of cones in the retina. These fish rely on their photopic visual system, as within the top few metres there is enough light available for a cone response. The deeper living species rely more on their rod receptors, and the proportion of cones is lower with rod cells at higher density. Behavioral studies on both shallow and deep living species may help to identify how the different retinal arrangements affect chromatic and overall light sensitivity.

5.5. Conclusions

This study has gone a long way to understanding the vision of a family of fish on which there was very little previous data. RH2, SWS2 and SWS1 genes appear to be expressed in a number of species. UV/violet sensitivity may be present as the SWS1 pigment regenerates *in vitro* with a $\lambda_{\text{max}}$ of 369 nm, but this has not been established by MSP. The retina is composed of double and single cones arranged in either a row or square mosaic depending on species, which may be linked to depth inhabited. The double cones are generally green sensitive ($\lambda_{\text{max}} \approx 489$-491 nm), expressing the RH2 gene. There may also be a population of double cones in the peripheral retina of *D. mawsoni* that express the SWS2 gene. However, MSP shows the SWS2 pigment is usually found in single cones. Closely related species that live at different depths, have not spectrally
tuned their opsin pigments but have increased rod receptor density to aid sensitivity to low light.

5.6. Future Work

Further PCR work is necessary to complete the SWS2 sequence. If more retinal tissue can be obtained, then further in situ hybridizations on whole flat mounted retina may help to determine whether SWS2 is indeed expressed in a population of double cones as hypothesized here. A thorough in situ hybridization based project that covered the whole retina may provide evidence for SWS1 expressing cones and establish the distribution of the three cone pigments. This may be more likely if a shallow species is studied, as the single cones of *D. mawsoni* were exceptionally small. The single cones of *T. hansoni* were twice the size and may be more useful for in situ detection.
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