VISUAL PIGMENTS, OIL DROPLETS AND OPSIN SEQUENCES FROM THE CANARY, *SERINUS CANARIA*

By

Debipriya Das, B.Sc.

Department of Visual Science
Institute of Ophthalmology
University College London
University of London

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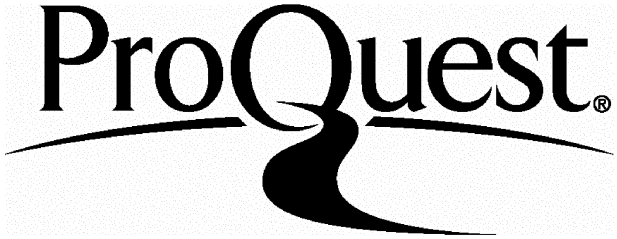

Abstract

The visual receptors in the retina of the passeriform bird *Serinus canaria* (the canary) have been examined microspectrophotometrically. The rods have a maximum absorbance close to 500 nm. Four spectrally different classes of single cone are also present: a long-wave sensitive cone with a photopigment P567, a middle-wave sensitive cone with a P501, a short-wave sensitive cone with a P442, and an ultraviolet sensitive cone with a P366. Also present are double cones possessing the P567 in both principal and accessory members. Typical combinations of photopigment and oil droplet occur in most cone classes, for instance, the MWS cones are paired with a Y-type droplet with a cut-off at 506 nm, SWS cones are paired with a C-type droplet with maximum absorbance below 410 nm and the UV cones contain a fully transparent oil droplet. In the double cones a pale droplet with variable absorbance (peaking at 410-415 nm) is associated with the principal member whereas the ellipsoid region of the accessory member contains only low concentrations of carotenoid. An ambiguity exists in the nature of the oil droplet found in the single LWS cone class; in some birds, LWS cones are paired with a typical R-type droplet with a cut-off at 578 nm, however the majority of canaries do not appear to possess R-type droplets. In most birds the LWS pigment is invariably paired with the P-type droplet which has previously been associated exclusively with the principal member of the double cone.

The visual pigment genes coding for all five canary opsins have also been isolated and sequenced and complement the spectral classes identified from MSP. From these DNA sequences, it has been possible to deduce the unique amino acid sequence of each of the opsin proteins and to identify putative spectral tuning sites.

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Declaration

I declare that this thesis submitted for the degree of Doctor of Philosophy is my own composition and the data presented herein is my own original work, unless otherwise stated.

Debipriya Das, B.Sc.

Dedication

I dedicate this thesis to my parents
in recognition of their constant
support and encouragement.

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Abbreviations

ATP	adenosine triphosphate
bp	base pair
°C	degrees Celsius
C-type	clear/colourless oil droplet
cDNA	complementary deoxyribonucleic acid
dATP	deoxy-adenosine-5'-triphosphate
dCTP	deoxy-cytosine-5'-triphosphate
ddATP	dideoxy-adenosine-5'-triphosphate
ddCTP	dideoxy-cytosine-5'-triphosphate
ddGTP	dideoxy-guanosine-5'-triphosphate
ddTTP	deoxy-thymidine-5'-triphosphate
dGTP	deoxy-guanosine-5'-triphosphate
di H_2O	deionised water
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxy-thymidine-5'-triphosphate
EDTA	ethylene-diamine-tetra acetic acid
g	gram(s)
IPTG	isopropyl-B-D-thiogalactoside
Kb	kilobase(s)
L	Longwave absorbing opsins (520-575 nm)
LWS	longwave 'red' sensitive (520-575 nm)
µg	microgram
µl	microlitre
M	molar
M_C	Middlewave-absorbing cone opsin (460-530 nm)
ml	millilitre
M_{Rd}	Middlewave-absorbing rod opsin (460-530 nm)
MSP	microspectrophotometry
MWS	middlewave 'green' sensitive (460-530 nm)
ng	nanogram
nm	nanometre
OD	optical density
P-type	pale/principal oil droplet

PCR	polymerase chain reaction
PEG	polyetheleneglycol
R-type	red oil droplet
RNA	ribonucleic acid
rpm	revolutions per minute
S_B	Shortwave 'blue'-absorbing opsin (430-470 nm)
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
S_V	Shortwave 'violet'-absorbing opsin (350-440 nm)
SWS	shortwave 'blue' sensitive (430-470 nm)
T-type	transparent oil droplet
TAE	Tris / acetate / EDTA buffer
TBE	Tris / borate / EDTA buffer
TEMED	N, N',N',N'-tetramethlethylenediamine
UV	ultraviolet
UVS	ultraviolet sensitive (350-400 nm)
VS	violet sensitive (350-420 nm)
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y-type	yellow oil droplet

1. GENERAL INTRODUCTION

1. THE CANARY, *Serinus canaria* (Order Passeriforme, family Fringillidae).

1.0. The wild canary

The canary is thought to have evolved from the Serin Finch, *Serinus serinus,* a small singing finch indigenous to islands off the northwestern coast of Africa, including Madeira, the Azores and the Canary Islands, for which it is named. Following the Spanish conquest of the Canary Islands in the 15th century, the Serin Finch was exported all over Europe. Many breeding populations were subsequently established, giving rise to the canary, *Serinus canaria* , from which all domestic varieties have been developed. The wild canary is a small bird (about 12.5 cm long) which inhabits open woods and scrubland. It is a gregarious bird, active for most of the day feeding on seeds and vegetation. Its plumage is dappled olive-green on the upper parts and yellow-green on the underside. Females are duller than males and often have a necklace of dark spots on the crop. The wild canary builds a cup-shaped nest in a tree or bush and cocks are distinguished from hens by their ability to sing.

1.1. The domestic canary

The original wild canary *Serinus canaria* is the ancestor of all the existing breeds of domestic canary. The changes which have occurred to its phenotype are due to the following;

- Domestication (selective breeding of birds showing unusual characteristics leading to the establishment of phenotypes different from that of the normal bird)
- Retention of mutations as they appeared
- Hybridization (cross breeding with a compatible related finch)

These three factors have led to the emergence of over 50 varieties of canary, some being developed for body type, colouration and/or song. In most essential characteristics, the original domestic canary resembles the wild canary. For example, it has a yellow ground (background) colour although the phenotype is often described as olive or green [this is merely a visual effect caused by black and brown (melanin) striations superimposed on a yellow ground colour giving the appearance of olive or green]. Cinnamon, white and fawn are a few of the plumage colours which have appeared as a result of mutations.

1.2 Ground colour and melanin pigmentation

The ground colour depends upon the genetic makeup of the bird and can be influenced by fat-based colouring (lipochrome) taken in the diet, whereas the black or brown melanin pigmentation is derived from protein constituents in the diet. The exact pattern of melanin deposits is controlled by a set of genes known as the variegation factors. There are three forms of the melanin pigment; phaeomelanin brown, eumelanin black and eumelanin brown. The maximum depth of a particular colour is pre-determined by the inheritance of secondary variegation factors. Walker and Avon (1993) observe that any canary deprived of dietary carotenoids will go white regardless of its ground colour. It is of interest to note that a study by Meyer (1971) found that the pigmentation of the feathers of quail maintained on carotenoid-free rations were not significantly altered and appeared similar to that of control quail.

In the wild, canaries are likely to feed on a wide variety of foodstuffs, depending on their habitat, which is mainly in the tree savanna, farm clearings with pollarded trees, gardens and villages. However the strong, hard, conical bills of canaries suggest that they are predominantly seed eaters and are often seen perched on the ground picking up seeds, especially on millet farmland in the vicinity of villages (Serle *et al.,* 1972). The bulk of the domestic canary diet consists of a mixture of canary seed, black rape, thistle and hemp seeds, each of which contain relatively large percentages of the yellow carotenoid, lutein, responsible for the yellow ground colour of the canary. Canary seed, rape, pinhead oatmeal and groats, are all high in protein and carbohydrate whereas other seeds such as hemp, maw, and niger have a higher fat content are given less frequently (e.g. in the winter).

1.3 Avian Eyes

Avian eyes are large and occupy most of the skull (Meyer, 1977). In some cases the two fundi actually touch or are separated by just a thin septum of bone (Martin, 1985). The two eyes often outweigh the brain giving an idea of the relative importance of vision to birds. A large eye improves visual acuity by allowing the formation of a large retinal image. The posterior nodal distance of an eye determines the size of the retinal area over which an image of an object is projected. The longer the posterior nodal distance, the larger the retinal image and the number of photoreceptors it is spread onto, thus increasing the amount of detail resolved (Miller, 1979). Amongst birds, nocturnal birds of prey have the largest eyes and sea-birds the smallest (Meyer, 1977). The avian eye is non-spherical. As in all vertebrate eyes the outermost covering of the avian eye, the sclera, is opaque except at its most anterior surface where it forms the transparent cornea. The sclera consists of a thick fibrous layer surrounding an inner cartilaginous cup. The cup of cartilage is attached to the underside of the ring of scleral ossicles and together these structures add strength and skeletal support to the eye respectively. Interior to the sclera is the uveal layer which has three regional divisions; the vascular choroid, the ciliary body and the iris. The retina lines the choroid and is the photoreceptive layer of the eye. It is divided into two main layers, the neural retina and the pigment epithelium.

1.4 Eye shape

There are three main shapes of avian eye; flat, globose and tubular (Walls, 1942). The main structural difference which determines the shape of the three eye types is the ring of scleral ossicles found in the corneo-scleral region. This ring of 10-18 overlapping boney plates acts to strengthen the junction between the anterior and posterior portions of the eye and maintains its non-spherical shape. Individual ossicles vary in shape according to the shape of the eye, being flat or slightly convex in flat eyes, and increasingly concave in globose and tubular eyes respectively (Duke-Elder, 1958). In small eyes the ossicles are made of compact bone, in large eyes, particularly the tubular type, the ossicles are hollow to reduce the weight on the eyes. As well as providing support, the scleral ossicles may aid the refractive apparatus during accommodation by preventing changes in the shape of the globe (Sillman, 1973). Most avian species posses the flat type of eye characterized by a short anteriorposterior (axial) diameter. Flat eyes are found in diurnal birds with narrow heads such as Galliformes and Columbiformes (Duke-Elder, 1958). In this type of eye the scleral ossicles form a flat ring. In Globose eyes the ring of scleral ossicles slopes backwards like the base of a cone giving the eyes a more rounded shape. As a result the axial and equatorial diameters are almost equal. Diurnal birds which need high resolution over large

distances often posses globose eyes. This is because the increased axial diameter increases the size of the image being thrown onto the retina giving a greater visual acuity. These birds have broader heads, examples of which are some Passeriformes, e.g. sparrows, the Corvidae and diurnal predators such as the Falconiformes. In Tubular eyes, the concave shape of the ossicles forms a definite 'waist' at the corneo-scleral region of the eye. This results in a longer axial than equatorial diameter and is the basis for the tubular shape. Species with large eyes such as the Strigiformes and some Falconiformes, generally have this type of eye (Martin, 1985) the increased axial length serving to increase visual acuity by increasing the visual field.

1.5 Morphology of the retina

The avian retina is avascular and generally thicker than that of other vertebrates (Duke-Elder, 1958). As in other vertebrate classes it can be divided into two main layers, the **inner neural layers** and the **outer pigmented layer**. The outer pigmented layer consists of the pigmented epithelium, (PE) which is a single layer of hexagonal cells. These cells perform a number of tasks, the most important being the active breakdown of the membrane discs shed from outer segments and the regeneration of the visual pigment. The PE also ferries oxygen and nutrients to the retina from the choroidal circulation. These cells have long ciliary processes that extend inwards towards the photoreceptor layer ensheathing each outer segment. Light absorbing melanin pigment granules found at the base of the pigmented epithelium cells, migrate along the ciliary processes (towards the photoreceptors) in response to light and retreat down towards the base of the cell in the dark (Duke-Elder, 1958). Any stray light that has not been absorbed by the photoreceptors is absorbed by the melanin granules. Under photopic conditions this enhances the spatial resolution of the retina by increasing the directional sensitivity of the cone photoreceptors. Movement of the melanin granules also aids the change from scotopic to photopic vision (Miller, 1979).

The inner nervous layers can be split into several additional layers: The **neuroepithelial layer** or the 'photoreceptor layer' (see below) contains the rod and cone photoreceptors, the cell somas of which are found in the **outer nuclear layer**. The **outer plexiform layer (OPL)**, is the region where the axon terminals of the rods and cones synapse onto the dendrites of bipolar cells and horizontal cells. The terminal processes of Müller cells form tight junctions around the myoid region of the photoreceptors forming the **external limiting membrane (ELM)**. The ELM is not a true membrane and the name relates to the fact that in retinal sections these junctions readily take up stain forming a thick line separating the outer nuclear layer from the outer plexiform layer. The **inner nuclear layer** contains the nuclei of bipolar,

amacrine, horizontal, and Müller cells. The dendrites of bipolar cells synapse with either rods or cones. Amacrine and horizontal cells send lateral processes to interconnect other retinal neurons and are therefore thought to be involved in the integration of information conducted by the bipolar cells from photoreceptors. Müller cells are retinal gliocytes, thought to add mechanical strength to the retina. The Müller cells lie in a compact row and have fibres which surround most neurons. The **inner plexiform layer** is the region where the bipolar axon terminals synapse onto ganglion cell dendrites. The **ganglion cell layer** as the name implies contains the nuclei of the ganglion cells. The axons of ganglion cells extend into nerve fibres which together form the **optic fibre layer** and leave the eye as the optic nerve see fig. 1.5a.

Since the avian retina contains two types of photoreceptor cell, rods and cones, it is said to be duplex in nature. The rods mediate vision under dim light or scotopic conditions whereas the cones mediate vision under photopic conditions of bright light and are also responsible for the ability to discriminate wavelengths (see later). Consequently, passerines and diurnal predator birds have cone-rich retinas with very few rods which are relegated to the periphery. Conversely, the retina of nocturnal birds such as the owl is rod dominated with very few cones (Bowmaker and Martin, 1978). The rod system is more sensitive than the cone system since many rods synapse onto a single rod bipolar cell, many of which synapse onto a single ganglion cell. In contrast the cone system compromises its sensitivity for greater resolving power by generally having a single cone connected to very few or a single bipolar which synapses onto a single ganglion cell. Consequently the cone dominated retinas of diurnal birds have a relatively thick inner nuclear layer due to the high concentration of bipolar cells. Moreover avian retina contains a particularly high concentration of amacrine and horizontal cells in the inner nuclear layer and the ordered layering of their terminal synapses adds to the overall retinal thickness. In contrast, the rod dominated retina of nocturnal birds has a much lower density of cells in the inner nuclear layer which, as a result, is even thinner than the outer nuclear layer (Sillman, 1973). The relatively large size of the eyes in birds plus the increased thickness of the inner nuclear layer suggests that much of the processing and integration of visual information is accomplished at the retinal level. This is in contrast to the situation in mammals where the complex processing goes on at higher centres in the brain.

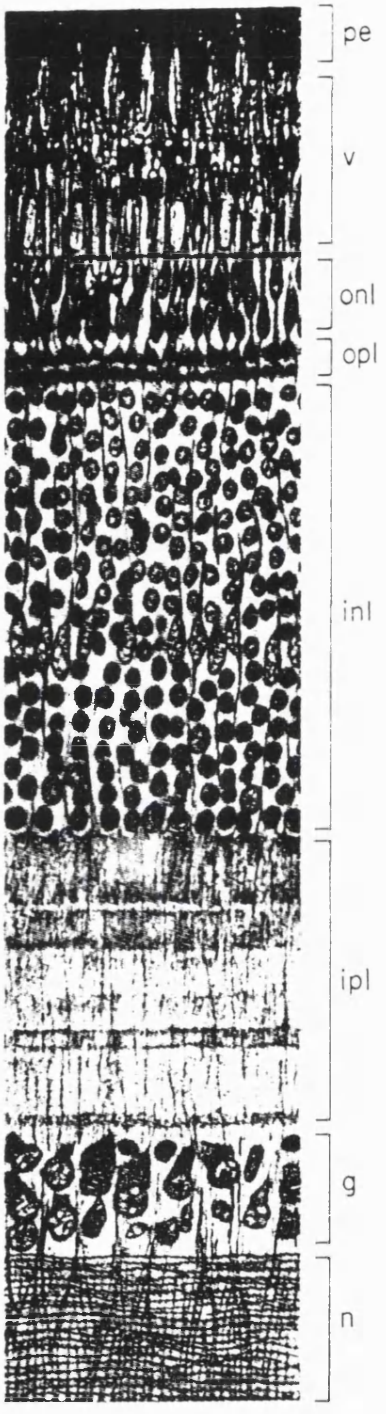


Fig 1.5a.

The retina of the chick; pe, pigment epithelium; v, visual cell layer; onl, outer nuclear layer; opl, outer plexiform layer; g, ganglion cell layer; and n, nerve fiber layer. (relabeled from Walls, 1942)

1.6 The Photoreceptor Layer

The photoreceptor layer of the avian retina contains three types of cell; rods, single cones and double cones (see fig 1.6a). These cells can be classified according to different features such as their morphology, oil droplet type and visual pigment.

Using the electron microscope Morris and Shorey (1967) identified several types of visual cell in the chick retina. These were; rods which lacked droplets and had a paraboloid, double cones comprising a chief member with an oil droplet plus an accessory member which had a large paraboloid and a small granular vesicle and two types of single cone both possessing an oil droplet but without a paraboloid. Morris (1970) later distinguished a third type of single cone on the basis of its electron density. Mariani and Leure-DuPree (1978) distinguished five morphological types of photoreceptor in the pigeon retina using the method of golgi impregnation. These were (1) Rods, (2) Straight cones, (3) Oblique cones (so called because of the lateral displacement of the inner segment) (4) Double cones; comprising a larger Principal member and (5) a short, fat accessory member.

1.7 Photoreceptor morphology

The photoreceptors in the avian retina are divided morphologically into an outer segment and an inner segment which are connected via a ciliary bridge. The cytoplasm of the inner segment is rich in cytoskeletal elements. Calycal processes are long finger-like outgrowths of the apical inner segment plasma membrane which lie parallel to each outer segment (Cohen, 1963). Their physiological function is unknown.

The inner segment

The inner segment has three regions; the ellipsoid, paraboloid and myoid, and houses the normal cellular organelles such as mitochondria and a nucleus. The part of the inner segment lying closest to the base of the outer segment is the ellipsoid, and mitochondria regulating the metabolism of the outer segment are numerous here. In the cone ellipsoids of many birds, some fish, amphibia, reptiles and non-placental mammals, there is also a large oil droplet. In birds and turtles, these oil droplets are often brightly coloured due to the presence of dissolved carotenoid pigments taken in the diet, (Meyer, 1971, Goldsmith *et al.,* 1984; Liebman and Granda, 1971). Since light reaching the pigment molecules in the outer segment must first pass through the oil droplet, they in effect act like colour filters and must alter the spectral quality of the light reaching the photoreceptors, and hence their spectral sensitivity (see later). Clusters of microdroplets (with a diameter of $\leq 0.5\mu m$) have also been found in the inner segments of some single cones in the pigeon retina (Pedler & Boyle, 1969). Hazlett *et al.* (1974) observed multiple oil droplets (greater than five) in the accessory cone inner segment of

the quail. The function of these numerous tiny droplets is unknown.

The myoid region contains organelles associated with protein synthesis such as free ribosomes, rough endoplasmic reticulum and golgi apparatus. The term myoid (meaning muscle-like) refers to the presence of contractile proteins in this region which are responsible for the photomechanical movements observed in some species. In the myoid of rods and the accessory member of the double cone there is a glycogen deposit known as the hyperboloid and paraboloid, respectively. These glycogen deposits are thought to act as an energy source for the photoreceptor cell metabolism. This may be particularly so in the avascular avian retina where the Müller cell glycogen is much reduced (Meyer, 1977). Amemiya (1975), has shown glycogen synthetase and phosphorylase activities within the chick accessory cone paraboloid in support of this theory. Electron microscope preparations show the paraboloid glycogen to be located between sacs of the smooth endoplasmic reticulum. In contrast, the glycogen in the rod hyperboloid lies free (Cohen, 1963). Both the hyperboloid and paraboloid are highly refractile bodies which may also serve to concentrate light onto the photosensitive outer segment (King-Smith, 1971). During both light adaptation and dark adaptation the rod hyperboloid and accessory cone paraboloid appear to show morphological changes, (Van Genderen Stort, 1887, as cited by Meyer, 1977). During light adaptation the rod inner segment lengthens while the accessory cone inner segment contracts causing the paraboloid to swell. King-Smith (1971) suggests that this may refract light onto the cone outer segment, the opposite phenomenon occurring during dark adaptation. The melanin granules in the interdigitating ciliary processes of the retinal pigment epithelium also migrate towards the outer segment during light adaptation.

The outer segment

One of a pair of centrioles in the inner segment gives rise to both the ciliary bridge and the outer segment itself. With such origins the outer segment is often described as a modified non-motile cilium. The outer segment of the photoreceptor is where phototransduction occurs and consists of a series of membranous folds.

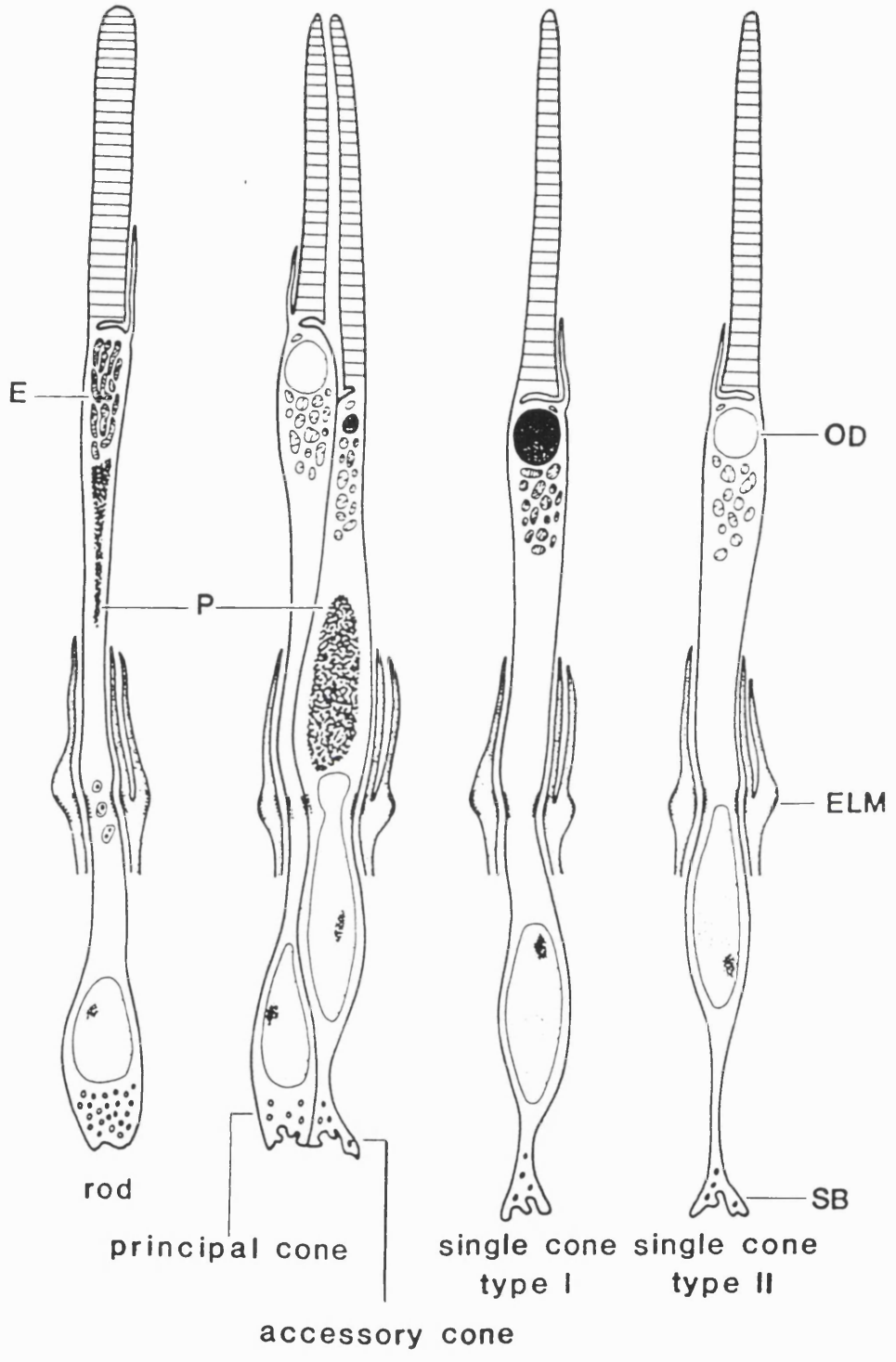


Fig 1.6a.

Schematic drawings of the five photoreceptor types identified in the retina of the chicken, *Gallus gallus,* in an electron microscope study by Morris and Shorey (1967). E, Ellipsoid; OD, oil droplet; ELM, external limiting membrane; P, paraboloid; SB, synaptic body (Adapted from Sillman, 1973).

The light sensitive outer segment plasma membrane is embedded with a very high number of visual pigment molecules. The overall sensitivity of a photoreceptor is dependent on the number of visual pigment molecules it contains and since these are integral membrane proteins a large membrane surface area is also required. Hence the pigmented membrane of the outer segment is highly folded into a series of flattened discs in order to occupy the smallest volume possible. These discs, formed at the base of the outer segment from evaginations of the plasma membrane are initially exposed to the extracellular space. In rods, the discs pinch off from the plasma membrane as they mature to form stacks of isolated discs with only the most basally located 20-30 discs remaining continuous with the plasma membrane. In cones the discs remain as continuous infoldings of the plasma membrane which are open to the extracellular space (Cohen, 1972). Young (1974), using the method of tissue autoradiography discovered that the rods and cones are in a state of continuous renewal throughout life. He showed that radioactive amino acids injected into rats and frogs were incorporated into precursors of visual pigment proteins in the inner segment myoid. From here, many of the proteins were subsequently located in a discrete band at the base of the outer segment, perpendicular to its long axis. Over a period of time, and in a temperature-dependent fashion (about eight weeks at 21°C; for comparison, this process is normally completed in about ten days in humans, and thirty days for cold blooded fish) the proteins were shown to have migrated from the base to the apex of the outer segment after which they were phagocytosed by the retinal pigment epithelium. Since the outer segment length remained unchanged, it was inferred that there must be a balance between disc shedding and disc synthesis. The importance of this process was noted by Bok and Hall (1971), who observed that failure to phagocytose the shed discs resulted in the death of the rod cells. The same process of disc synthesis and destruction is thought to occur in cones too (Anderson & Fisher, 1975). Soon after this, La Vail (1976) reported that disc shedding in rat rods followed a rhythmic cycle with major bursts of disc shedding coinciding with periods of subjective light. Cone disc shedding has also been investigated in the lizard, goldfish and chicken where it was found to occur after the onset of darkness in contrast to the situation in rods (Young, 1977, 1978; O'Day and Young, 1978). It has been difficult to draw any firm conclusions since other species have shown bursts of disc shedding from rods and cones during both light and dark onset. It does appear that the time of day affects photoreceptor metabolism although exactly how has yet to be established.

Rods and cones contact horizontal and bipolar cells via chemical synaptic terminals known as the spherule and pedical, respectively. The smaller rod spherules contain a single deep cleft which accommodates the dendrites of second order neurons. In contrast to this, the larger cone pedicles have a flattened base with many shallow

invaginations. These synapses often contain densely staining synaptic ribbons surrounded by synaptic vesicles.

1.8 Double cones

As well as single cones, double cones have also been observed in the retinae of all vertebrate groups except placental mammals. In the avian retina, double cones consist of two closely associated but independent cones which differ in their size, shape and structure. The larger Principal cone is long and thin and contains an oil droplet in the inner segment ellipsoid. The smaller accessory cone is broader and shorter and may have either a small droplet or a low concentration of carotenoid (Bowmaker & Knowles, 1977; Jane & Bowmaker, 1988). The double cones are isolated from single cones by Müller cell processes, and by the interdigitating RPE processes which ensheath their outer segments (Mariani and Leure-DuPree, 1978). Double cones have been identified in the retinae of several avian species (see later) including the American crow, the great blue heron, the red-tailed hawk and several owl species (Braekevelt, 1993b, 1994; Braekevelt *et al.,* 1996). Some microspectrophotometric studies on avian retinas have reported the notable absence of double cones; for example in the tawny owl, (Bowmaker & Martin, 1978), the emu and tinamou (Sillman *et al.,* 1981) and the Humboldt penguin (Bowmaker & Martin, 1985). However, this could merely be due to the Principal and Accessory members of double cones becoming separated during tissue preparation (see later). The significance of double cones has not yet been fully understood although, in contrast to the single cones they have been suggested to be exclusively involved in brightness discrimination in birds.

1.9 Receptor mosaics in avians

The spacing of receptors has been investigated in vertebrates, notably in teleost retinae (Engström, 1963) and the gecko (Dunn, 1966) where definite receptor mosaics have been seen. Engström (1958), observed a pattern in the great Tit, *Parus major,* in which four double cones formed a square which surrounded a single cone, each such grouping being interspersed with rods and other single cones. Another study by Morris (1970) also found a uniform distribution of receptors in the chick retina although in this case the receptor arrangement was in a hexagonal lattice.

2. COLOUR VISION

Animals possessing colour vision have the ability to distinguish different wavelengths irrespective of brightness. The two main prerequisites for colour vision are (i) the presence of at least two photoreceptor classes absorbing in separate regions of the spectrum but with overlapping visual pigment sensitivities and (ii) a nervous system capable of integrating the information from both classes of receptor. Photoreceptors can be thought of as photon counters, the strength of the neural signal from a rod or cone being modulated by the number of photons absorbed (Lythgoe and Partridge, 1989). Colour vision is achieved by neurally comparing the quantum catches of photoreceptors possessing maximal sensitivities in slightly different spectral locations; the precise location of λ_{max} being dependent on the particular opsin expressed within the photoreceptor outer segment, Mollon (1993).

The degree of colour vision within a species is largely determined by its ancestry, the visual tasks that it has to perform and the spectral range of light available in its environment (Bowmaker, 1991a). Land animals and aquatic species living close to the surface are exposed to a very broad spectral range of wavelengths, although in practice this is limited to wavelengths of between 350 to 800 nm, due the photochemistry of the visual pigments (wavelengths longer than about 850 nm are too low in energy to photoisomerize visual pigments, whereas wavelengths shorter than 300 nm have enough energy to destroy proteins), and the transparency of the pre-receptoral tissue (e.g. the human lens which absorbs all wavelengths below 400 nm, Wald, 1945). For example, in response to their restricted wavelength environment consisting of mainly blue-green light, fish living in shallow coastal waters have evolved just two cone pigments with maximal sensitivities at about 430 to 530 (Lythgoe, 1979; Lythgoe and Partridge, 1991) (Bowmaker, 1991b). By way of contrast, diurnal birds are terrestrial animals living in a broad spectral range of light. Several avian species have been shown to possess four cone visual pigments with maximum sensitivities ranging from about 360 nm to 570 nm (Chen and Goldsmith, 1986; Jane and Bowmaker, 1988).

2.0. Colour vision in birds

By simply observing the habits of birds it becomes apparent that they must possess the ability to see colours. For example, birds such as the peacock, English robin and the pheasant show a sexual dimorphism in which the plumage of the male is brightly coloured, implying that colours are important in courtship displays and mating behaviour. Avian pollinators such as humming birds, visit brightly coloured flowers whereas other birds are attracted to coloured berries or seeds suggesting that colour

may be important in feeding. The avian retina contains a relatively high percentage of cones compared to most vertebrate retinas (Walls, 1942). It is for this reason that research into colour vision has often used the avian retina since it is the cones which are responsible for colour discrimination (under photopic conditions).

2.1. The monopigment hypothesis

The mechanism of bird (and reptile) colour vision was unclear for many years due to the presence of oil droplets within the cone inner segments which were believed to subserve colour vision by virtue of their ability to selectively filter certain wavelengths (Krause, 1893, as cited by Crescitelli, 1972). It was thought that a colour vision system could be achieved with only one cone pigment in conjunction with a set of oil droplet colour filters which had distinct absorption characteristics (King-Smith, 1969). By only permitting the transmission of specific wavelengths of light to their respective outer segments, the oil droplets could divide cones (containing the same visual pigment) into several different types, each with a unique overall spectral absorbance. Support for the monopigment theory came from several studies which were unable to demonstrate more than one cone pigment in the avian retina. For example, extractions from the retinas of several avian species yielded a single cone visual pigment, iodopsin (P562) (Crescitelli *et al.,* 1964; Wald *et al.,* 1955; Bridges, 1962 and Sillman, 1969). Microspectrophotometric studies of pigeon, chicken and gull retina also failed to detect any cone pigment other than iodopsin (Liebman, 1972). However, cone pigments are notoriously evasive to chemical extraction, and microspectrophotometric absorbance measurements are difficult in the avian retina for several reasons including; (a) the very small dimensions of avian cone outer segments (generally about 2μm in diameter) and (b) the sparcity of intact cones since, upon removal of the retina from the eye cup, the outer segments tend to break off and remain attached to the pigment epithelium (Bowmaker, 1984). Hence, the observation of only one cone pigment in a biochemical or microspectrophotometric study does not necessarily preclude the existence of other cone pigments.

Results from later microspectrophotometric studies provided indisputable evidence for the occurrence of more than one cone pigment in the avian retina. Two cone pigments were identified in the chicken with λ_{max} at about 569 nm and 497 nm (Bowmaker and Knowles, 1977) and three pigments were found in the pigeon with λ_{max} at about 567 nm, 515 nm and 460 nm (Bowmaker, 1977). In the same year, using selective bleaching and recording of early receptor potentials, Govardovskii and Zueva (1977) found four cone sensitivities in both chicken and pigeon and deduced that there may be four cones with λ_{max} at about 561, 509, 465 and 415 nm. Thus the monopigment hypothesis was finally invalidated.

TECHNIQUES USED IN COLOUR VISION STUDIES

2.2. Visual pigment extraction

The oldest method of studying visual pigments consists of extracting them from the retina into solution and has been used to establish their absorption characteristics. Since the visual pigments are water insoluble they are first brought into aqueous solution by the addition of detergents. The unusually high proportion of cones in the avian retina has made it a particularly useful tool for researchers attempting to chemically extract cone visual pigments, which were previously elusive to the standard methods of extraction. Based on the observed products of bleaching, all the extracted avian visual pigments appear to be based on the retinal chromophore, as is the case in mammals where dehydroretinal-based pigments have also never been observed. In contrast to the chicken rod pigment, the cone pigments are positively charged at neutral pH, and are unstable in the presence of hydroxylamine (even in the dark). Cones show a greater susceptibility than rods to hydroxylamine attack and also display a faster pigment regeneration on addition of 11-*cis*- retinal, both of which, are thought to reflect a more exposed retinal binding pocket in cones, than in rods (Wang *et al.,* 1992).

Wald (1937) was the first to successfully isolate a presumptive cone pigment prepared from chicken retinas in digitonin extracts. Wald, named this pigment, iodopsin, extending the rhodopsin-porphyropsin terminology (Greek; iodes = violet-like). Iodopsin, with λ_{max} at about 562 nm (Bliss, 1946; Wald *et al.,* 1955), is now known to be the pigment contained in the longwave sensitive cones. Iodopsin has also been extracted from the retina of the domestic turkey (Crescitelli *et al.,* 1964). Other groups working on the pigeon retina appear to have extracted longwave pigments differing in their λ_{max} , for instance, Bridges (1962) reported the extraction of a photosensitive pigment with λ_{max} at 544 nm (c.f. 562 nm) which, infact, appears to be a chloride shifted longwave cone pigment (see below). Sillman (1969) failed to find any cone pigments in a study of 20 species of birds including the pigeon. However the failure to extract cone pigments from birds is not uncommon and there are many reasons as to why some pigments seem to be easier to extract (Meyer, 1977). It would be rash, therefore, to conclude that they are not present from these results alone.

Iodopsin in digitonin extracts, receptor suspensions and in individual receptor cells, is sensitive to the concentration of chloride ions (Knowles, 1976). More importantly, the λ_{max} of iodopsin shows a blue-shift of approximately 40 nm to 520 nm upon removal of the chloride ion, the exact value depending on the amount of residual chloride ion. The chloride depleted pigment also shows a greater susceptibility to hydroxylamine attack in the dark (Fager and Fager, 1979). The requirement for chloride ion in

particular is thought to be due to the ion size. Knowles (1980) suggests that the very low (mM) concentrations of chloride ion required to restore the pigment to its original λ_{max} indicate that the effect is not due to a conformational change in the protein. Instead he suggests that the longwave-shift back to 569 nm may be caused by a single chloride ion binding at a site in close enough proximity to the retinal chromophore to be able to directly affect its electron distribution and hence the pigment absorption. Infact, this is now known to be incorrect. The amino acids which participate in the chloride binding site of longwave sensitive opsins have now been identified as His-197 and Lys-200, using the numbering system used for longwave opsins [or His-181 and Lys-184 according to bovine rod opsin numbering] (Wang *et al.,* 1993). Both residues are located in the extracellular loop connecting transmembrane helices IV and V. This is close to one of the cysteines forming the disulphide bond (**Cys-203** - Cys-126 [or **Cys-187** - Cys-110]) which is essential for the correct folding of the opsin molecule (Karnik and Khorana, 1990). This disulphide linkage lies towards the end of helix III which houses the Schiff's base counterion (Glu-129 [Glu-113]). Therefore, it seems that the chloride binding site may function by exerting an indirect effect on the chromophore (Wang *et al.,* 1993).(See later)

Fager and Fager (1981) demonstrated the presence of two shortwave absorbing pigments from digitonin extracts of the chicken retina. Chicken blue ($\lambda_{max,}$ 449 nm) and chicken violet ($\lambda_{max,}$ 417 nm) cone pigments shared with iodopsin the properties of rapid regenerability and hydroxylamine lability, but showed no chloride sensitivity. Using similar hydroxylamine bleaching experiments the absorbance maximum of the chicken green cone pigment was found to be at 500-508 nm (Okano *et al.,* 1989; Yen and Fager, 1984). A rather different approach was that of Wang *et al.* (1992) who employed molecular cloning techniques to extract a presumptive chicken green cone opsin. Chicken green opsin cDNA was first expressed in cultured cells before being reconstituted *in vitro* by the addition of 11-*cis*- retinal and solubilizing in digitonin. From this method, the chicken green cone pigment was found to absorb maximally at 495 nm.

In contrast to cone pigments, the avian rod pigment appears to be much more amenable to extraction and shows a relative constancy in its spectral location. All of the avian rod pigments so far extracted show a maximal absorbance of between 500 - 506 nm (Crescitelli, 1958; Sillman, 1969; Okano *et al.,* 1989).

2.3. Studies using electrophysiology

Much data on spectral sensitivity has been obtained using the electroretinogram (the mass electrical response of the retina to light). Electroretinographic responses of this

sort are a consequence of the complex interaction between photoreceptors (both rods and cones) and therefore will not accurately represent the absorption spectra of individual visual pigments (Govardovskii and Zueva, 1977). It is possible to isolate a single cone mechanism by selective adaptation (see Chen and Goldsmith, 1986), however, the light filtering effect of the coloured oil droplets in avian cones as well as possible interactions between cone classes within a retina could still mask the effective absorption of a visual pigment.

Donner (1953) found four modulator units in pigeon retinal ganglion cells having maximal sensitivities near 480 nm. Ikeda (1965) used the ERG to measure the sensitivity of the pigeon retina to spectral colours. The cone response was isolated from the rod response by high levels of light adaptation and flickering stimuli. The results showed a broad spectral sensitivity curve peaking at around 547 nm. This peak was consistent with Bridges' (1962) isolation (from visual pigment extraction) of a presumptive cone pigment with λ_{max} at 544 nm. Using a 547 nm monochromatic background light, Ikeda isolated a 605 nm peak in the spectral sensitivity curve. The 547 nm adapting light had bleached out all the cones except the red cones (iodopsin, P562) which will have been screened by their red droplets (King-Smith, 1969) to produce the sensitivity function observed. Granda and Yazulla (1971) recording from cells in the pigeon's nucleus rotundus found single units with peaks at 540 nm and 600-620 nm. Yazulla and Granda (1973) reported opponent units in the nucleus rotundus with a peak excitatory response at 420 and 530 nm and a peak inhibitory response at 570 and 440 nm.

From ERG responses and using red and orange background lights to bleach the longwave receptors, Graf and Norren (1974) and Norren (1975) isolated spectral sensitivities with maxima at 410-420 nm and 480 nm in three different birds; the pigeon, chicken and the daw. The 480 mechanism was probably caused by the filtering effect of C-type droplets on the blue cones (P467). Although this evidence seemed convincing, it did not constitute unequivocal evidence for the presence of a short-wave pigment. Govardovskii and Zueva (1977) used a combination of selective bleaching and recordings of the early receptor potential to determine the visual pigments in chicken and pigeon retina. They demonstrated the presence of four cone sensitivities in the chicken and the pigeon with maxima at 507 and 562 nm and two previously unknown short-wave mechanisms at 413 and 467 nm. These short-wave mechanisms were shown to be visual pigments and not the bleaching products of rhodopsin. The 413 nm (violet) mechanism agreed with the maxima obtained by Graf and Norren (1974) and Norren (1975) and resulted from the combination of T-type droplets with the violet (P413) sensitive cones. With respect to the P413, the necessity for

transparent ocular media (cornea, lens, and vitreous) is evident and this was also demonstrated to be the case, with the ocular media being transparent down to 340 nm in both the chicken and pigeon. Chen and Goldsmith (1986), using the electroretinogram, measured the spectral sensitivity functions of several passerine birds under different states of adaptation to coloured lights. The passerine species examined had four peaks of photopic sensitivity at 370, 450, 480 and 570 nm with some birds displaying a maximum at 510 nm. The authors stress that each of these peaks represent the combined effect of absorption by a visual pigment and the transmission of its oil droplet(s).

2.4. Psychophysics

While microspectrophotometric data provide the initial evidence that an animal has the photoreceptors to perceive certain wavelengths and electrophysiological studies measure the responses of the photoreceptors to various wavelength stimuli, only a behavioural study can provide evidence that an animal is actually sensitive to particular wavelengths. Blough (1957) using a behavioural method, determined the spectral sensitivity of the pigeon in the light adapted eye and found a maximum at 565 nm. Comparing this curve to the absorption spectrum of iodopsin Blough inferred that the lowered sensitivity at short wavelengths was due to the filtering effect of oil droplets.

Determination of the hue discrimination function of an animal is one of the four traditional approaches used in psychophysical determinations of colour vision, the others being; colour mixture, chromatic adaptation and spectral sensitivity (Boynton, 1963; Jacobs, 1981). Minima in the hue discrimination function correspond to regions of the spectrum where an animal perceives a rapid change in hue, and hence, where colour discrimination is at its best. These 'interhue transition points' (colour boundaries) may also indicate the number of hues which an animal perceives (Wright, 1972). This hue discrimination function has been determined in the pigeon where the maximum wavelength discriminability appears to be at 500, 540-550 and 600 nm (Wright, 1972; Schneider, 1972; Bloch and Martinoya, 1971). Blough (1972) and Wright and Cumming's (1971) data on colour naming in the pigeon also support the existence of a peak in discrimination at 540 nm and 600 nm. These findings are not in general agreement with those of Hamilton and Coleman (1933) due to certain experimental anomalies (See Wright, 1972 for a discussion).

Wessels (1974) in an extensive behavioural study, used a two choice apparatus to obtain the spectral sensitivity curve of the daw, *Corvus monedula.* His results gave early indications of a possible tetrachromatic colour vision system, with four maxima in the red, green, blue and violet regions of the spectrum. Using a mathematical model

Wessels estimated the four cone mechanisms in the daw to have maximal sensitivities at 563, 508, 455 and 414 nm respectively. Jitsumori (1976) used an anomaloscope and a colour matching procedure where pigeons were given the task of matching a yellow light to different mixtures of red and green light. Although the function obtained was similar to that obtained from trichromatic humans, the pigeons did not make very good matches with the red and green combinations implicating tetrachromacy as a possible explanation (as cited by Wallman, 1979). Using behavioural methods, the spectral sensitivity function of a passeriform bird, the red-billed leiothrix (Pekin robin), *Leiothrix lutea,* was found to have four peaks at 370, 460, 530 and 620 nm (Burkhardt and Maier, 1989; Maier, 1992). These peaks, reflecting the four underlying cone mechanisms, correlate well with the calculated effective spectral sensitivity functions from individual cones (Maier and Bowmaker, 1993) suggesting that like most birds, the red-billed leiothrix is tetrachromatic. In contrast to Wessels' (1974) findings which suggested a violet sensitive cone mechanism (414 nm) in the daw, the Pekin robin appeared to have a cone mechanism sensitive to even shorter ultraviolet wavelengths (370 nm).

The wavelength discrimination function of the pigeon has been shown to consist of four minima suggesting the possibility of a pentachromatic system in this bird (Emmerton and Delius, 1980). Results from colour mixing experiments also suggest the possibility of a five pigment system in the pigeon (Palacios and Varela, 1992). Remy and Emmerton (1989) demonstrated behaviourally that the pigeon is able to perceive UV light. Using frontal and lateral stimulation they were able to stimulate the red and yellow fields separately and found differences in their sensitivity. The results showed that the yellow field was 2.4 log units more sensitive to wavelengths below 500 nm, especially in the blue and ultraviolet spectral range. The extent of the ultraviolet sensitivity varied for individual pigeons. A peak at 400 nm was seen in the sensitivity of the yellow field which had previously been seen in electrophysiological studies of the pigeon (Wortel *et al.,* 1984).

2.5 Immunocytochemistry

Since the early eighties, antibody technology has made possible the immunocytochemical labelling of photoreceptor cells in the retina (Szél *et al.,* 1986). Visual pigment specific monoclonal antibodies such as OS-2 and COS-1, bind to the C-terminal domains of opsin proteins. For example, the antibody, OS-2, has been raised against rhodopsin which can recognize a common epitope (specific antigenic determinants) in all chicken visual pigments. COS-1 selectively binds specific classes of cone outer segment within a retina and, in birds, recognizes middle-to-longwave sensitive cones while OS-2 recognizes the blue sensitive cones. The photoreceptor

ratios and patterns of distribution found in these antibody labelling studies appear to correlate well with those obtained using microspectrophotometric and electrophysiological methods (Röhlich and Szél, 1993).

3. MICROSPECTROPHOTOMETRY

Until the early 60s, before the development of the technique of microspectrophotometry, the only direct way of establishing the visual pigments present in a species was by pigment extraction. In summarizing some of the disadvantages of studying visual pigments through extracts, Knowles and Dartnall (1977) mention that they [extracts] require laborious analysis by partial bleaching in order to demonstrate the visual pigment, are not suitable for demonstrating minority pigments in a retina due to the abundance of the rod pigment, do not reflect the variety of pigments in the living retina since cone pigments are not as robust as the rod pigment and do not survive the extraction procedure, only allow the relation between an extracted pigment and its cellular origin to be guessed and provide no information as to the orientation of pigment molecules, or the location of structures such as oil droplets. The technique of microspectrophotometry does not have these drawbacks and has been described as 'a premier tool in visual pigment study' (Carlson, 1972). One reason for this is that it is the only method available which allows *in situ* measurements from isolated photoreceptor cells; a minute beam of monochromatic light is passed transversely through the outer segment and the transmittance and hence the absorbance of the visual pigment is calculated at each wavelength in the spectrum (See Liebman, 1972 for a review). Thus the problems associated with visual pigment extraction such as the possible modification of a visual pigment by a detergent or the spectral masking of one visual pigment by another are avoided. Another virtue of MSP is that information about the cell morphology can be directly correlated with pigment type. This is particularly advantageous in the case of intact cones in reptilian and avian retinas, since the absorbance spectrum of pigment in the outer segment can be measured and correlated with the associated droplet in the inner segment. Before MSP there was no convenient way to measure both elements in the same cell.

3.0. The Liebman microspectrophotometer

In the dual beam system, the beam of monochromatic light is partitioned or 'chopped' into a sample beam and a reference beam. The two beams are collected and demagnified (by passage through a condenser lens) in order to achieve the same dimensions as a typical outer segment (approximately 2 μm in diameter in birds) before reaching the specimen plane of a high-magnification microscope. One of the beams is passed through a rod or cone outer segment (sample beam) while the other is passed through an area free of cellular material (reference beam). During a scan, a photomultiplier sequentially measures the relative intensities of the sample and reference beams at each wavelength; the ratio of photocurrent thus obtained is a measure of the transmittance

and hence absorbance of the cell. The sample and reference beams are separated in time and measured alternately. The reference signal is fed back to control the gain of the photomultiplier by regulating the dynode voltage in a negative feedback loop. Hence, the voltage is raised in response to diminishing levels of short-wavelength light from the tungsten light source, causing a subsequent increase in photomultiplier sensitivity. This ensures that the reference beam output is held constant as the spectrum is scanned and that the sample beam transmission reflects changes in the proportion of light absorbed by the cell, rather than inherent variations in the output of the light source. The sample and reference beams are not identical since they originate from different points on the lamp filament, pass through differing parts of the optics and strike different points on the photomultiplier cathode. In order to allow for these constant differences, an initial baseline recording with both beams in a cell-free area of the preparation is made before measurements commence. The absorbance spectrum of a cell is then recorded, followed by a second baseline. Both baseline recordings are separately subtracted from the cell absorbance yielding two spectra for each cell.

The technique of MSP has advanced greatly since its early days when Caspersson (1940; as cited by Carlson, 1972) first introduced a photometric method for determining the absorption spectra of nucleic acids in cells. Chance *et al.* (1959) developed the technique further, using it to measure the very low absorbance of cytochromes, the respiratory enzymes in mitochondria. Hanaoka and Fujimoto (1957) were the first to apply the techniques used by Chance *et al.* (1959) to the study of the photosensitive visual pigments in the carp retina. Working with visual pigments presented an obvious problem which was not apparent in the previous applications of the technique - that of photosensitivity. The high light levels that Chance *et al.* (1959) used to obtain a good signal to noise ratio from the weakly absorbing cytochromes in mitochondria would immediately bleach the photosensitive visual pigments. Using a 3 μm spot of light they were able to make absorbance measurements photoelectrically and showed the carp had five pigment types. Although their results have proved difficult to interpret, several ideas from their original experimental set-up have been adopted in the design of more modern microspectrophotometers (see Liebman, 1972). Liebman (1972) highlights the difficulty faced in measuring visual pigment absorbance spectra as follows;

> 'To obtain good spectral resolution and detectivity for weakly-absorbing cells we need to use high light exposures to reduce the relative noise fluctuations - but the more light we use, the more pigment is bleached with increasing danger of spectral distortion or, in the limit, complete destruction of the pigment being measured!'

Due to its small size, a single outer segment contains a low pigment concentration with the result that a significant proportion of pigment will be bleached during a scan, leading to distortions in the measured absorbance spectrum. (This is in contrast to bulk pigment extractions, where the number of pigment molecules can greatly exceed the number of photons absorbed during a scan, leaving the absorbance relatively unaltered [Liebman and Entine, 1964]). The intensity of a measuring beam has to be high enough to produce an optimal signal to noise ratio but low enough to avoid destroying the pigment during a scan. The highest ratio of signal detection to pigment bleaching is obtained when measurements are made using the minimum number of photons.

In an attempt to overcome the low pigment densities in outer segments, early investigators made measurements with the measuring beam passing axially through the length of the outer segments. Although axial measurements offer the advantage of an increased path-length, the relative gain in the signal to noise ratio is, at best, only a factor of 2 (due to the increased measuring area in transverse measurements - see below) and is perhaps outweighed by the associated optical problems; since the measuring beam must pass through the neural layers of the retina (including the inner segment) before reaching the outer segment, there is the serious problem of light-scattering which intensifies with time. This scattered light, being wavelength dependent, produces a high short wave absorbance and since it is absorbed by the surrounding receptors, may even give the false impression of a pigment mixture within a cell (Liebman, 1972). Apart from a very small reflection from the receptor surfaces, no comparable light scattering problems are encountered in transverse measurements owing to the greatly reduced pathlength. The reduced path length (2 μm as opposed to 20 μm) can be compensated for by using light polarized perpendicular to the long axis of the outer segment in order to exploit the dichroic properties of the pigments. Additionally, the measuring beam can be passed through a greater cell area without affecting the fraction of pigment bleached. Therefore the size of the measuring beam can be increased to fit the dimensions of an outer segment, further improving the signal to noise ratio.

3.1 Studies on avian retinas utilizing microspectrophotometry

Relatively few avian species have been examined by microspectrophotometry. The chicken, *Gallus gallus* (Bowmaker and Knowles, 1977; Bowmaker *et al.,* 1997), pigeon, *Columba livia* (Bowmaker, 1977; Bowmaker *et al* ., 1997), tawny owl, *Strix aluco* (Bowmaker and Martin, 1978), duck, *Anas platyrhynchos* (Jane and Bowmaker, 1984), penguin, *Spheniscus humboldti* (Bowmaker and Martin, 1985), Pekin robin, *Leiothrix lutea* (Maier and Bowmaker, 1993), Japanese quail, *Coturnix*

coturnix japonica (Bowmaker *et al.,* 1993), budgerigar, *Melopsittacus undulatus* (Bowmaker *et al* ., 1997), and the zebra finch, *Taeniopygia guttata* (Bowmaker *et al* . , 1997), have all been shown to posses several cone pigments. However, the primitive, so called paleognathous birds, such as the emu, *Dromiceius novae-hollandiae* , and tinamou, *Nothoprocta,* spp., have so far been shown to contain a single cone pigment, a P560-570 (Sillman *et al* , 1981).

Results from microspectrophotometry have been particularly useful, providing absorbance data on both cone visual pigments and their adjacent droplets. By taking into consideration the filtering effect of the associated oil droplet, the effective spectral sensitivities of each cone can be derived. On the assumption that the gross photopic sensitivity will be a summation of the sensitivity of the individual cone types (in the same ratio that they are present in the retina), Bowmaker and Knowles (1977) were able to derive the photopic sensitivity of the chicken. This was found to be remarkably similar to the photopic sensitivity curves obtained from behavioural and electrophysiological methods.

One of the drawbacks of microspectrophotometry is that it is not possible to state unequivocally that all the visual pigments in a retina have been identified and measured, due to the random sampling of tissue associated with the technique. For example, four cone pigments have been reported in the chicken retina (Govardovskii and Zueva, 1977; Fager and Fager, 1981) although only two were measured by microspectrophotometry (Bowmaker and Knowles, 1977).

3.2. The photoreceptors found in avian species

The retina of diurnal birds is dominated by droplet-containing cones which constitute up to 80% of the total photoreceptor population (Maier and Bowmaker, 1993). Many birds possess at least five spectrally distinct cone classes; four classes of single cone and one class of double cone. A total of four different cone pigments occur in avian retina; thus birds have the potential for tetrachromacy (Chen and Goldsmith, 1986; Bowmaker, 1991a). Typical pigment-droplet combinations are shown in table 3.2a The relative proportions of the different cone classes are similar in many avian retinas. In passerine birds 30-50% of the total cone population is made up of double cones. The LWS and MWS cones are found in roughly equal numbers making up 15-20% of cones. The remaining 5-20 % of cones consist of SWS and UVS cones and contain the C- and T-type droplets respectively (Bowmaker, 1991a).

Table 3.2a

Typical pigment-oil droplet combinations found in bird retinas.

Cone type (single / double)	**Pigment λ_{max} (nm)**	**Associated oil droplet**	**Droplet λ_{cut} (nm)**
single **LWS 'red' cone**	550-570	(red) **R-type**	560-570
single **MWS 'green' cone**	500-515	(yellow) **Y-type**	500-510
single **SWS 'blue' cone**	440-460	(clear) **C-type**	420-440
single **(U)VS 'violet' or 'ultraviolet' cone**	360-420	(transparent) **T-type**	-
double cone **(Principal member)**	550-570	(principal) **P-type**	480-485 sh
double cone **(Accessory member)**	550-570	(accessory) **A-type**	triple peaked (low concentrations of carotenoid)

Terminology based on classification suggested by Bowmaker (1991a)
LWS=longwave sensitive
MWS=middlewave sensitive
SWS=shortwave sensitive
(U)VS=(ultra)violet sensitive

Early MSP studies in the retinas of the chicken, pigeon and gull demonstrated the presence of only one cone pigment, the P562 (unpublished: see Liebman, 1972). This result appeared to agree with the monopigment hypothesis of avian colour vision based on a set of oil droplet colour filters. However, at that time it was known from microspectrophotometric studies that another Sauropsidan, the turtle, *Chelonia mydas,* (which also contained coloured oil droplets in its cones) possessed not one, but at least three cone pigments, P562, P502 and P440 (Liebman and Granda, 1971). It was not long before evidence for the presence of multipigment colour vision systems was also found in birds.

Columbiformes;

3.3. The pigeon, *Columba livia*

Using an improved Liebman microspectrophotometer, Bowmaker (1977) was able to demonstrate that the pigeon retina contains rods, double cones and four types of single cone (each associated with a particular droplet type). The four pigments are; the P576, found in longwave sensitive single 'red' cones and both principal and accessory members of double cones, the P514 found in the middlewave sensitive 'green' cones, the P461 found in the shortwave sensitive 'blue' cones and the P503 in rods. The observation of the P503 and P567 confirmed results previously reported by Liebman (1972) but the middle-wave P514 and short-wave P461 had not been previously reported. In a more recent study, Bowmaker *et al.* (1997) have re-examined the pigeon retina, and confirm the presence of these pigments, placing the improved 'red', 'green' and 'blue' cone λ_{max} values at 568, 507 and 453 nm respectively, and the rod λ_{max} at 506 nm. These pigments were combined with oil droplets in a logical fashion; the P568 was combined with an R-type droplet, the P507 with a Y-type droplet and the P453 with a C-type droplet. However R- and Y-type droplets found in the dorsal retina appeared to have a cut-off at longer wavelengths than in the ventral retina. Additionally, a fourth, previously unreported violet sensitive cone population was identified, containing a P409 paired with a T-type oil droplet. Much evidence has accumulated from behavioural and electrophysiological studies suggesting that the pigeon can perceive ultraviolet light (see colour vision chapter). Using ERG responses, cones with maximal sensitivity at 415 nm (Graf and Norren, 1974) and 366 nm (Vos Hzn, 1994) have been isolated, suggesting that the pigeon may be pentachromatic. Additionally, a behaviourally established spectral sensitivity function including measurements down to the ultraviolet region has been obtained for the pigeon indicating a very high sensitivity between 325-360 nm (Kreithen and Eisner, 1978). In support of pigeon UV-sensitivity is the observation that the ocular media of the pigeon are highly transparent at short wavelengths (Govardovskii and Zueva, 1977; Emmerton and Delius, 1980; Jane and

Bowmaker, 1988). Using operant conditioning methods to plot the wavelength discrimination function of pigeons Delius and Emmerton (1979) and Emmerton and Delius (1980) demonstrated four minima indicative of five visual pigments, further adding to the possibility of pentachromacy in the pigeon. Bowmaker *et al.* (1997) note that although a fifth cone class with a λ_{max} below about 380 nm was not identified from their microspectrophotometric study, the possibility that it exists cannot be excluded. It is possible that these cones form a small population which are not uniformly distributed in the retina, and therefore were missed by the random sampling inherent to the technique of microspectrophotometry.

WATER FOWL:

Anseriformes;

3.4. The duck, *Anas platyrhynchos*

A microspectrophotometric study of the photoreceptors in the duck retina has demonstrated the presence of rods and both single and double cones (Jane and Bowmaker, 1988). Four cone pigments have been identified; the P420, P452, P502 and P570 each of which occur in single cones, with the P570 occurring in both principal and accessory members of double cones. The rods contain a P505 pigment. The shortwave P420 may be similar to a neural channel, with maximal sensitivity between 410-420 nm, which has been measured in the daw (Wessels, 1974; Norren, 1975) and the chicken (Govardovskii and Zueva, 1977) using electrophysiological and psychophysical methods. An oil droplet is present in the ellipsoid region of the inner segment of single cones and the principal member of double cones, whereas the accessory member does not contain an oil droplet, although low concentrations of carotenoid are still found in the ellipsoid region, as is found in the chicken (Bowmaker and Knowles, 1977). Five types of oil droplet were measured (R, Y, P, C, and T), their distribution being uniform, with no clear red/yellow fields as is found in the pigeon (Bowmaker, 1977). Typical pigment-droplet combinations are found in the cones of wild and domesticated duck. In single cones, the P570 is found with an R-type droplet, the P502 is with a Y-type droplet, the P452 with a C-type droplet and the P420 with a T-type droplet. The P570 is paired with a P-type droplet in the principal member of double cones. Unlike in the pigeon, the ocular media of the duck show a high shortwave absorbance, being transparent only down to about 400 nm. Thus, it is unlikely that ultraviolet sensitive cones (λ_{max} = < 400 nm) are present in duck retina (Jane and Bowmaker, 1988).

Passeriformes;

3.5. The Red-billed leiothrix (Pekin robin), *Leiothrix lutea*

The behavioural spectral sensitivity function for the Red-billed Leiothrix shows four spectrally distinct sensitivity peaks with maxima at 370, 460, 530 and 620 nm (Burkhardt and Maier, 1989; Maier, 1992). Behavioural studies under selective chromatic adaptation also suggest the presence of four different cone mechanisms (Maier, 1990, as cited by Maier, 1992). Microspectrophotometric examination of the photoreceptors in the retina of the Red-billed Leiothrix has clarified the presence of four cone pigments with λ_{max} at 568, 499, 454 and an ultraviolet sensitive pigment at about 355 nm, paired with R-, Y-, C- and T-type droplets, respectively. The transmission of the ocular media has been found to decrease at ultraviolet wavelengths, reducing to 50% at about 315 nm (Maier, 1994). This will reduce the effective sensitivities of both the 'ultraviolet' and 'blue' sensitive cones at short wavelengths (Maier and Bowmaker, 1993). Rods contain a P500, and both members of the double cones contain the P568. The droplet complement within the cones of the Red-billed Leiothrix resembles that found in many passeriform birds (Bowmaker and Knowles, 1977; Goldsmith *et al.,* 1984; Partridge, 1989; Bowmaker, 1991a). The principal member of the double cone contains a large droplet with highly variable absorbance which is sometimes even lower than the low density carotenoid spectrum of the accessory droplet (Maier and Bowmaker, 1993).

3.6. The zebra finch, *Taeniopygia guttata*

A microspectrophotometric analysis of the retina of the zebra finch, *Taeniopygia guttata,* (another representative of the *Passeriformes*) has revealed the presence of rods, double cones and four spectrally distinct classes of single cone with λ_{max} s at 568, 506, 430, and 360-380 nm respectively (Bowmaker *et al.,* 1997). The P567 is found in single 'red' cones (paired with an R-type droplet), and in both members of double cones, paired with a P-type droplet in the Principal member. In cones, the P507 is paired with a Y-type droplet. Interestingly, the λ_{max} s of the rods (506 nm) and 'green' cones (507 nm) are almost identical - a fact that has been observed in several other avian species (see table 3.12a). The λ_{max} of the 'blue' cones (430 nm) is considerably shorter than is seen in most birds including the Red-billed leiothrix, *Leiothrix lutea,* (the only other member of the *Passeriformes* to have been examined microspectrophotometrically), where the 'blue' cone λ_{max} was found to be at 453 nm (Maier and Bowmaker, 1993). The C-type droplets in the zebra finch had relatively low densities (0.1). The fourth class of 'ultraviolet' sensitive cones contained a pigment absorbing maximally between 360 and 380 nm (the precise λ_{max} being difficult to determine due to insufficient data), which was paired with a T-type oil droplet.

Behavioural experiments, looking at mate choice in the zebra finch, have also indicated their sensitivity to ultraviolet light (Bennet *et al.,* 1996).

Psittaciformes;

3.7. The budgerigar (shell parakeet), *Melopsittacus undulatus*

A recent microspectrophotometric examination of the budgerigar retina has demonstrated the presence of rods containing a P509, double cones and four spectrally distinct classes of single cone with λ_{max}s at 564, 508, 444 and 371 nm respectively (Bowmaker *et al.,* 1997). In the single cones the P564 was paired with an R-type droplet, the P508, with a Y-type droplet , the P444 with a C-type droplet and the P371 with a T-type droplet . Similar to the situation in the pigeon retina (Bowmaker *et al.,* 1997), two varieties of Y-type droplet were identified, although no correlation with the retinal region was found. The 'blue' sensitive cones absorb maximally at slightly shorter wavelengths (444 nm) than is seen in most birds (cf. 450 nm, see table 3.12a), and the density of the associated C-type droplets was relatively low (0.1). The P564, found in both members of the double cones, was paired with a P-type droplet in the Principal member. The P-type droplets had a λ_{Tcut} ranging from about 410 to 450 and occasionally displayed a 480 nm shoulder which in some droplets formed an actual 480 nm absorbance peak, indicating the presence of mixtures of carotenoids. The rod outer segments were found to be larger, and more common in the budgerigar retina, compared to the zebra finch (a Passeriform bird), which is thought to reflect the slightly more crepuscular behaviour of budgerigars (Bowmaker *et al.,* 1997).

DOMESTIC JUNGLE FOWL:

Galliformes;

3.8. The chicken, *Gallus gallus*

The chicken is a domesticated form of the jungle fowl. Bowmaker and Knowles (1977) obtained microspectrophotometric evidence for the presence of rods and five types of cone, a double and four single cones in the chicken retina. Three pigments were measured, a P569 (found in both members of the double cones and three types of single cone, characterized by the droplet), a P497 found in one class of cones and a P506 in rods. The P569 was found in association with an R-type droplet in single 'red' cones, a P-type droplet in the principal member of double cones and an A-type droplet in the accessory member of double cones (using droplet classification suggested by Bowmaker, 1991a). The P569 was reportedly found paired with C-type droplets in a selection of single cones too, a combination not usually seen in other birds. Perhaps these 'single' cones were actually Principal cones, containing non-dense P-type droplets which had become separated from the Accessory member. The other pigment

found in single cones, the P497, was associated with a Y-type oil droplet, and formed the 'green' cone population. In their latest re-examination of the chicken retina, Bowmaker *et al.* (1997) confirm the presence of these pigments, and identify two additional cone pigments; a shortwave sensitive P455, paired with a C-type oil droplet and an ultraviolet sensitive P418, paired with a T-type oil droplet. These findings constitute the first microspectrophotometric evidence that the chicken, like many other birds, is tetrachromatic. The improved data also suggest that the rod and 'green' cone λ_{max}s (506 nm and 507 nm, respectively) are much closer than previously reported.

Other data obtained using a variety of different methods has pointed to tetrachromacy in the chicken. By recording early receptor potentials, Govardovskii and Zueva (1977) deduced that there were four cone sensitivities in the chicken (and pigeon) with absorption maxima at about 413, 467, 507 and 562. Both violet (λ_{max} = 417 nm) and blue (λ_{max} = 449 nm) pigments were later extracted from the chicken retina (Fager and Fager, 1981). Moreover, the genes encoding the chicken rod opsin and the chicken red, green, blue and violet opsins have been isolated and sequenced from cDNA libraries, proving that these pigments are expressed in the chicken retina (Okano *et al.,* 1992).

3.9. The Japanese quail, *Coturnix coturnix japonica*

Another member of the *Galliformes,* the quail, is a close relative of the chicken and consequently shows a similar photoreceptor complement. Its retina contains rods (λ_{max} = 505 nm) and four classes of single cone with λ_{max}s at 567, 505, 458 and 420 nm which are paired with R-, Y-, C-, and T-type droplets, respectively (Bowmaker *et al.,* 1993). The P567 is found in both principal and accessory members of double cones paired with a P-type droplet and A-type droplet (containing low concentrations of carotenoid) respectively.

A NOCTURNAL BIRD:

Strigiformes;

3.10. The tawny owl, *Strix aluco*

The tawny owl is one of the most nocturnal members of the *Strigiformes* (owls and barn owls) and has a retina dominated by rods containing a P503, with only 20% of cones (Bowmaker and Martin, 1978). Double cones were reported to be absent from the tawny owl retina. However, *intact* double cones are rarely seen in retinal preparations due to the Principal and Accessory members becoming detached during tissue preparation. Moreover, double cones have been identified in light and electron-microscopy studies of the retinas of the Great Horned Owl, *Bubo virginianus,* and the barred owl, *Strix varia* (Braekevelt, 1993a; Braekevelt *et al.,* 1996). The low

proportion of cones in general, including the putative double cones in the tawny owl retina would contribute to their apparent 'absence'. The single cones contain three pigments, a P555, P503 and a P463 and four types of oil droplet suggesting that the owl retina represents the remnant of a more highly developed ancestral photopic system of its diurnal ancestors (Bowmaker and Martin, 1978). The longwave absorbing pigment, the P555, found in 90% of cones is 10-15 nm shorter in comparison to that seen in most passerine species (see above). A cone mechanism peaking at about 555 nm has also been observed in the Great Horned Owl, *Bubo virginianus* (Jacobs *et al.*, 1987), indicating that this may be a common feature amongst the *Strigiformes.* In the tawny owl, the P555 is paired with a very pale yellow droplet with a 485 nm shoulder, which does not fit into the usual droplet classification (single 'red' cones are usually associated with an R-type droplet). It is suggested that this droplet may be closely related to the P-type droplets found in the principal member of double cones (Bowmaker, 1991a). Extending this interpretation further, it is possible that these 'single red cones' *are* infact detached Principal members of double cones. 1% of cones, assumed to contain the P555, were associated with a 'browny' coloured oil droplet resembling very dilute concentrations of the carotenoid astaxanthin found in R-type droplets. It is possible that these cells formed the single red cone population, however without any data to verify the pigment contained within the outer segments of these cells, any interpretations are merely speculative. The 'green' P503 pigment in cones, is identical in λ_{max} to the pigment found in rods and is found in 5-10% of cones paired with a typical Y-type droplet. 5% of cones contain a 'blue' P463 pigment and are associated with a T-type droplet. The generally low absorbance of oil droplets would correlate with the nocturnal habit of owls in that a higher density of carotenoid in the droplet will further reduce the sensitivity of cones in an already dim light environment.

AN AQUATIC BIRD:

Sphenisciformes

3.11. The Humboldt penguin, *Spheniscus humboldti*

Microspectrophotometric analysis of the photoreceptors in the retina of the Humboldt penguin has revealed the presence of rods (containing a P504) and five types of single cone characterized by their oil droplet (Bowmaker and Martin, 1985). The single cones contain three pigments, a P403, P450 and a P543. Similar to the situation in the retina of the tawny owl (Bowmaker and Martin, 1978), the longwave cone pigment absorbs at much shorter wavelengths (λ_{max} = 543 nm) than most passerine species. Moreover, the oil droplets in the penguin retina also do not fit the oil droplet classification scheme outlined in the table above. Unlike any other avian retina so far examined, there is a

total absence of R-type droplets. A very pale droplet (resembling the C-type droplet of the Bowmaker, 1991a classification) is found in all three cones. Of the total cone population, 20% of cells consist of the P543 in combination with a Y-type droplet. Another 20% of cones consists of the 'blue' P450 and 'violet' P403 pigments in combination with either a very pale or T-type droplet. Although double cones were not observed in the penguin retina, 60% of the cone population consisted of the P543 in combination with a pale droplet (probably the P-type), perhaps indicating that these were infact the Principal members of double cones. As a result of the domination of the Humboldt penguin retina by P543 containing cones, this bird will be relatively insensitive to long wavelengths. However, this appears to be an adaptation to the aquatic habitat of *Spheniscus,* which consists of blue water bodies in which there is relatively little light above wavelengths of 575 nm (Bowmaker and Martin, 1978).

3.12. PALEOGNATHUS BIRDS:

The Emu, *Dromiceius novaehollandiae,*

The Tinamou, *Northoprocta perdicaria, Northoprocta cineruscens*

These birds are generally flightless. Microspectrophotometric examination of the retina of these birds suggest that they possess a rather different complement of cone types to the neognathus birds described above (Sillman *et al.,* 1981). The retinas of paleognathus birds appear to consist solely of single cones containing just one longwave sensitive pigment with maximal absorbance between 565-570 nm. Although double cones were notably absent, these structures are rarely found with both Principal and Accessory members intact and attached to each other. There appear to be three classes of single cone, two of which contain either an R-type or Y-type droplet, dense enough to act as a cut-off filter. No C- or T-type droplets were identified. The third class of cones completely lacks an oil droplet in the ellipsoid region of the inner segment. Again, it is possible that these cones were infact the accessory members of double cones which became detached during tissue preparation.

Table 3.12a

Summary of avian pigments obtained from MSP

Avian species	pigment λ_{max} (nm)					
NEOGNATHUS BIRDS	ultra-violet	violet	blue	green	red	rod
[a] ***Sphenisciformes:*** Humboldt penguin *Spheniscus humboldti*	-	403	450		543	504
[b] ***Procellariformes:*** Manx shearwater *Puffinus puffinus*	-	402	452	P	P	505
[c] ***Anseriformes:*** Mallard duck *Anas platyrhynchos domesticus*	-	420	452	502	570	505
[d] ***Galliformes:*** domestic chicken *Gallus gallus*	-	418	455	507	569	509
[e] Japanese quail *Coturnix Japonica*	-	419	456	505	569	505
[f] ***Columbiformes:*** pigeon *Columba livia*	(366)	409 (410)	453	507	568	506
[g] ***Psittaciformes:*** budgerigar *Melopsittacus undulatus*	371	-	444	508	564	509
[h] ***Passeriformes:*** Peking robin *Leiothrix lutea*	355	-	453	501	567	500
[i] zebra finch *Taeniopygia guttata*	*ca* 360 - 380	-	430	506	568	507
[j] ***Strigiformes:*** tawny owl *Strix aluco*	-	-	463	503	555	503
PALEOGNATHUS BIRDS						
[k] Emu *Dromiceius novae-hollandiae*	-	-	-	-	567	502
[l] Chilean tinamou *Northoprocta perdicaria*	-	-	-	-	566	500
[m] Brushland tinamou *N. cinerascens*	-	-	-	498	564	504

References for table 3.12a

a Bowmaker and Martin, 1978

b Bowmaker, unpublished

c Jane and Bowmaker, 1988

d Bowmaker and Knowles, 1977; Bowmaker *et al.*, 1997

e Bowmaker *et al.*, 1993

f Bowmaker, 1977; Bowmaker *et al.*, 1997; (366), Vos Hzn *et al.*, (410), Graf and Norren, 1974

g Bowmaker *et al.*, 1997

h Maier and Bowmaker, 1993

i Bowmaker *et al.*, 1997

j Bowmaker and Martin, 1978

k

l Silman *et al.*, 1981

m

3.13 Microspectrophotometric, behavioural and ERG evidence for UV cones in avian retina

As discussed in the previous section the majority of bird species which have been examined using microspectrophotometry contain a near ultraviolet sensitive class of cones, associated with a T-type droplet (see table 3.12a). Based on the results from the relatively few species examined, it appears that the λ_{maxs} of the UVS pigments tend to cluster around three distinct spectral regions.

in the violet region between 415 - 420 nm, as exemplified by species from the Orders *Galliformes,* and *Anseriformes,*

in the violet region between 400 - 405 nm, as shown by species from the orders *Columbiformes, Sphenisciformes* and the *Procellariformes*

and below about 380 nm in the near ultraviolet region, as exemplified by members of the Order *Passeriformes* and the *Psittaciformes* (as represented by the budgerigar).

'Violet' or 'ultraviolet' sensitive cones in an individual bird retina appear to be mutually exclusive, except perhaps in the pigeon. Wavelength discrimination function studies (Wright, 1979; Emmerton and Delius, 1980) and electrophysiological studies (Graf and Norren, 1974; Norren, 1975; Romesky and Yager, 1976; Wortel *et al.,* 1984; Vos Hzn *et al.,* 1994) seem to suggest that the pigeon possesses both 'violet' and 'ultraviolet' sensitive cone mechanisms, maximal at about 410 and 365 nm, respectively. However, there has been no evidence to substantiate this from microspectrophotometry. So far, only cones responsible for the putative 'violet' sensitive channel have been observed (Bowmaker *et al.,* 1997). The possibility that a population of P365 containing 'ultraviolet' cones does exists in the pigeon retina but was not found due to the sampling error associated with this technique, cannot be excluded. Having said this, without a thorough knowledge of the neural pathways or processing which occurs in the avian retina, it may be incorrect to assume that every cone 'mechanism', isolated from electrophysiological or behavioural studies, must be the direct consequence of a distinct population of cones. The 365 nm peak in the pigeon sensitivity may equally be the result of neural comparisons of the signals between say, the 'violet' cones and another subset of cones.

3.14. Evidence for ultraviolet sensitivity in birds

Much data evidencing the sensitivity of birds to near UV light has accumulated since it was first demonstrated in the humming bird, *Colibri serrirostris* (Huth and Burkhardt,

1972, as cited by Maier, 1994), and in the pigeon, *Columba livia,* (Wright, 1972), using behavioural methods. Chen and Goldsmith (1986) isolated a UV channel with maximal sensitivity at about 370 nm from 15 species of mostly passerine birds using electrophysiology. Since then UV sensitivity has been indicated in over 31 avian species and appears to be a common trait amongst birds (Bennet and Cuthill, 1994 - see their table 1 for a list). Some behavioural studies suggest that the sensitivity to UV light in birds is extraordinarily high, which, considering the size and sparcity of UV cones, is surprising (Kreithen and Eisner, 1978; Maier, 1992). For example, Maier (1992) found the Pekin robin, *Leiothrix lutea*, to have a sensitivity about ten times higher in the UV than in the 'human-visible' spectrum. However, it is possible that the apparent relatively high sensitivity of the UV cones in these behavioural experiments was due to the selective adaptation of the other cones to the ambient light, (Maier, 1992). Goldsmith (1994) mentions this problem of interpreting behavioural results;

> '...in birds behavioral assessments of spectral sensitivity may depart sharply from the properties of the retina as determined by the sizes, relative numbers, and sensitivities of individual classes of cones..'

He goes on to say that measures of 'photopic luminosity' made in a behavioural context may just reflect the presence of a pathway (fixed or dynamic) the biological function of which may be totally unconnected to the procedures used to reveal it, and might not be related to the relative brightness under which the colour discriminations were made.

It is possible that birds are merely sensitive to UV radiation and use it in achromatic brightness discriminations. Alternatively, ultraviolet wavelengths could be processed by birds, as part of a colour discrimination channel and used in their colour vision system (Bennet and Cuthill, 1994). That this is the case is suggested by the few avian behavioural spectral sensitivity functions which have been obtained. For example, the behavioural sensitivity function of the Pekin Robin, *Leithrix lutea,* shows typical 'peaks' and 'troughs'. The peaks in such data are usually interpreted to represent the underlying cone mechanism, whereas the troughs are thought to reflect antagonistic processes between different receptor types, indicating that UV cones in this bird are part of its colour opponent system. A study by Emmerton and Delius (1980) on the pigeon, suggested that this bird is capable of distinguishing approximately 7-16 nm wavelength differences, well into the near ultraviolet (360-380 nm), beyond which recordings were not made. Similarly, the humming bird was found to be capable of distinguishing 10 nm differences down to 420 nm, beyond which, measurements were not taken (Goldsmith, Collins and Perlman, 1981).

3.15. UV vision in birds

UV sensitivity is widespread amongst birds and other vertebrates, such as mammals, birds, fish, reptiles and amphibia (see Jacobs, 1993, Tovee, 1995 for a review). Primates are atypical in being UV blind. Since DNA and proteins absorb energy at ultraviolet wavelengths, even low doses of near UV radiation can produce retinal damage (see Ham and Mueller, 1989, for a review). This implies that sensitivity to UV light must confer some significant evolutionary advantage(s) for animals, far outweighing these damaging effects. The fact that many animals have maintained UV sensitivity suggests that it may play a role(s) in their existence. The uses of UV light may vary depending on the ecology of a certain species. In birds UV receptors are probably integrated into a colour opponent system but UV radiation will be focussed elsewhere the eye, making the determination of spatial detail impossible. The very low packing density of UV cones however, suggests that their main function is probably not to do with spatial resolution, but rather with 'wide angle' analysis. It is thought that UV wavelengths may be used in birds for regulating circadian rhythms, navigation, foraging and sexual displays.

3.16. The Rayleigh effect: wavelength dependent scattering

On passing through a medium, light is scattered by the molecules present. If these particles are small compared to the wavelength of the light (as in the case of dust particles, or oxygen molecules) then, according to Raleigh's law, the degree of scattering is proportional to the inverse of the fourth power of the wavelength, (Born and Wolf, 1970; Lythgoe, 1979). In other words 'ultraviolet' wavelengths are susceptible to scattering. Consequently, objects will appear degraded which limits the use of ultraviolet wavelengths in spatial resolution and contrast (Lythgoe, 1979).

3.17. Possible uses for UV sensitivity

UV light in orientation:

Many birds orientate themselves with the aid of a "sun compass"; the position of the sun's disk in the sky, and a knowledge of the time of day give the bird a sense of direction (Schmidt-Koenig, 1979, 1990). Any mechanism which allows the determination of the position of the sun, such as the ability to detect the plane of polarized light and/or spectral and intensity gradients across the sky, may be useful for navigational purposes.

Plane polarized light

Plane polarization, due to light scatter, is wavelength dependent and occurs at right angles to the incident light (Born and Wolf, 1970). Thus, concentric rings or *'e-*

vectors' of plane polarized light occur around the sun. UV wavelengths are strongly polarized and some insects possess UV receptors which are sensitive to these *e-vectors,* enabling them to orientate with respect to the sun (von Frisch, 1967; Wehner, 1989). The evidence for polarization sensitivity in the homing pigeon has been ambiguous (Kreithen and Keeton, 1974; Coemans *et al.,* 1994; Martin, 1991). However separate experiments on migratory passerines such as the Savannah sparrow, *Passerculus sandwichensis,* have suggested that polarized skylight patterns do provide information about geographic compass directions during daylight hours (e.g. Able and Able, 1993). However, in order to detect plane polarized light of a particular orientation, the chromophore of each visual pigment in an individual receptor must be aligned in the same orientation (Land, 1991). Since the visual pigment molecules in vertebrate cones are not systematically ordered it is unclear how UV polarization might be detected.

Intensity and spectral gradients along the sky

As well as producing plane polarized light, Rayleigh scattering also produces intensity and spectral gradients along the sky. These gradients could be used by migratory birds as alternative cues for estimating the position of the sun for orientation purposes. Since ultraviolet- and short wavelengths are more likely to be scattered than longer wavelengths, they have a relatively even intensity across the sky. Long wavelengths actually undergo an increase in intensity towards the horizon (due, to long optical pathlengths). Because of this, the ratio of intensities of any two wavelengths will vary with the angular distance from the sun (Coemans *et al.,* 1994). It is suggested that the UVS cones in the pigeon, could be used in conjunction with the LWS cones to analyse the spectral composition of a patch of sky, in terms of the proportion of long wavelengths in a background of ultraviolet wavelengths (Coemans *et al.,* 1994). In support of their theory the authors cite the sparse and 'dispersed' distribution of UV cones in the pigeon retina which would thus be ideally suited to the analysis of 'wide angle' chromatic information such as colour gradients in the sky, rather than in the analysis of spatial detail, such as finding seeds. A recent study looking at the UV cones in the retina of the budgerigar, has demonstrated a semi-random distribution, in support of this (Wilkie *et al.,* 1997).

Tests on migratory silvereyes, *Zosterops lateralis,* (Order *Passeriformes)* under light of different wavelengths has indicated that orientation is indeed a light-dependent process. These birds show maximal migration in the twilight hours of dawn and dusk, when there is a high proportion of light of short wavelengths (Lythgoe, 1979). In tests, birds were able to orient in the appropriate direction under wavelengths ranging from about 400 - 570 nm, although red light (633 nm) disrupted the magnetic orientation, leading the authors to suggest that orientation requires a minimum threshold energy (Wiltschko,

1993).

3.18. UV light and visual acuity

There is evidence to suggest that many of the foodstuffs eaten by birds, either absorb or reflect strongly in the UV, suggesting that birds employ UV light for foraging. Some seeds have a UV reflecting-waxy coating which, upon removal can affect the rate at which the seeds are taken by birds (Bennet and Cuthill, 1994). The strong, hard, conical bills of canaries suggest that they are predominantly seed eaters and are often seen perched on the ground picking up seeds, especially on millet farmland in the vicinity of villages (Serle *et al.,* 1972). It is possible that UV sensitivity in the canary could be used in locating and perhaps distinguishing seeds on the basis of UV reflectance. Although not predominantly seed eating birds, pigeons were found to possess poor UV sensitivity in a seed-detection task (Emmerton and Remy, 1983), but were reasonably good at discriminating shapes under UV light (Emmerton, 1983). Perhaps the extremely low spatial resolution of UV cones in the pigeon retina precludes the detection of very small objects such as seeds. Although regional differences occur in the pigeon retina, (the dorso-posterior 'red field' possessing a preponderance of R- and Y-type droplets and the remaining 'yellow field', a higher proportion of P-, C- and T-type oil droplets, Bowmaker, 1979) in both tasks, the red field is more likely to have been used since it 'views' the ground. If UV sensitivity were of any real importance in foraging responses, such as pecking at seeds, one would expect a much higher proportion of T-type droplets (and hence UV sensitive cones) in the red field (Bowmaker, 1977).

Fruits, such as berries, have been found to reflect in the UV (Burkhardt, 1982), whereas most leaves do not, possibly making the small fruit easier to spot (Kevan, 1978; Burkhardt, 1982). Although most reports of the wild canary cite that it is a seed eater, and make no reference to fruits, the diet of domestic canaries is often supplemented with a variety of foods such as apple, carrot, cucumber, orange, leaves from plants of the brassica family, lettuce and various wild plants, with no obvious ill-effect (Walker and Avon, 1993). Presumably the wild canary is a fruit-eater too, and may well use its UV sensitivity in distinguishing different berries on the basis of their UV reflectance. Moths and butterflies, eaten by some birds, also reflect in the UV (Kevan, 1978; Eiser, Anshansley and Eisner, 1978; Silberglied, 1979), as do many flowers, which are frequented by avian pollinators (Goldsmith, 1980).

Behavioural studies on the Eurasian kestrel, *Falco tinnunculus,* suggest that it uses its UV sensitivity in a novel foraging technique. Its prey consists of small mammals, such as voles, which scent their runways with urine and faeces which strongly absorb UV

light, compared to the surrounding vegetation (Viitala *et al.,* 1995). In experiments kestrels appear to spend more time in areas treated with artificial urine than in non-treated areas suggesting that they use these markings as a means to identify possible areas of high vole density, in which hunting is more likely to be fruitful (Viitala *et al.,* 1995). Although urine and faeces may have other distinguishing attributes besides UV absorbance, such as strong odour for example, which could be alternative cues for hunting, kestrels were unable to distinguish vole scent marks under human-visible light - indicating the importance of UV absorbance.

3.19. Intra- and interspecies communication

Birds are probably one of the most obvious examples of body colour displays, the bright plumage of many species being used in sexual displays and for intimidating rivals. The plumage of the female wild canary is usually duller than that of the male and it is also possible that differences in the plumage UV reflectance of male canaries also exists. The sexual selection hypothesis suggests that UV vision may also have a role to play in signalling between birds, since much evidence has been obtained for the presence of UV-reflecting plumage in different birds (Burkhardt, 1982; 1989; Burkhardt and Finger, 1991). Finger, *et al.,* (1992) elucidated a type of feather which due to its structure, consisting of randomly orientated keratin granules, scatters short (particularly ultraviolet) wavelengths of light.

Reports of ultraviolet light being used in mate choice and/or species recognition have come from behavioural experiments on two passerine species, the Pekin robin, *Leiothrix lutea,* (Maier, 1994b) and the zebra finch, *Taeniopygia guttata,* (Bennett *et al.,* 1996). In these experiments female birds were allowed to view males, either through UV-transparent filters or UV-opaque filters. On the basis of the number of ritualised display hops by females, it was inferred that they prefer a male partner viewed through a UV-transparent filter implying that UV wavelengths are important in mate-choice. However these results do not prove that wavelengths in the UV region of the spectrum are especially significant in mate-choice decisions since the effect of removing other wavelength bands was not tested. For example, it is possible that a similar result would have been obtained if a different band of wavelengths was excluded - say, wavelengths below 500 nm. All than can safely be said is that removal of UV wavelengths alters the spectral quality of light available to female zebra finches, and hence their response but whether this is effect is unique to UV wavelengths, has yet to be verified.

4. OIL DROPLETS

A classic difference between rod and cone photoreceptors is the presence of an oil droplet in the distal ellipsoid region of the cone inner segment. These spherical globules can be coloured or colourless and are unaffected by light. They are present in most vertebrate divisions (see table 4.0a), but are notably absent from teleost fish and placental mammals (Walls, 1963; Rodieck, 1973). Droplets are usually only associated with cones and are seen in both the single cones and the Principal member of the double cones in the retina of passerine birds and diurnal reptiles. The small Accessory member of chicken double cones sometimes contains a small oil droplet (Bowmaker and Knowles, 1977) but in other birds and turtles no droplet or a low concentration of carotenoid is seen (Bowmaker, 1977; Lipetz and MacNichol, 1982). Rods are devoid of droplets with the exception of the primitive reptile, *Sphenodon*, certain geckos and lungfish (Walls, 1942).

4.0. Phylogeny of droplets

The "lowest" vertebrates to possess oil droplets are sturgeons and some lungfish which possess Colourless droplets (see table 4.0a). This fact is often used in the argument that Colourless droplets evolved first, but as Walls (1942) points out, these fish have nocturnally adapted eyes; hence the argument that the Colourless droplets of sturgeons have evolved from previously coloured droplets, is equally feasible. Frogs are considered to be the most primitive vertebrates containing coloured (yellow) oil droplets. In the mammals, Colourless droplets have been noted in monotremes (*Ornothorynchus*) and several Australian marsupials. However, droplets have never been reported in placental mammals, holosteans and teleosts. As seen in table 4.0a, in most cases oil droplets are either Colourless or very pale. However, one of the most striking features of turtle and diurnal bird retinas is the presence of brightly coloured oil droplets. Located towards the distal end of the cone inner segment, the oil droplet is directly in the path of in coming light. In such a position a brightly coloured oil droplet will alter the spectral composition of the light reaching the photosensitive pigment and hence the overall spectral sensitivity of the cone.

The droplet colours are a result of varying concentrations of lipid-dissolved carotenoid or mixtures of carotenoid taken in the diet (Wald, 1948; Meyer, 1971a, 1977; Goldsmith *et al.,* 1984). In the case of diurnal birds and turtles the carotenoid concentration in droplets is sufficiently high to permit the droplets to act as bandpass or cut-off filters, absorbing the shorter wavelengths of light and only transmitting the

Table 4.0a

A summary of the phylogenetic distribution of oil droplets. Adapted from Walls (1942).

	HABITAT	**DROPLET COLOUR**
FISHES;		
Chondrosteans	benthic	- colourless
Holosteans	diurnal	- *absent*
Dipnoans and Cladistians	nocturnal	- colourless if present
Teleosts	strongly diurnal	- *absent*
AMPHIBIANS;		
Anurans;		
Frogs	nocturnal / diurnal	- yellow and colourless
Toads	nocturnal	- *absent*
Urodeles;	fossorial	- *absent*
REPTILES;		
Sphenodon	nocturnal, fossorial	- pale yellow
Crocodilians	nocturnal	- *absent*
Turtles	**diurnal**	**- red, orange, yellow, colourless**
Lizards	diurnal / fossorial	- yellow and colourless, or just colourless
Geckos	nocturnal / diurnal	"
Snakes	crepuscular / fossorial	- *absent*
BIRDS;		
Most birds	**strongly diurnal**	**- red, orange, yellow, colourless**
Many birds	crepuscular or nocturnal	- yellow, but mostly colourless
Nocturnal Birds	nocturnal	- colourless
MAMMALS		
Monotremes (nocturnal)	nocturnal	- colourless
Marsupials	nocturnal	- colourless
Placentals;		
Ungulates and large Carnivores	nocturnal / diurnal nocturnal	- *absent*
Insectivores, Rodents and Lagomorphs	diurnal/ nocturnal	- *absent*
Primates	diurnal	- *absent*

longer wavelengths (Liebman and Granda, 1975). The other component of oil droplets, neutral lipids, seem to be the only component in Colourless droplets.

4.1. Biochemical Analyses on oil droplets

The oil droplet content of avians and reptiles has been extensively studied because they possess the most vivid and greatest assortment of colours. Capranica (1877 as cited by Meyer *et al.,* 1965) analysed the oil droplets of frogs, reptiles and birds, by subjecting them to reactions with various chemical substances such as iodine and acids. From these reactions he concluded that the various droplet hues were due to dilutions of a single substance, lutein, found in egg yolk, milk and animal fat. Kühne and Ayres (1878 as cited by Meyer *et al.,* 1965) subsequently obtained not one, but three distinct coloured extracts from the chicken retina using saponification and solubility procedures. These substances which they called chlorophane [yellowish-green], xanthophane [orange-yellow], and rhodophane [red] were found to be very stable and insensitive to light and could not be crystallized, despite several attempts. However, these findings were questioned by Walls and Judd (1933) who argued that the true chemical properties of oil droplets *in vivo* may have been altered during the extraction procedure.

It was not until Wald (1937) and Wald & Zussman (1938) succeeded in purifying and crystallizing three retinal extracts from chicken retina that the first breakthrough in the understanding of oil droplet pigment composition was achieved. They classified the pigments as carotenoids. Carotenoids can be defined as follows; " A subgroup of polyene pigments, yellow to red in colour, of aliphatic and alicyclic structure. They consist of isoprene units (usually eight) , the conjugated double bonds constituting the chromophoric system." (Meyer *et al.,* 1965). Wald and Zussman (1938) designated the red carotenoid as astacene, the orange as a xanthophyll ester and the yellowish green as an unidentified hydrocarbon later identified as an alcohol and given the trivial name, galloxanthin (Wald, 1948; Strother and Wolken, 1960). However, astacene does not occur *in vivo* in the retina (Kuhn *et al.,* 1939, as cited by Meyer *et al.,* 1965) and it was subsequently found to be the readily formed autoxidation product of astaxanthin which is the naturally occurring red pigment (Strother and Wolken, 1960; Meyer *et al.,* 1965).

4.2. Objective measurements on oil droplets

Biochemical analyses on retinal extracts are somewhat limiting in that the association of a particular carotenoid with a coloured droplet is merely by inference. This problem has been approached by investigating the spectral absorption characteristics of oil droplets. Early observations of cone oil droplets reported the presence of a great variety of

colours including blue and violet but these colours were probably due to the uncorrected microscope objectives in use at that time (Walls and Judd 1933). By using apochromatic lenses it has been possible to describe oil droplet colours accurately. The first objective measurements of oil droplet absorption spectra were made by Roaf (1929), working on the retina of the domestic hen. Roaf formed the image of a droplet on the slit of a microspectroscope and photographed the resulting spectrum. He discovered that the droplets acted as 'cut off' filters, transmitting long wavelengths and absorbing short wavelengths of light and that they could be categorized, depending on the wavelength at which they started to absorb as follows; those starting to absorb at 574 nm (Red droplets), at 512 nm (Yellow droplets), and at 455 nm (pale green droplets).

Strother and Wolken (1960), undertook a microspectrophotometric study of oil droplet pigments with a view to identifying their respective carotenoids. They noted broad absorption peaks, the width increasing with the maximum absorbing wavelength. It was suggested that each globule contained a mixture of carotenoids, with one predominant carotenoid (Hanaoka and Fujimoto, 1957; Strother and Wolken, 1960). Yellow globules were thought to be a mixture of lutein and zeaxanthin (optical isomers) whereas astaxanthin and galloxanthin appeared to be the sole carotenoid consituents of the Red and yellowish-green coloured droplets, respectively (Meyer *et al.*, 1965).

Up until the mid 1970s, most oil droplet spectra obtained using direct microspectrophotometric methods did not show the expected carotenoid fine structure but rather took the from of 'structureless cut-off absorbance curves reminiscent of commercial colour filters' (Liebman and Granda, 1975). It was soon realized that this was due to technical limitations of microspectrophotometers which were designed to measure the low absorbances (less than 1.0) of the visual pigments. Therefore, a droplet which has a very high carotenoid absorbance can only be accurately measured in the region where the absorbance rises rapidly to about 1.0. Beyond this value, the measured transmittances will be higher than the actual transmittances due to stray light, (produced by light leakage around droplets) dominating these measurements. The recordings show broad flat absorbance curves (with a maximum absorbance less than 1.0) throughout the spectral region 'where true specimen density exceeds instrument capability' (Liebman and Granda, 1975).

A different procedure solving this problem was pioneered by Liebman and Granda (1975) who reduced the concentration of carotenoid in individual droplets by diluting them in a colourless solvent, mineral oil. The resulting carotenoid concentration was

low enough for accurate measurements, permitting the characteristically peaked carotenoid spectra to be seen. They obtained the density spectrum of the original droplet by determining the ratio of the radius of the droplet after dilution to that before dilution and multiplying the square of this value by the measured optical density of the diluted droplet. Using this technique in the turtle retina they were able to show the single peak spectrum of astaxanthin in Red and Orange droplets and the three-fingered spectrum of zeaxanthin in Yellow droplets. More importantly they were able to quantitatively establish that the pigments in turtle oil droplets are extremely dense. Their calculations suggested that the absorbance of the Red droplet in *Pseudemys* was between 50-90 absorbance units corresponding to a 1 M concentration of astaxanthin.

Such high oil droplet densities do however, seem improbable when compared to the much lower oil droplet densities obtained from birds. For instance, in a study by Goldsmith *et al.,* (1984) the calculated *in vivo* optical densities of oil droplets (following mineral oil expansion and dilution) were shown to be over 20 in the Red droplets, 8 or less in the Yellow and between 1-4 in the Pale and Colourless droplets. In contrast to turtles, Orange droplets were uncommon amongst birds and showed a varying carotenoid composition; sometimes they were comprised of a low concentration of astaxanthin (as seen in the common tern) whereas in other cases they appeared to contain a mixture of two carotenoids (as seen in the mourning dove) (Goldsmith *et al.,* 1984). The Yellow droplets typically displayed a three-banded absorption spectrum similar to lutein or zeaxanthin. Pale droplets were found to be a spectrally heterogeneous class with colours ranging from greenish-yellow to yellow when viewed under the microscope. Both the Pale and Colourless droplets were characterized by carotenoid(s) absorbing at 385 and 402 nm. However, the Pale droplets contained a mixture of two chromophores; galloxanthin (C_{27}), the 402 nm chromophore and ε-carotene. The 385 nm chromophore (C_{25}), typical of Colourless droplets, was assigned a trivial name, fringillixanthin denoting the family Fringillidae (finches), where it was initially observed.

Bowmaker *et al.,* (1993), looked at the development of carotenoids in the oil droplets of carotenoid-free Japanese quail chicks, *Coturnix coturnix japonica* , following the introduction of a *normal diet.* After 24 hours on a normal diet, the spectra of droplets classified as R-types, showed a longwave λ_{max} at 490 nm and an additional secondary peak at 440 nm, which was still visible after three days. This suggests that, in the quail, the R-type droplets probably do not consist solely of astaxanthin, but of a mixture of carotenoids - a situation which is similar to the Orange droplets reported in some other avian species, (see above, Goldsmith *et al.,* 1984). After a week, the fine spectral detail of the oil droplet carotenoids was no longer visible due to the relatively high density of

accumulated carotenoid and associated problems of light leakage around droplets.

4.3. Lipid oil droplet components

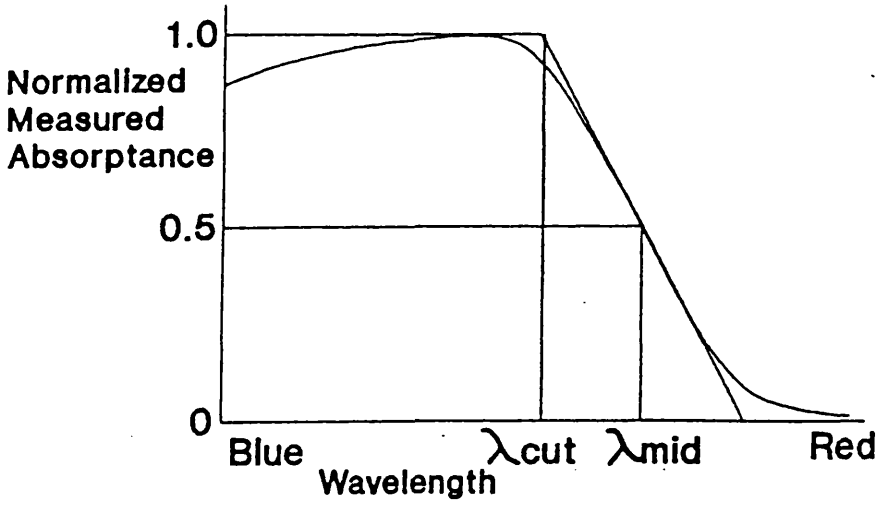
Carotenoids are not the only oil droplet constituents. Johnson and Hudson (1976), in a detailed study of the chemical composition of oil droplets reported the presence of a complex mixture of neutral lipids particularly enriched in polyunsaturated fatty acids such as linoleic and arachidonic acid. High quantities of cholesterol, glycerol ester, mono-, di- and triacylglycerols were also isolated. The authors suggest that such a lipid mixture, like most mixed solvents, would provide maximum solubility for the oil droplet carotenoids.

4.4. Embryonic development of oil droplets

During early embryogenesis, oil droplets are formed by the accumulation of cytoplasmic lipids which eventually produce a microscopically observable globule. In the chick retina, this occurs before the cone outer segment membranes have developed (Meyer *et al.*, 1965, 1966). The oil droplets remain Colourless until the final stages of embryonic development when the accumulation of xanthophillic carotenoids produces the bright colours seen under the light microscope. The molecular basis underlying the segregation of specific carotenoids (and hence, 'colours') into particular cone types has not been investigated. Johnson and Hudson (1976) suggest the possibility that the exact composition of the mixed lipids may be important in determining the carotenoid(s) which will eventually 'fill' a droplet due to the varying solubilities of carotenoids in different lipid mixtures. Alternatively, the carotenoids could be initially segregated by selective membrane transport mechanisms specific to the different cones. The complete array of oil droplets colours are present at the time of hatching (Cooper and Meyer, 1968) and it is therefore assumed that the carotenoids are normally stored and provided from the yolk.

4.5. The classification of oil droplets by cut-off wavelength

Unfortunately there is no common terminology to characterize avian oil droplets (see table 4.5a). This is partly due to different workers using different methods to define and calculate cut-off wavelengths. The classification of Goldsmith *et al.*, (1984) is based on the relative droplet sizes and their carotenoid constituents. Bowmaker and Knowles (1977) classified the droplets according to their λ_{T50} (the wavelength at which 50% transmission occurs). Although this measurement provides no information as to the chemical identity of the droplet carotenoids, the λ_{T50} is useful for calculating the effect of a droplet on the adjacent visual pigment absorptance (fraction of light absorbed) and hence the effective spectral sensitivity of the cone. Lipetz (1984a) characterized droplets according to two variables, their λ_{mid} (corresponding to an absorptance of half the measured maximum) and their λ_{cut} (the wavelength corresponding to the intersection between the tangent to the absorptance curve at λ_{mid} and the maximum absoptance value) (see below). Bowmaker (1991a) suggested a simpler classification system that combines the terminologies used by Lipetz and Goldsmith.



A normalised absorptance spectrum of an oil droplet measured using MSP.
λ_{mid} = the wavelength corresponding to half the maximum measured absorptance.
λ_{cut} = the wavelength corresponding to the intersection of the tangent to the absorptance curve and at λ_{mid} and the maximum absorptance value. From Partridge, 1989.

Table 4.5a
Summary of various terminologies used to classify avian oil droplets.

Cone type (single or double)	Pigment λ_{max}	Droplet type & appear-ance	Identified droplet carotenoid	λ_{cut} (nm) a	b	c	Terminology suggested by Bowmaker, 1991
single	550-570 nm	R (red)	astaxanthin	560-570	Red	R	**R-type** (red)
single	500-515	O (orange)	uncertain	500-570	C	O	**Y-type** (yellow)
single	500-515 nm	Y (yellow)	zeaxanthin	500-510	C	Y_2	**Y-type** (yellow)
single	440-460 nm	C (colour-less or pale yellow)	galloxanthin (405 nm) or x (375 nm)	420-440	A	P_2	**C-type** (clear or colourless)
single	360-420 nm	T (trans-parent)	no carotenoid	-	Clear	-	**T-type** (transparent)
chief member of double	550-570 nm	P (pale)	galloxanthin (405 nm) or x (375 nm) + ε-carotene	480-485 sh	B_1	P_1? P_2 Y_1	**P-type** (principal)
Accessory member of double	550-570 nm	-	-	triple peaked	B_2	-	**A-type** (accessory)

X an apocarotenoid observed only in MSP measurements.
a values from *Leiothrix lutea* (Maier and Bowmaker, 1993).
b terminology of Bowmaker (1977) and Bowmaker and Knowles (1977).
c terminology of Partridge (1989).
? uncertainty in the agreement between classification systems.
Adapted from Varela *et al.*, 1993

4.6. Distribution of oil droplets in the Retina

The proportions of the different oil droplet colours varies in different regions of the retina. The proportion of Red droplets is greater in the dorsal retina than in the ventral retina in many birds. This is exemplified by the pigeon retina where there is a large yellow field dominated by cones containing Yellow droplets and few Red droplets, and a smaller posterior dorsal red field dominated by cones containing multiple Red or single Orange droplets (Pedler and Boyle, 1969). In other birds such as the common tern (a plunge diver) the Red and Yellow droplets appear to vary together across the retina (see later). In the turtle, however, no such regional differences have been observed. The transmission characteristics of oil droplets often varies with the retinal region. The λ_{T50}'s of all the droplets in the yellow sector appear to be 10 nm shorter than those measured in the red sector (King-Smith, 1969; Bowmaker, 1977). There is also a gradual change in the absorbances of the (B_1) droplet in the chief member of the double cone; The λ_{T50} of these droplets becomes progressively shorter from the center of the red field (554 nm), to the center of the yellow field (500 nm) to the periphery of the yellow field (460 nm). The effective maximum sensitivities of the double cones will also show a corresponding displacement to longer wavelengths (Bowmaker, 1979).

Oil droplets lie at different levels in the retina (Waelchli, 1883, as cited by Bowmaker, 1979). Meyer *et al.,* (1966) showed three differently coloured oil droplets to be intimately associated with 3 specific cones in the chicken retina. From this observation they inferred that the droplets must also lie at different depths in the retina. They found that the 'Yellow' droplets (equivalent to the 'B_1' or 'P-type' droplets of more recent classification systems) occupying the tall chief member of the double cone, form the most scleral layer, the Red oil droplets occupying the shortest cones, form the most vitreal layer while the Accessory droplets form an intermediate yellowish-green layer.

4.7. Theories of oil droplet function

Narrow band sensitivity channels and improved hue discrimination

Bowmaker (1977) summarizes the general effects of an oil droplet in a given cone as follows;

- to displace the effective maximum sensitivity of the cone to a wavelength much longer than the λ_{max} of the visual pigment.
- to reduce the band width of the spectral sensitivity of the visual pigment by cutting off the shorter wavelengths.
- to reduce the absolute sensitivity of the cone at its λ_{max} from that of the visual pigment.

For example, these effects are displayed by an R-type droplet in the red sector of the pigeon retina, which cuts off light below 560 nm. An R-type droplet displaces the effective maximum sensitivity of the P567 to 619 nm, reduces the absolute sensitivity at 619 nm to about 38% of the λ_{max} and reduces the band width of the spectral sensitivity to about 30% of that of the P567 (Bowmaker, 1977). Where the droplets have a high enough optical density as is seen in the Y-type and C-type (or C- and A-type droplets of the Bowmaker and Knowles (1977) classification) there will be a similar effect on the spectral sensitivity of their respective visual pigment. The other droplets with lower optical densities will have some affect but to a lesser degree.

Bowmaker and Knowles (1977) suggest that in cones containing the Red and Orange-yellow oil droplets, narrow-band sensitivity channels are produced which serve a similar function at the receptor level, to the opponent mechanisms operating at higher neuronal levels in fish and mammals which improve hue discrimination. The left and right hand limbs of a 'narrowed' absorbance spectrum are, by definition, steeper and subsequently show a greater rate of change than the normal absorbance spectrum. Therefore, in certain regions of the spectrum where there is significant overlap between two different cone absorptance curves, there will be a larger difference in the photon capture (and hence, sensitivity) at any particular wavelength, due to the more rapidly changing slopes of the individual absorptance curves. It is thought that this may improve hue discrimination (Wright, 1979).

Oil droplets as secondary lenses

Young and Martin (1984) addressing the fact that Colourless oil droplets have a widespread occurrence in fish, amphibians and marsupials suggest that in addition to spectral filtering, these structures probably have other functions based on their optical properties. Using a theoretical approach based on wave optics they showed that oil droplets could act as converging microlenses, collecting and funnelling light into the outer segment. It is suggested that the increased photon capture of the visual pigment would improve visual sensitivity, contrast detection and motion detection.

Detection of plane of polarized light

Young and Martin (1984) also observed that due to their small size, much light will be diffracted around droplets or scattered away from the receptor axis. It was suggested that such oil droplet *scatter* in the *double cones* in the avian retina could be used to analyse the plane of polarization of incident light. The lack of melanin screening between the Principal and Accessory outer segments of the double cones implies that light can pass from one cell to another. It has been suggested that the light scattered by the P-type droplet of the Principal member of the double cone pair may pass

transversely into the Accessory cone outer segment lying adjacent to it. Since vertebrate photoreceptors are dichroic to transverse (but not longitudinal) light (Harosi and MacNicol, 1974), the signals from the Accessory cone could vary according to whether the e-vector of the scattered light was perpendicular or parallel to the outer segment membranes. However, it is likely that, in addition to the proposed transverse cross excitation from light scattered by the Principal droplet, light will also pass *longitudinally* into the Accessory cone outer segment. How would these two different modes of excitation be differentiated at the Accessory cone synapse ? Unless the Accessory cone outer segment is excited *only* by transverse droplet scattered light it is difficult to see how the two inputs would not be confused. Another question is, how would the level of intensity of incident light be monitored, and distinguished from changes in polarization ?

Dietary manipulation of oil droplet carotenoids

Oil droplet carotenoids cannot be synthesized by birds *de novo* , and must be ingested from plant sources (or other animals which have them stored). Hence by manipulating the diet, it is possible to produce carotenoid-free birds whose oil droplets are all identical (i.e. colourless due to absence of carotenoid) (Meyer, 1971a; 1971b; Wallman, 1979). Several experiments attempting to clarify the effect of oil droplets on avian colour vision have utilized carotenoid deprived Japanese quail (Meyer, 1971b; Wallman, 1979; Bowmaker *et al.,* 1993). Results seemed to show that these carotenoid-free birds were not completely colour blind and could respond to a red/green grating mainly on the basis of luminance information (in contrast to normal birds which responded principally to wavelength information). It was suggested that this was evidence for an indirect role, for oil droplets, in colour vision; although coloured droplets were not essential for colour vision, if they were depleted, colour vision deteriorated, and if they were replaced colour vision improved. However, carotenoid deprived birds are in a general state of poor health (they have matted feathers and show great feather loss, for example) which raises the question of whether such sick animals are likely to behave 'normally' anyway.

Prevention of absorption by the *cis* peak of visual pigments

The absorption spectrum of a visual pigment has a primary α absorption peak in the visible region, a secondary β or *cis* peak (due to the 11-*cis* isomer of retinal), near 340 nm in the near ultraviolet region and a γ band (due to the protein component of the visual pigment) in the far ultraviolet, near 280 nm. There is some evidence to suggest that the relative intensities of the α and *cis* peaks depend upon the λ_{max} of the α band; the longer this value is, the higher the relative intensity of the *cis* peak. Absorption in the *cis* band can also produce excitation and breakdown of the photopigment

(Spekreijse *et al.,* 1972) and must be considered in visual systems since its sensitivity extends well in to the 'blue' region of the spectrum (although the peak lies in the near ultraviolet). This would mean that a 'red' or 'blue' light (via absorption in the *cis* band) could excite a longwave sensitive cone to almost the same degree, leading to ambiguities in interpretation of the signal (this is distinct from regions of the spectrum where the long- and shortwave pigments actually overlap). One way to prevent absorption by the *cis* peak of visual pigments would be to use an oil droplet cut-off filter which could effectively absorb short wavelength light before it reaches the outer segment of long- and middlewave sensitive cones (Wolbarsht, 1976). However, oil droplet cut-off filters are also present in shortwave sensitive cones (in which absorbance in the *cis* band should not pose as great a problem) suggesting another secondary role for these structures such as light gathering (see later). The violet/ultraviolet sensitive cones will presumably also be affected by absorption in the *cis* band, but combination with any shortwave absorbing oil droplet would clearly not be suitable. This raises the question of how ambiguous signals, resulting from absorption in the *cis* peak, are avoided in violet/ultraviolet cones.

Reduction of chromatic aberration

The eye is focused for light in the yellow/red part of the spectrum. Therefore, without an appropriate filtering mechanism, if an object is illuminated by white light, the out of focus blue light will form a blue halo around the image, which will reduce acuity (Wolbarsht, 1976) . In this respect oil droplets are analogous in function to the yellow macular pigment found in the inner layers of primate retina which serves to reduce chromatic aberration by absorbing light of short wavelengths. (Presumably in animals which do not possess oil droplets, the macular pigment and/or the lens also contribute to reducing the effects of *cis* band absorption).

4.8. Correlation of oil droplet complement with visual ecology

When combined with their respective visual pigments, the R- and Y-type droplets in the retinas of several diurnal birds have been found to produce maximum cone sensitivities in the 550-570 nm and 600-640 nm regions (Bowmaker and Knowles, 1977; Bowmaker, 1977; Maier and Bowmaker, 1993). It is possible that these spectral regions have some significance to birds. For example, the chicken is a domesticated form of the jungle fowl, whose photic environment consists mainly of the dappled shade light of a forest canopy. Bowmaker and Knowles (1977) observed that the maximum sensitivity of the absorptance spectra of red (606 nm) and green (533 nm) cones in the chicken retina, corresponded to regions of the spectrum in which there was the greatest variation in reflected light from different types of vegetation (see their figure 10). The narrow-band cone sensitivities caused by the R- and Y-type droplets may

allow birds to discriminate between different colours of vegetation (Bowmaker and Knowles, 1977). However, R-type and Y-type oil droplets have also been well documented in sea birds, for example, whose photic environment would not be considered as consisting solely of the maximum irradiances from various plant matter (see below).

4.9. Distribution of oil droplets in the retina

Red and Yellow droplets often show a similar distribution across the retina. Birds which need to look down into water, such as the common tern, possess R- and Y-type droplets in the dorsal half of the retina [which views the ground] (Muntz, 1972), whereas the ventral retina (which views the sky) contains a higher proportion of P-type droplets (Goldsmith *et al.,* 1984). P-type and C-type droplets distributed ventrally are in a good position to effectively filter damaging short wavelengths of light from the sky (Kirschfeld, 1982) and eliminate the dazzling blue light of the sky, thus enhancing contrast and increasing visual acuity (Duke-Elder 1958).

4.10. Oil droplets in nocturnal and crepuscular birds

Since coloured oil droplet filters sacrifice sensitivity, it is not surprising that crepuscular and nocturnal birds such as the owl, and nightjar have very few cones with only pale yellow or Colourless droplets (Lythgoe, 1979). The wood thrush hunts in the understorey of dense woods and has to find food in low light levels. Therefore too much pigmentation in the oil droplets may be a disadvantage. This bird has been suggested to have T-type oil droplets in the dorsal (ground-viewing) retina enabling it to use as much of the available light as possible (Goldsmith *et al.,* 1984). The ventral retina contains Colourless droplets.

Birds that feed 'on the wing' such as swifts, swallows and martins may require greater contrast sensitivity to see their silhouetted prey against the sky. It is thought that the degree of contrast reduction of dark objects against the sky between droplet types is as follows; Red > Orange > Yellow > Pale > Colourless > Transparent. The low proportion of coloured oil droplets observed in the retinas of these birds is assumed to be related to the loss in contrast associated which would occur with long wave cut-off filters. Terrestrial birds have heavily pigmented P-, C- and T-type droplets which enhance the contrast of light objects seen against the sky (such as a white cloud), due to the selective reduction of shorter wavelengths making the sky itself appear darker. However, this would seem disadvantageous since objects, such as insects and other birds, which are darker than the sky, would not be seen as clearly (Muntz, 1972).

Aquatic birds have added problems associated with viewing objects through the water

surface. Lythgoe (1979) lists some of the reasons making this a difficult task. The uneven water surface distorts the image, glare from the sky reduces visual contrast and there are also brightness reductions due to the magnifying effect of the air water interface. Light welling up from the water is richest in short wavelength light so a visual system most sensitive to these wavelengths is best suited to viewing objects through the water surface. The *reduced* number of Red and Orange oil droplets in the retinas of birds which pursue fish under water (such as the razor bill, shag and shearwater) has been suggested to be an adaptation to increase sensitivity to shorter wavelengths of light. This may make them adept at seeing objects deep beneath the water surface (Muntz, 1972). In contrast, plunge divers such as Gulls and Terns which just feed from the water surface or a little below it have numerous Red and Orange droplets. Red droplets enhance the contrast between a white sea-bird and the sea surface. It has been suggested that this may be important in the 'scout and cluster' feeding strategy used by some sea birds which requires them to be able to see each other through the atmospheric haze (Lythgoe, 1979). Any mechanism which improves vision by cutting out scattered short wavelength light is useful. Supporting this view is the observation that birds which just live on the water, such as ducks, but do not fly above it (and therefore do not need to look through it) do not have this development of Red droplets (Muntz, 1972).

4.11. Variation of oil droplet complement and hormonal states

A very different approach is to investigate the role of the oil droplets in the behaviour of birds. Pézard (1931; 1957a; 1957b; 1964, as cited by Meyer, 1977) demonstrated that hormonal states affect not only colour preferences in chickens but also the frequency of the different types of droplets. His data suggested that capons (castrated male cocks) had fewer Red droplets, thereby being less sensitive to the red combs of other chickens, and are thus less aggressive. However, Dücker (1970, as cited by Meyer, 1977) and Mayr (1970, as cited by Meyer, 1977) tested Pézards results and were unable to show any correlation between colour preference and oil droplet frequency distribution.

To summarize, oil droplets share many functions with the yellow filters in the cornea, lens and macular of the vertebrate eye. These include; screening from the damaging effects of ultraviolet light (Kirschfeld, 1982), reducing chromatic aberration (Muntz, 1972), and improving contrast (Muntz, 1972). In addition to this oil droplets may act as microlenses, focusing light into the outer segment and could also be polarization analysers, (Young and Martin, 1984). Some authors have correlated the complement of oil droplets with the visual ecology of birds, (Muntz, 1972 and Lythgoe, 1979). A few general deductions can be made from these reviews: nocturnal birds have very few

coloured oil droplets; birds that catch insects on the wing have few Red and Orange droplets as do birds living on the water surface (e.g. the duck) whereas plunge divers and white sea-birds have many red and Orange droplets.

5. VISUAL PIGMENTS

Vision depends upon the detection of light. In order to be detected, light reflected from objects must first be absorbed. This is accomplished by the photosensitive visual pigment molecules embedded in the folded membranes of rods and cones. Visual pigments are members of the super-family of G protein coupled receptors which all have seven transmembrane segments joined by extra-cellular and intra-cellular loops. All receptors in this family function through the activation of a guanine nucleotide binding protein (G protein) and an effector enzyme which changes the levels of a second messenger in the cell cytoplasm (Saibil, 1990). All visual pigment molecules consist of a protein moiety, opsin, which is bound to a prosthetic group, retinal, the aldehyde of vitamin A (see later). Vitamin A exists in two forms, A1 and A2 (the latter containing an extra double bond in the cyclohexane ring). Consequently, visual pigments can be classified into two groups;

- Rhodopsins, based on the aldehyde of vitamin A1 (retinal) and found in all vertebrates and some invertebrates, and
- Porphyropsins, based on the aldehyde of vitamin A2 (3-dehydroretinal) and found in some teleosts, amphibians and reptiles.

(Insect visual pigments are sometimes based on another prosthetic group, 3-hydroxyretinal, and a cephalopod mollusc, the firefly squid, *Watasenia scintillans,* is the only animal known to possesses a visual pigment with a 4-hydroxyretinal chromophore, (Kito *et al.,* 1992)

Any opsin will form two spectrally distinct visual pigments when combined with either retinal or 3-dehydroretinal since the extra double bond in the cyclohexane ring of the latter causes a bathochromic shift to longer wavelengths (Bowmaker, 1991b).

5.0. The phototransduction pathway

The purpose of a visual pigment (rhodopsin/porphyropsin) is firstly, to signal the absorption of a photon of light (i.e. photoexcitation) and secondly, to amplify the signal via the activation of the G-protein, transducin. The following section will concentrate on phototransduction mechanisms in the rod photoreceptor since most of what is known about the phototransduction pathway has been obtained from studies focusing on rods. However, similar mechanisms are thought to occur in cones, the main differences being quantitative (see Farber, 1995 and Yau, 1994 for reviews).

All the enzymatic steps in phototransduction occur on the rod disc-membrane surfaces and in the adjacent thin sheets of cytoplasm. The plasma membrane of the rod cell contains cyclic GMP sensitive ion channels (Lamb and Pugh, 1990) and an electrogenic sodium-calcium ion exchanger (Cook and Kaupp, 1988) which set the receptor potential. The receptor potential is normally slightly depolarized in its resting state due to the constant inflow of cations. The photoactivated rhodopsin binds to transducin, catalysing the exchange of GDP (bound to the α-subunit of transducin) for GTP. Following nucleotide exchange, the activated transducin dissociates into two portions; a βγ-subunit and an α-subunit. The Tα-GTP subunit then activates the enzyme, phosphodiesterase, which hydrolyses cyclic GMP.

Cyclic GMP levels

In the dark, the cyclic GMP concentration in the cytoplasm of the rod cell is high, keeping the cyclic GMP sensitive cation channels open and allowing the continuous entry of Na^+ and Ca^{2+}. As this occurs, the ion-exchanger pumps, located in the rod cell plasma membrane, simultaneously extrude Ca^{2+} and K^+ in exchange for Na^+, keeping the Na^+ level high. However, once activated, the phosphodiesterase hydrolyses cyclic GMP, reducing its concentration in the cytoplasm. This causes the cyclic GMP sensitive cation channels to close, preventing the influx of cations. The resulting decrease in the level of cations, hyperpolarizes the rod cell. During this sequence of events, rhodopsin (whilst in its activated state) is free to activate other molecules of transducin, further amplifying the response.

Calcium levels

Following the closure of cation channels, the ion exchanger pump continues to expel Ca^{2+} which results in a fall in its intracellular concentration. The drop in the level of Ca^{2+} stimulates the enzyme, guanylate cyclase, which begins to resynthesise cyclic GMP, restoring it to the high concentrations required to reopen the cation channels and reestablish sensitivity.

The deactivation of rhodopsin is accomplished through phosphorylation by the enzyme, rhodopsin kinase, and the later binding of arrestin. The inherent GTP-ase activity of the α-subunit of transducin enables it to reassociate with the βγ-subunits and return to its resting state, while the phosphodiesterase returns to its inactive state.

5.1. Classification of vertebrate visual pigments

A visual pigment can be classified by looking at the degree of homology between the amino acid sequence of its opsin (or the nucleotide sequence of the opsin *gene)* and that of other opsins representing each of the different classes. Phylogenetic trees of visual pigments have been constructed by aligning several opsin sequences and using the amino acid identity as an approximation of evolutionary distance (Okano *et al.,* 1992; Johnson *et al.,* 1993; Hisatomi *et al.,* 1994; Yokoyama, 1994 and Chang *et al.,* 1995). For example, Okano *et al.* (1992) classified vertebrate visual pigments into four groups based on a comparison of the amino acid identities of 13 vertebrate opsins. These were;

longwave (L) pigments (including human red/green and chicken red opsins),
shortwave (S) pigments (including human blue and chicken violet opsins)
middlewave (M_1) pigments (consisting of the chicken blue opsin).
middlewave (M_2) pigments (including vertebrate rod opsins and chicken green opsin)

Yokoyama (1994) constructed a phylogenetic tree from comparisons between 26 vertebrate opsins, which divided vertebrate visual pigments into the following four groups.

RH1, consisting mainly of vertebrate rod opsins (460-530 nm)
RH2, consisting of green cone opsins (460-530 nm)
SWS, consisting of blue and violet cone opsins (350-470 nm)
LWS/MWS, consisting of longwave absorbing opsins (520-560 nm)

The apparent difference between this clustering of opsins and that shown by Okano *et al.* (1992) is merely due to the greater number of opsin sequences used to construct the tree (Yokoyama, 1994).

Throughout the following section, a combination of the notation used by Okano *et al.* (1992) and Yokoyama (1994) to describe opsin groups will be used. These are as follows [as presented in Bowmaker and Hunt, (1997)];

L = **Longwave absorbing opsins** (520-575 nm)
M_C = **Middlewave-absorbing cone opsin** (460-530 nm)
M_{Rd} = **Middlewave-absorbing rod opsin** (460-530 nm)
S_B = **Shortwave 'blue'-absorbing opsin** (430-470 nm)
S_V = **Shortwave 'violet'-absorbing opsin** (350-440 nm)

5.2. The structure of opsin

The opsin part of the visual pigment consists of a single polypeptide chain of 340-500 amino acids which threads its way through the photoreceptor membrane to form seven α helical transmembrane regions connected by alternating, cytoplasmic and extracellular straight chain loops (Dratz and Hargrave, 1983; Findlay and Pappin, 1986). The overall length of opsins differs due to variations in the lengths of the connecting loops and carboxy- and amino-terminal regions. Each helix is made up of 26 predominantly hydrophobic amino acids with only the 18 core amino acids lying within the membrane (Baldwin, 1993; Schertler *et al.,* 1993). In the tertiary structure the seven helices form a bundle within the membrane creating a hollow cavity on the extracellular side which forms the retinal binding pocket.

5.3. Features of opsin (using bovine rod opsin numbering)

- Lysine-296, located in the centre of helix VII in bovine rod opsin, is the site of attachment of 11-cis retinal (Bownds, 1967; Wang *et al.,* 1980). [see later]
- Glutamic acid-113, located towards the extracellular end of helix III is the counterion for the protonated Schiff's base (Sakmar *et al.,* 1989; Zhukovsky and Oprian, 1989; Nathans, 1990a). [see later]
- The amino-terminal sequence of opsin (exposed on the extracellular surface of the membrane, or inside the disk, in rods), contains one or two (in the case of some M_C and M_{Rd} opsins) Asp residues which are sites of glycosylation (Hargrave, 1982) which is important for anchoring the nascent polypeptide in the membrane.
- The carboxy-terminal region, (exposed on the cytoplasmic surface of the membrane) contains numerous Ser and Thr (hydroxyl) residues which are sites of phosphorylation by rhodopsin kinase (Hargrave, 1982; Thompson and Findlay, 1984).
- Two palmitoylated cysteines occur in the carboxyl terminal segment which are thought to anchor the tail into the lipid bilayer, forming a fourth cytoplasmic loop (Ovchinnikov *et al.,* 1988).
- Regions of the cytoplasmic connecting loops are thought to contain residues which interact directly with membrane-bound G-proteins (Hargrave, 1982; Applebury and Hargrave, 1986; König *et al.,* 1989; Franke *et al.,* 1992). For example, Glu-134 and Arg-135 (using the numbering according to bovine rod opsin) are thought to interact directly with the G-protein, transducin (Franke *et al.,* 1990).
- Correct folding of the opsin requires two cysteines which form a disulphide bridge; Cys-110 and Cys-187 are highly conserved and are located in the extracellular loops linking helices II & III, and IV & V, respectively (Karnik and

Khorana, 1990).

- Proline, is found in five of the seven transmembrane regions of bovine rhodopsin and is thought to produce a slight bend in the helices (since proline produces a 20° kink) which may form the walls of the retinal binding pocket (Dratz and Hargrave, 1983).

5.4. The Schiff's base linkage

Like typical aldehydes, retinal undergoes a condensation reaction with primary amines to form an 'aldimine' bond (aldehyde + imine), commonly known as a Schiff's base linkage. In visual pigments, retinal is covalently attached, via such a bond, to the ε-amino group of a Lysine in the seventh transmembrane helix of the opsin (Lys-296 according to bovine rod opsin numbering). The structure of retinal consists of a conjugated chain of single and double bonds which produce a shell of delocalised π electrons around the molecule. Changes in the degree of delocalisation of these electrons can vary the wavelength of maximal absorption of retinal. When unbound, both opsin and retinal absorb in the ultraviolet at about 300 and 380 nm respectively. However, when bound, the protonated form of the Schiff's base shifts the λ_{max} up to about 440 nm (Knowles and Dartnall, 1977). This may be due to increased delocalisation of the π electrons in the retinal molecule so that they occupy shallower potential wells and require less energy for excitation (Hubbard and Sperling, 1973). The additional variation in the λ_{max}s of visual pigments (in the range of 350-620 nm) is thought to arise from further interactions between retinal and (genetically determined) amino acid residues in its immediate vicinity. Charged residues in particular probably affect the conformational structure of retinal. Identification of these residues or 'spectral tuning sites', has been difficult since the detailed 3-dimensional crystallographic structure of a vertebrate pigment has yet to be elucidated. Most models are based on the 3-dimensional folding pattern of bacteriorhodopsin (an integral membrane protein from the archaebacterium, *Halobacterium halobium*).

5.5. The Schiff's base counterion, Glu-113

A negative counterion is required to stabilize the protonated Schiff's base since it would require much energy to maintain a positive charge in the hydrophobic retinal binding pocket (Applebury and Hargrave, 1986). Kropf and Hubbard (1958) originally suggested that rhodopsin in the photoexcited state could be stabilized by an electrostatic interaction between the negative charge from an amino acid side chain and the delocalised positive charge from the protonated Schiff's base. Honig *et al.* (1979) proposed an external point charge model in which two negative carboxylate residues interacted with the 11-*cis* -retinal; one carboxylate residue was thought to be the counterion for the protonated Schiff's base nitrogen while the other provided a point

charge positioned close to carbon atoms 12 and 14 of the retinal.

Subsequent site-directed mutagenesis and Raman spectroscopy studies on bovine rod opsin have shown that the counterion for the protonated Schiff's base is a single negatively charged Glutamic acid residue (Glu-113) lying towards the extracellular side of the third transmembrane helix, (Sakmar *et al.,* 1989; Zhukovsky and Oprian, 1989; Lin *et al.,* 1992). Substituting Glu-113 with an uncharged polar amino acid, Gln, causes a dramatic shift in the absorption maximum of bovine rod opsin from 500 nm to 380 nm. This can be interpreted as follows: replacement of the charged imine counterion with a neutral residue causes the Schiff's base nitrogen to remain unprotonated, resulting in the shift to 380 nm. However, this spectral shift is sensitive to pH and a red-shift back to 490 nm occurs at high pH when the Schiff's base becomes protonated due to the acidic environment. In this case, another carboxylate group in the opsin or possibly an anion from the solvent such as Cl^- may serve as the counterion for the acid protonated Schiff's base. Additionally Glu-113 may also catalyse hydrolysis of the Schiff's base in the photoactivated rhodopsin as shown by the slower rate of hydrolysis in the Gln-113 mutant. Mutating Glu-113 to Asp results in a slightly red-shifted pigment (from 500 to 505 nm, respectively) suggesting that electrostatic interactions occur between the counterion and retinylidene Schiff's base; Aspartic acid has a shorter side-chain and therefore increases the distance (and decreases the interaction) between counterion and Schiff's base imine. Hence, less of a charge effect is exerted on the protonated Schiff's base nitrogen, causing the slight red-shift. The two point charges originally postulated by Honig *et al.,* (1979) are the two oxygen atoms of the carboxylate anionic side chain of glutamic acid. These carbonyl oxygens stabilize the positive charge on the Schiff's base nitrogen.

Of the other carboxylic acid residues examined, Glu-122, also lying in the third transmembrane helix, appeared to contribute most to the opsin shift, a 20 nm shift to 480 nm occurring in mutants substituted with Gln (polar uncharged side chain). However, substituting Glu-122 with Ala (a non-polar uncharged side-chain) causes only a small 5 nm shift to 475 nm suggesting that the effect of Glu-122 on the absorption spectrum of rhodopsin may be steric in nature rather than providing a point charge or neutralizing the counterion (Sakmar *et al.,* 1989; Zhukovsky and Oprian, 1989). This site appears to be important in the spectral tuning of avian M_{Rd} pigments (see later).

5.6. Putative spectral tuning sites

5.7. L opsins

Chloride sensitivity

The spectral properties of several longwave sensitive cone pigments have been shown to be influenced by the binding of chloride ions (Cl^-). For example in Cl^- depleted media the green absorbing pigment (P521) of the Tokay gecko, *Gecko gecko* undergoes a blue shift to approximately 500 nm; upon addition of Cl^- ions, the pigment shifts back to it's native λ_{max} of 521 nm. This effect is not seen in the gecko blue pigment [P 467] (Crescitelli, 1977; Crescitelli and Karvaly, 1991). A similar chloride sensitivity is also seen in the chicken longwave sensitive pigment, iodopsin, which displays a shift in the λ_{max} to 520 nm in the absence of Cl^- ions but shifts back to the native λ_{max} of 565 nm upon addition of Cl^- ions (Knowles, 1976; Fager and Fager, 1979; Shichida *et al.,* 1990). Kleinschmidt and Harosi (1992) demonstrated Cl^- sensitivity in the longwave absorbing pigments of four species of fish and one amphibian *in situ,* under physiological conditions. This was in contrast to previous studies which had used detergent extracted pigments. The effects of several other anions were also tested; only Br^- was also able to induce a red-shift back to the native λ_{max} of the pigment. The addition of lyotropic ions (salts possessing strong dehydrating properties) to the chloride depleted media resulted in blue shifts of the pigment λ_{max} relative to the Cl^- depleted state. On the basis of their results the authors suggested that anion sensitivity is a common feature of all vertebrate longwave pigments. Wang *et al.,* (1993) demonstrated similar chloride ion induced shifts in the human red and green pigments. Using site-directed mutagenesis they were able to identify two positively charged amino acid residues, (using human longwave opsin numbering) His-197 and Lys-200, [or His-181 and Lys-184 according to bovine rod opsin numbering] which participate in the chloride binding site. These residues are conserved in all vertebrate longwave absorbing pigments but are notably absent from rod opsins and shortwave cone opsins which are instead substituted with the residues Glu-197 and Gln-200 and consequently do not bind Cl^- ions. Of the two residues, His-197 is thought be dominant in producing the Cl^- dependent shift. Both residues are thought to be located on the extracellular side of the membrane in the loop connecting transmembrane helices IV and V. This is close to Cys-203 [Cys-187] which forms the disulphide bond with Cys-126 [Cys-110] which is essential for the correct folding of the opsin (Karnik and Khorana, 1990). Since this disulphide linkage lies towards the end of helix III, housing Glu-129 [Glu-113], the Schiff's base counterion, the chloride binding site may exert an indirect effect on the chromophore (Wang *et al.,* 1993).

The chloride binding site can only account for 15-25 nm of the 'red' shift in longwave pigments (Wang *et al.,* 1993) implying that there are other important residues which are responsible for the remaining shift in λ_{max}. The human long- and middlewave opsin genes have been cloned and their nucleotide sequences are very similar showing 98% identity (Nathans *et al.,* 1986). Both genes consist of six exons (exons 1 and 6 are identical) and from the predicted translation, their respective opsins have been shown to differ at only 15 sites out of a possible 364 amino acids. Surprisingly, none of the 15 differences involve charged residues although seven of them are non-homologous substitutions (i.e. involve the introduction or removal of a hydroxyl group). Some or all of the 15 differences between the human red and green opsin sequences may account for the rest of the 'red' shift and explain the 31 nm difference between the two pigment λ_{max}s.

By comparing gene sequences from New and Old World primates, Neitz *et al.* (1991) proposed that the variation in spectral sensitivity of cone pigments in the MW to LW regions could be explained by the substitution of a single non-polar amino acid for an hydroxyl bearing amino acid at just three sites (using numbering of L opsins), 180, 277 and 285. It was suggested that the three substitutions were linearly additive, with (green to red) Ala-180 to Ser (Exon 3), Phe-277 to Tyr (Exon 5) and Ala-285 to Thr (Exon 5) red-shifting the peak by 6, 9 and 15 nm respectively. These results agree with the findings of Chan *et al.* (1992) from a study of site directed mutants in bovine rhodopsin (λ_{max} = 500 nm). Although bovine rod opsin is shorter than the human red/green genes by 16 amino acids (due to the extra exon in the red and green genes), it contains the same substitutions that are found in the human green opsin, Ala-164, Phe-261 and Ala-269 (using bovine rod opsin numbering). When these residues are substituted with the corresponding hydroxyl bearing residues from the red pigment, Ser-164, Tyr-261 and Thr-269, red shifts of 2, 10 and 14 nm from the native λ_{max} are observed. These three polar amino acid substitutions are present in the chloride sensitive L opsins of other non-mammalian vertebrates including the P563 of the blind cave fish, *Astyanax fasciatus,* (Yokoyama and Yokoyama, 1990; Yokoyama, 1995), the P571 of the chicken, *Gallus gallus,* (Kuwata *et al.,* 1990), the P570 of the goldfish, *Carassius auratus,* (Johnson *et al,* 1993) and the P561 of the chameleon, *Anolis carolinensis,* (Kawamura and Yokoyama, 1993), indicating that these sites are highly conserved.

In their extensive study using chimaeric opsins and single and multiple point mutations, Asenjo *et al.* (1994) demonstrated that a total of seven amino acids were responsible for the entire 31 nm difference in the absorption spectra of the human red and green opsins. In addition to previously identified residues at sites 180, 277 and 285, amino acid

residues 116, 230, 233 and 309 were also implicated in the spectral shift (see table 5.7a). These findings are in agreement with several other studies (Winderickx *et al.,* 1992; Deeb *et al.,* 1992; Merbs and Nathans, 1993; Williams *et al.,* 1992; Merbs and Nathans, 1992). Position 116 is thought to exert an indirect effect on the chromophore due to its extra-membrane location, in the loop connecting transmembrane segments II and III. From other non-human primate species so far examined it appears that, at site 233, pigments with λ_{max} above 560 nm possess a non-hydroxyl bearing amino acid (Ala) whereas pigments with λ_{max} below 540 contain a hydroxyl bearing amino acid (Ser/Thr) (Ibbotson *et al.,* 1992; Williams *et al.,* 1992). A similar situation exists in the *longer* -wave pigment(s) of some New World monkeys and also the blind cave fish, *Astyanax fasciatus* (Yokoyama and Yokoyama, 1990; Neitz *et al.,* 1991).

Table 5.7a
Amino acids identified by Asenjo *et al.* (1994) as being involved in the 31 nm difference in the absorption spectra of the human red and green opsins.

no. in bov. rod	no. in hum LWS/ MWS	Residue found in L_G opsin, λ_{max} = 532 nm	Residue found in L_R opsin, λ_{max} = 563 nm	Change? polar/ non- polar
100	116	Tyr	Ser	hom
164	180	Ala	Ser	+ OH
214	230	Thr	Ile	- OH
217	233	Ser	Ala	- OH
261	277	Phe	Tyr	+ OH
269	285	Ala	Thr	+ OH
293	309	Phe	Tyr	+ OH

5.8. M_{RD} and M_C opsins

Generally speaking the M_{Rd} and M_C opsins of non-mammalian vertebrates share a 70% (amino acid) sequence homology compared to a 40% homology shared with the L and S opsins. Detailed microspectrophotometric analyses of the retinas of birds suggests that the λ_{max}s of rods and green cones are very similar [± 1 nm], (Bowmaker *et al.,* 1997; Maier and Bowmaker, 1993; Bowmaker *et al.,* 1993; Bowmaker and Martin, 1985). For example, the chicken green and rod visual pigments absorb maximally at 507 and 506 nm, respectively (Bowmaker *et al.,* 1997). This is reflected in the high homology between the amino acid sequences of the two opsins (Wang *et al.,* 1992; Okano *et al.,* 1992). One of the few amino acid differences is the charged, Glu-122, residue in chicken rod opsin which is replaced by an uncharged, Gln, in chicken green cone opsin and may be important in spectral tuning. As mentioned before, site-directed mutagenesis studies in bovine rod opsin have shown that an identical Glu-122 to Gln substitution causes a 20 nm blue shift from the native λ_{max} of 500 nm (Sakmar *et al.,* 1989; Zhukovsky and Oprian, 1989; Nathans, 1990a). However, the two green opsins from an A_2 species, the goldfish, *Carassius auratus,* absorb maximally at 505 nm and 511 nm (when reconstituted with the A_1 chromophore) but despite showing strong homology to the amino acid sequence of the chicken green opsin, do not display the Gln-122 substitution (Johnson *et al.,* 1993). In contrast, the blue-shifted P467, in the retina of the Tokay gecko, *Gekko gekko* , (which is actually found in rod-like cells, Crescitelli *et al.,* 1977) also shows strong homology to the amino acid sequence of the chicken green opsin and does have the Gln-122 substitution (Kojima, 1992). Therefore it seems that site 122 may not be significant in the tuning of all M_C and M_{Rd} pigments, although it may be important in birds. Hunt *et al.* (1996) have identified several possible tuning sites within rod opsins, based on the results of an extensive study of the rod pigments of cottoid fish. Three of these potential sites, positions 83, 261 and 292, face the retinal binding pocket, and may be important in spectral tuning.

5.9. S_B AND S_V OPSINS

Using a comparative phylogenetic approach, Chang *et al.* (1995) have identified four putative amino acid replacements thought to be involved in the tuning of shortwave (S_B and S_V) pigments. These residues represent amino acid changes that have been accepted by organisms and occur naturally. These sites, corresponding to positions 124, 289, 292, and 307 (using bovine rod opsin numbering) represent non-polar to polar amino acid changes, and lie close to the protonated Schiff's base end of the chromophore see table 5.9a.

Table 5.9a
Putative amino acid replacements thought to be involved in the spectral tuning of shortwave (S_B and S_V) pigments (Chang *et al.*, 1995).

no. in Bov. rod	no. in LWS cone	Helix	L	M_C/ M_{Rd}	S_B	S_V 420 nm	S_V 415 nm	Change
124	140	III	Gly	Ala	**Ser**	**Thr**	**Thr**	**+OH**
289	305	VII	Ala	Ala/Thr	**Ser**	**Thr**	**Thr**	**+OH**
292	308	VII	Ala	Ala	**Ser**	**Ser**	**Ala**	**+OH**
307	323	VII	Val	Val/Ile	**Val**	**Cys**	**Cys**	-

L = Longwave absorbing opsin
M_C = Middlewave-absorbing cone opsin
M_{Rd} = Middlewave-absorbing rod opsin
S_B = Shortwave 'blue'-absorbing opsin
S_V = Shortwave 'violet'-absorbing opsin

Positions 289 and 307, located in helix VII tend to be substituted with non-polar residues, Ala-289 and Val-307 in vertebrate L opsins, but polar residues Thr and Cys respectively, in S_V opsins (see table 5.9a). At site 292, most vertebrate opsins tend to possess a non-polar Ala, the exception being the shortwave 'blue' opsins which possess a polar, Ser (Johnson *et al.,* 1993). A non-polar Ala/Gly at site 124 in helix III is present in all vertebrate opsins except the shortwave 'violet' opsins in which it is replaced by a polar Thr residue.

Chang *et al.* (1995) use proton, rather than electron, movements to explain differences in λ_{max}. They suggest that in S_B and S_V opsins, polar residues are concentrated around the protonated Schiff's base end of retinal keeping the positive charge fixed over the Schiff's base nitrogen. It is suggested that this preferentially stabilizes the ground state of the molecule (over the excited state), increasing the energy required for photon absorption. By way of contrast, L opsins tend to have polar residues placed along the whole length of the conjugated chain of the chromophore (Chan *et al.,* 1992). As a result, the positive charge may be drawn towards the β-ionone ring, stabilizing the excited state over the ground state and reducing energy required for excitation .

Yokoyama (1994) based on the results of another evolutionary analysis of opsin sequences identified other putative sites involved in the tuning of the S_B and S_V pigments as summarized in table 5.9b. From table 5.9b the homologous substitutions at sites (using bovine rod opsin numbering) 247 (Glu to Asp) and 325 (Lys to Arg) are only found in the chicken 455 nm absorbing, S_B opsin but are not within transmembrane regions. Amino acid replacements at sites 125 (Leu to Gly), 156 (Gly to Val) and 265 (Trp to Tyr) are all located within transmembrane regions and are present in both members of the S_V group of opsins (the human 420 nm, and the chicken 415 nm absorbing opsins). The substitutions at positions 67 (Lys to Arg), 114 (Gly to Ala) and 202 (Ser to Tyr) are all homologous but are only found in the chicken 415 nm absorbing S_V opsin. Although these three positions are not located within the transmembrane helices, sites 114 and 202 are only one residue away from helices III and V respectively, and site 67 three residues away from helix II.

In a later paper, again using a phylogenetic approach, Yokoyama (1995) suggested a total of 55 polymorphic amino acids which could be involved in blue-shifting the wavelength of maximum absorption. Forty of these residues were located within or near transmembrane regions. Amino acid replacements located within the transmembrane helices and involving polar to non-polar changes have been presented in table 5.9c. Of these five candidates, residue 90 lies in close proximity to retinal and the Schiff's-base linkage. Trp-265 in bovine rod opsin has been shown experimentally to

cause a 15 nm blue shift when substituted with Tyr (Nakayama and Khorana, 1991) and lies between sites 261 and 269 which are involved in tuning the longwave-absorbing opsins. Site 224 faces away from the retinal binding pocket and is probably the least likely candidate.

Neither of the studies mentioned above have included the mouse or bovine blue cone opsin sequences.

Table 5.9b.
Putative spectral tuning sites suggested by Yokoyama (1994), to be involved in the tuning of the S_B and S_V pigments.

no. in Bov. rod	no. in LWS cone	Helix	L	M_C/ M_{Rd}	**S_B 455 nm** chicken	**Sv 420 nm** human	**Sv 415 nm** chicken	Change
247	263	-	Glu	Glu	**Asp**	Glu	Glu	-
325	341	-	Lys	Lys	**Arg**	Lys	Lys	-
125	141	III	Leu	Leu	Leu	**Gly**	**Gly**	-
156	172	IV	Gly	Gly	Gly	**Val**	**Val**	-
265	281	VI	Trp	Trp	Trp	**Tyr**	**Tyr**	**+OH**
67	83	-	Lys	Lys	Lys	Lys	**Arg**	-
114	130	-	Gly	Gly	Gly	Gly	**Ala**	-
202	218	-	Ser	Ser	Ser	Ser	**Tyr**	-

Table 5.9c
Amino acid replacements, located within the transmembrane helices and involving polar to non-polar changes suggested by Yokoyama, (1995).

no. in Bov. rod	no. in LWS cone	Helix	L	M_C/ M_{Rd}	S_B	S_V	Change polar / non-pol?
90	106	II	Ala	Gly	Gly	Ser	**+OH**
167	183	IV	Trp	Cys	Ala	Val	-
204	220	V	Met	Val	Val	Thr	**+OH**
224	240	V	Leu/Ile	Gly	Gly	Thr/ser	**+OH**
265	281	VI	Trp	Trp	Trp	Tyr	**+OH**

Testable hypotheses and Aims of the project

The complement of cones and rods in the retina of a bird can be studied, using the technique of MSP. The photoreceptors can be classified in terms of the wavelength values at which the visual pigments (in their respective outer segments) absorb. In cones, the effect of the oil droplet cut-off filter on the overall sensitivity of the adjacent visual pigment can also be calculated. Such data from microspectrophotometric studies may give us an indication of the potential spectral sensitivity and wavelength discrimination of the species.

Many birds have at least four spectrally distinct cone visual pigments which are associated with coloured oil droplets acting as selective cut-off filters. Although long-, middle- and shortwave sensitive cones have been documented in most of the avian species examined to date, the precise spectral location of the fourth class of cones can vary, with species possessing either a 'violet' or 'ultraviolet' sensitive cone class.

The canary (serin finch), *Serinus canaria,* is a member of the Order Passeriformes. Although the singing capability of male canaries has been studied extensively, no data are available regarding the colour vision of this species. Previous MSP studies on other passeriformes suggest that, in addition to long-, middle- and short- wave absorbing cone photoreceptors, birds from this Order possess a class of *ultra* violet ($\lambda_{max} < 370$ nm, as opposed to violet; $\lambda_{max} \approx 400$ nm) absorbing cones. It is therefore likely that the canary also possesses an ultraviolet class of cones.

If the canary possesses an ultraviolet sensitive class of cones it is possible to deduce, at the amino acid level, the underlying mechanism responsible for ultraviolet sensitivity. This can be achieved by isolating retinal cDNA and amplifying the ultraviolet opsin gene using degenerate primers designed from the chicken violet sequence. The resulting cDNA sequence can be used to deduced the amino acid sequence of the opsin by doing a simple translation. The predicted ultraviolet opsin sequence thus obtained can then be aligned to other published opsin sequences in order to construct a phylogenetic tree, and identify putative ultraviolet specific spectral tuning sites.

The bright colours of oil droplets in bird and turtle retina are due the high concentrations of oil droplet carotenoid(s). Canaries have long been noted for their bright yellow feather colouring (although many other coloured varieties are found) and this in part, is related to the carotenoid concentration in feathers. It may be interesting to investigate whether canaries with bright-yellow feather colouring have a higher oil

droplet carotenoid concentration than non-yellow canaries with lower feather carotenoid content. This question can be addressed by surveying different inbred genetic strains of canary containing varying degrees of carotenoid in their feathers.

AIMS OF THE PROJECT:

To determine the following:

- the complement of photoreceptors in the canary retina.
- whether the canary possess an ultraviolet sensitive class of cones containing a typical T-type oil droplet.
- (if the canary has an ultraviolet sensitive cone pigment) which amino acid residues in the opsin protein confer ultraviolet sensitivity.
- the phylogeny of the canary ultraviolet sensitive opsin
- whether clear (yellow), variegated and heavily variegated canaries have progressively higher carotenoid concentrations in their retinal oil droplets.

6. METHODS AND MATERIALS

MICROSPECTROPHOTOMETRY

6.0. Experimental animals

In total, twenty-five male canaries were purchased commercially. The three inbred genetic strains of canary which were used were as follows; 'clear yellow birds' (clear meaning the feathers totally devoid of any melanin pigmentation), 'variegated birds' (feathers showing light melanistic pigmentation) and 'heavily variegated birds' (birds with heavy melanistic pigmentation, usually brown in colour). Examples of these three strains are shown in figures 6.0a, 6.0b, and 6.0c. All birds were maintained on a 12 hour light/dark ratio and a standard diet until required in experiments.

6.1. Microspectrophotometry: Preparation of tissue

Birds were dark adapted for at least 1 hour prior to being sacrificed. All subsequent procedures were carried out under dark red illumination (Kodak safelight No. 2) to protect the tissue from bleaching light. Birds were decapitated and both eyes enucleated. One eye was wrapped in foil and stored until the following day in a light-tight container at 4° C. The other eye was hemisected by cutting along the circular ridge of the scleral-ossicular juncture. The anterior half of the eye and the vitreous were discarded and the resulting eye cup placed in a solution of chilled calcium-free avian Ringer's (pH 7.1). Using the pecten as a guide, the eye cup was cut into quadrants corresponding to the retinal areas; ventro-nasal, ventro-temporal, dorso-nasal, and dorso-temporal . Each quadrant was separately placed in an appropriately labelled petri dish of calcium free Avian Ringer's Solution (154 mM NaCl; 5 mM KCl; 17.5 mM $Na_2HPO_4.2H_2O$; 7.5 mM $NaH_2PO_4.2H_2O$; 10 mM glucose)

For each preparation a small piece of retina (approximately $1mm^2$) was cut out and lifted onto a large coverslip. The tissue was gently teased out and dispersed using a pair of razor blades, the object being to create a definite 'edge' of outer segments when later viewed under the microscope. A drop of Ringer's solution containing 5% dextran was added to the tissue which was then sealed under a second smaller coverslip. The dextran increases the viscosity of the solution, thus hindering tissue movement without affecting the osmotic balance of cells. Excess solution was blotted from the coverslip before sealing the edges with a film of paraffin wax to prevent evaporation and associated problems of cell mobility. The preparation was secured in a slide holder and placed on the microscope stage. Measurements began approximately 30 minutes post mortem.

Fig 6.0a

A Clear Yellow Buff 'Border' Canary.
(from Walker and Avon, 1993).

Fig 6.0b

A Variegated 'Irish Fancy' Canary.
(from Walker and Avon, 1993).

Fig 6.0c

A Heavily Variegated Buff 'Gloster Consort' Canary.
(from Walker and Avon, 1993).

6.2. Microspectrophotometric measurements

A modified Liebman dual beam microspectrophotometer under computer control (Liebman and Entine, 1964; Knowles and Dartnall, 1977, pp. 562-566; Mollon *et al.*, 1984; Bowmaker *et al.*, 1991) was used to make measurements from photoreceptors. The retinal preparation was viewed under infrared illumination with the aid of an infra-red image converter. Due to the very small diameter of the cone outer segments (less than 2μm) the sample and reference beams were adjusted to approximately 2μm in square cross section. The beams were passed transversely through cells and polarized so that the *e*- vector of the sample beam was perpendicular to the long axis of the outer segment. This was done to take advantage of the dichroic ratio of the visual pigment, maximizing the proportion of light absorbed. In all instances measurements were only made from isolated intact cones where both outer and inner segments, and the oil droplet were clearly distinguishable.

Absorbance measurements

The microscope stage was rotated until the structure to be measured (usually a receptor cell outer segment or oil droplet) was superimposed on the sample beam while the reference beam passed through an adjacent tissue-free area of the preparation. Once cells were satisfactorily aligned, the condenser focus was slightly adjusted to correct its focus for longitudinal chromatic aberration in the infra-red. A consistent adjustment was achieved by fractionally defocusing the beams (with the pellicle removed) before each measurement. The background illumination was extinguished and the monochromator of the microspectrophotometer driven to 750 nm by a stepping motor. The beams were directed towards the photomultiplier and the electronics switched on. The infra-red filter was removed and once the feedback circuit began to regulate and the voltage had settled down to approximately -5.0 V (equivalent to 100% transmission), a scan was executed. Thus, no bleaching light reached the photoreceptors until the moment of measurement. During the scan, the monochromator descended from 750 nm to 370 nm in 2 nm steps, immediately returning to long-wavelengths on the interleaved odd number wavelengths. Transmission data were automatically converted to absorbance (optical density) and displayed on the VDU as plots of absorbance (optical density) Vs wavelength. Individual records were stored on disk for later analysis. After each scan, the cell was checked to make sure it had not drifted out of the beam during measurement.

In order to keep the effects of bleaching minimal, only one absorbance spectrum was usually taken from each outer segment, except in the case of SWS and UVS cones where bleaching is less of a problem. In these instances, measurements were repeated three times (equivalent to six spectra, after subtraction of three baselines), in order to

reduce the unavoidable noise at short wavelengths and obtain a clearer cumulative signal after averaging. Following each measurement of the absorbance spectrum of an outer segment, a second estimate of the baseline absorbance spectrum was obtained by placing the beams in a nearby area, free of cellular material. Putative outer segments were bleached by exposure to white light to verify the presence of photolabile pigment, the length of exposure varying with the cell type and pigment λ_{max}. The exposure times for rod and cone outer segments were as follows: 2 mins for rods (λ_{max}, 500 nm) and LWS cones (λ_{max}, 562 nm), 3 mins, 5 mins and 10 mins for MWS (λ_{max}, 502 nm), SWS (λ_{max}, 449 nm) and UVS (λ_{max}, 419 nm) cones, respectively. The longer exposure time required for SWS and UVS cones is partly due to the tungsten light source of the monochromator which progressively emits less light at shorter wavelengths. Bleached outer segments were subjected to the same number of measurements as the intact visual pigment to equalize the weighting of data during subsequent averaging and creation of a difference spectrum.

6.3. Observing the retina in the light

Once a retinal preparation had been exhausted of all measurable outer segments the background light was switched on (thus bleaching all pigments) in order to observe the oil droplets. Under such illumination, it was possible to distinguish the dense R- and Y-type, droplets which appeared red and orange-brown in colour, respectively. The P-, C- and T-type droplets were impossible to distinguish by colour, although the P-type droplets could sometimes be identified by their relatively large size.

6.4. Analysis of individual records

A standardized computer program was used to estimate the wavelength of maximum absorbance (λ_{max}) for each outer segment. First, the two absorbance spectra from a cell were averaged. Then a 2 nm averaged curve of the outward and return traces was created and the resulting points converted to 'relative absorbance' by re-expressing them as percentages of the peak absorbance (the latter being taken as the mean of seven points centered around the highest individual point on the curve). Twenty individual points on the long-wavelength limb of the relative absorbance curve (corresponding to a 40 nm portion of the original trace with relative absorbance values ranging from 45-90%) were referred to a standard template curve to obtain an estimate the λ_{max}. Each of these 20 points had its own unique % absorbance which was matched to the same % absorbance value on the template curve. The distance in frequency (expressed as wavenumber) between the spectral location of a particular % absorbance value on the template curve and its λ_{max} should be the same as the distance between equivalent points in the sample absorbance record. After adding the difference to each point, 20 different estimates of the λ_{max} were obtained and the average of these was then taken

to be the λ_{max} of the recorded absorbance spectrum.

A further estimate of the λ_{max} was obtained by fitting 25 consecutive points from either side of the highest point in the absorbance curve to the template curve and averaging the resulting estimates. Of the two methods, the right-hand limb estimate is the more reliable for several reasons such as its rapidly changing slope which makes noise less of a problem. It is also less likely to suffer from the effects of distortion due to photoproduct formation (MacNichol *et al.,* 1987), or from wavelength-dependent light scattering, both of which are greater at short-wavelengths.

It has been found that as the λ_{max}s of visual pigments become shorter there is an associated narrowing of bandwidth of the absorbance curve making it necessary to employ distinct templates to analyse the different classes of photoreceptor. Several investigators have suggested different ways of presenting absorbance data to alleviate this problem. For instance, Barlow (1982) suggests that the absorbance spectra of different classes of photoreceptor have approximately the same shape when plotted against the forth root of the wavelength. To this end, Mansfield (1985) has similarly suggested plotting absorbance curves on a scale of F/F_{max}, where F is the frequency at a particular point and F_{max} is the frequency at which maximum absorbance occurs for a visual pigment. The template curve which was used for analysis was Dartnall's standard spectrum for rhodopsin (Knowles & Dartnall, 1977, p.76) placed with its λ_{max} at 502 nm and expressed on an abscissal scale of log frequency. When expressed on such an abscissa most absorbance curves of visual pigments have almost the same shape (Mansfield, 1985; Bowmaker *et al.,* 1991) except at short wavelengths when the relationship breaks down (Hárosi, 1994). Therefore, for UVS pigments, the Hárosi template was used, with a half band width of roughly 5000 cm^{-1}.

The size of cone outer segments often varied with the spectral class of receptor, (the LWS cones being larger than the MWS cones which were larger than the SWS and UVS cones) and associated differences in the quality of records were seen. Normally, only the records that passed rigid selection criteria were selected for detailed analysis, resulting in the disposal of approximately 50% of records. The different selection criteria normally employed for the spectral classes of photoreceptor are shown in table 6.4a.

Table 6.4a.
The different selection criteria employed for the spectral classes of photoreceptor.

	Standard deviation from right hand limb estimate	Difference between two λ_{max} estimates	% shortwave absorbance	Transverse density at the λ_{max}
LWS cones	< 7 nm	< 5 nm	10-50	> 0.010
MWS cones	< 7 nm	< 5 nm	20-70	> 0.010
SWS cones	< 15 nm	< 10 nm	-	> 0.005
Rods	< 5 nm	< 5 nm	10-50	> 0.016

The UVS cones were scarce and often had very small outer segments (approximately 1μm in diameter) which made recording from them difficult. Additionally, the microspectrophotometer was only sensitive down to 360 nm with a low signal to noise ratio at short-wavelengths. Due to these problems, none of the above selection criteria were imposed on UVS cone records. Instead, putative UVS outer segments were identified by a rise in absorbance at wavelengths below 420 nm which could be reduced (i.e. bleached) by a 10 minute exposure to white light, and the presence of a transparent oil droplet in the inner segment.

6.5. Analysis of oil droplet spectra

Due to the small diameter (2-4 μm) of oil droplets in avian and reptilian cones, light leakage occurs around the droplet edges (Liebman and Granda, 1975; Goldsmith *et al.*, 1984). Coupled with the high carotenoid concentrations found in oil droplets, this light leakage leads to characteristically 'flat-topped' oil droplet spectra. The oil droplets were classified according to their 'cut-off' wavelength (λ_{cut}) calculated by the method suggested by Lipetz (1984).

Measurement of the canary Lens

The Canary lens was measured by Dr. Ron Douglas at City University. The left and right lens of a canary were removed and immediately scanned (250-700 nm) using a Shimadzu UV-240 recording spectrophotometer coupled to an integrating sphere (for details, see Douglas and McGuigan, 1989).

METHODS AND MATERIALS

MOLECULAR GENETICS

The initial goal of this part of the project was to clone and sequence the UVS, SWS, MWS, LWS and rod opsin genes from *Serinus canaria,* using a PCR-based approach.

6.6. Isolation of retinal cDNA

In order to PCR amplify just the coding regions of canary opsin genes it was necessary to isolate canary cDNA. Messenger RNA was prepared from canary retinae followed by first-strand cDNA synthesis using the 3' RACE kit. PCR amplification was then performed on the isolated cDNA using primers specific to the target opsin gene.

6.7. Isolation of messenger RNA

The QuickPrep *Micro* mRNA Purification Kit (27-9255-01: Pharmacia P-L Biochemicals) was used for mRNA extractions. A brief summary of the method is given below:

6.8. Extraction of tissue:

Freshly excised canary retinae were homogenized in 1.2 ml of Extraction Buffer until a uniform suspension was obtained. 2.4 ml of Elution Buffer was added to the extract and briefly homogenized before centrifuging at top speed for 1 minute. The supernatant, consisting of a cleared cellular homogenate, was transferred to a microcentrifuge tube containing pelleted Oligo(dT)-Cellulose.

6.9. Selection for mRNA using Oligo(dT)-Cellulose chromatography:

The Oligo(dT)-Cellulose was resuspended in the extract by inverting the tube manually for 3 minutes. The tube was briefly centrifuged for 10 seconds and the supernatant discarded. The pelleted material was washed sequentially with five 1 ml aliquots of High-Salt Buffer and Low-Salt Buffer. After the fifth wash with Low-Salt Buffer the Oligo(dT)-Cellulose pellet was resuspended in 0.3 ml of Low-Salt Buffer and the resulting slurry transferred to a MicroSpin Column within a microcentrifuge tube. The column was washed with three 0.5 ml aliquots of Low-Salt Buffer, with a 5 second centrifugation between each addition of buffer. The polyadenylated mRNA (poly $(A)^+$ mRNA) was eluted with two applications of 0.2 ml of pre-heated (65°C) Elution Buffer.

6.10. Precipitation of poly $(A)^+$ RNA:

µl of Glycogen Solution and 40 µl K Acetate Solution were added to the 400 µl of

sample. 1 ml of 95% ethanol (chilled to -20°C) was added to the sample before placing at -80°C for 30 minutes. The precipitated mRNA was pelleted by centrifuging the tube at 4°C for 5 minutes. The supernatant was decanted and the precipitated RNA redissolved in 40 µl of DEPC-treated water.

6.11. Quantitation of poly $(A)^+$ mRNA:

The concentration of mRNA was determined using the following formula:

Concentration of RNA = A_{260}/A_{280} X 40 µg/ml

A typical yield of poly $(A)^+$ RNA was approximately 100 µg/ml.

6.12. First strand cDNA synthesis from poly $(A)^+$ RNA

First strand cDNA was synthesized from poly $(A)^+$ RNA using the 3' RACE system for Rapid Amplification of cDNA Ends (18373-019: GIBCO BRL). Briefly, 1 µg of poly $(A)^+$ RNA, in 13 µl of DEPC-treated water, was added to 1 µl of Adapter Primer (10 µM) in a 0.5 ml microcentrifuge tube. The solution was mixed and heated to 65°C for 10 minutes before chilling for 2 minutes on ice. 2 µl of 10X synthesis buffer, 1 µl of 10 mM dNTP mix and 2 µl of 0.1 M DTT were added and the mixture equilibrated to 42°C for 2 minutes. 1 µl of SUPERSCRIPT Reverse Transcriptase was added to the mixture and the tube incubated at 42°C for 30 minutes. The reaction was collected by brief centrifugation and chilled on ice before adding 1 µl of RNase H. The tube was mixed and incubated for 10 minutes at 42°C. The cDNA was stored at -20°C.

6.13. Amplification of the target cDNA using the Polymerase Chain Reaction (PCR).

Fragments of opsin genes from the isolated retinal cDNA were amplified using the Polymerase Chain Reaction (PCR) method (see Saiki *et al.* 1985; Innis and Gelfand, 1990 for reviews).

1µl of a 1 in 10 dilution of first strand cDNA was used as the template in a typical 25 µl PCR mix, comprising the following components:

Reaction component	µl
NH_4 buffer	2.5
DNTP	1
forward primer	1
reverse primer	1
Taq DNA Polymerase	1
$MgCl_2$	1.5
H_2O	16
cDNA / gDNA	1

Briefly, the reaction components (except the enzyme) were heated in a tube to 94°C and then allowed to cool so that the primers hybridized to the single stranded DNA, which was consequently 'primed'. The pair of oligonucleotide primers were designed from nucleotide sequence flanking a particular region of the target gene; the forward and reverse primers hybridize to opposite strands of the DNA and are oriented so that PCR amplification is directed across the region between the two primers. To begin PCR amplification the thermostable enzyme, *Taq* DNA Polymerase, was added to the 'primed' target DNA. Primer extension was carried out at 72°C, effectively doubling the amount of target DNA. Following repeated cycles of denaturation, priming and extension, the target DNA underwent an exponential amplification. In order to allow for possible *Taq* polymerase incorporational errors during DNA synthesis (approximately 1 error per 1000 bases), individual PCRs and subsequent cloning were performed in triplicate. Therefore, the final sequence of an individual fragment was determined from the best of three consensus sequences from three independent PCR amplifications.

6.14. OPTIMIZATION OF PCRs :

Magnesium concentration

Magnesium titrations were carried out with new sets of primers to establish its optimal concentration since the Mg^{2+} concentration can affect the enzyme fidelity and activity, primer-template specificity, and strand dissociation temperature.

The DNTPs

A pre-made dNTP mixture containing each nucleotide (dATP, dCTP, dGTP and dTTP) at a concentration of 2.5 mM was used for PCRs.

Primers

A final concentration of 12.5 μM of each primer was typically used in PCR reactions since higher concentrations are likely to favour mispriming and non-specific bands. Several pairs of primers were used to direct PCR amplification from canary cDNA/gDNA. Degenerate primers were initially derived from a comparison of the conserved regions of published opsin gene sequences from chicken, human, and/or goldfish (Okano *et al* , 1992; Johnson, 1993; Nathans, 1986). However, once canary sequence had been obtained, it was possible to design species-specific primers. When designing new primers a few general rules were followed. These were; to avoid palindrome sequences, runs of more than three G's or C's - since this could promote mispriming at G+C rich regions, mismatches at or near the 3' end - since this is where the polymerase binds (although slight mismatches at the 5' end are tolerated), and complementary at the 3' end since this could promote the formation of primer-dimers. Most primers were designed to be between 18 to 20 bases in length, and to have a GC nucleotide content of 50-60%. See table 6.14a for a list of the primers used in PCRs. Where sufficient homology existed between the nucleotide sequences of opsin genes, primers designed from two different templates were sometimes combined, e.g. Bg 295+, a MWS primer, when paired with bl 599-, a SWS primer amplified a fragment of the LWS opsin gene! - see later in results.

Thermo-cycling parameters

Annealing: In order to estimate the annealing temperature (T_m) of a particular primer, 2°C was added for every A/T and 4°C for every G/C nucleotide, the ideal annealing temperature being the T_m minus 5°C. However, slightly higher annealing temperatures were often used to increase the stringency and specificity of the PCR. An annealing time of one second was found to be adequate for most primers.

Extension: An extension temperature of 72°C (the optimal temperature for *Taq* polymerase) was used. In order to increase the specificity of the reaction, the extension time was varied with the size of product expected. For example, a 30 second extension time was adequate for products of up to 2 Kb in size, whereas in the case of small fragments (≤ 300 bp) the extension step could be left out completely. Cycling was usually followed by a final 1 minute extension at 72°C.

Table 6.14a
A list of the primers used in PCRs to amplify canary opsin genes. The 5' position refers to the base pair number in the equivalent chicken opsin gene (cDNA).

Original species & gene	Primer Name	5' Posn	Original opsin gene template and Sense strand sequence of primer	Fwd/ Rev
			UV OPSIN GENE	
chicken violet	v 88+	88	5'- TGG GCC TTC TAC CTM CAG -3'	fwd
chicken violet	v 205+	205	5'- TAC ATY CTG GTS AAC RTS TC -3'	fwd
budgerigar/ canary UV	bcv 833+	833	5'- CGA CCT GCG CCT CGT CAC CA -3'	fwd
budgerigar/ canary UV	bcv 273-	273	5'- GAC GAA GAC GGT GAA GAT GCA-3'	rev
chicken violet	v 788-	788	5'- TAG GGS ACG TAG CAS ABR CAG A -3'	rev
chicken violet	v 854-	854	5'- ATG GTG ACR AGS CGK ARG TCC A -3'	rev
			SWS OPSIN GENE	
chicken blue	bl 34+	34	5'- GAG GAY TTC TAC ATC CCC AT -3'	fwd
chicken blue	bl 230+	230	5'- CSC ACC TKA AYT ACA TCC TGG T -3'	fwd
chicken blue	bl 572-	572	5'- TGC AGC CCC TCC GGG ATG TA -3'	rev
chicken blue	bl 599-	599	5'- GTG TAC CAG TCC GGC CCG CA -3'	rev
chicken blue	bl 824-	824	5'- TAT GGC GCC CAG CAY ACC ARG AA -3'	rev
			CONSENSUS PRIMER (all opsin genes)	
all chicken opsin genes	consensus 1040-	(rod) 1040	5'- TGG CTG GWG GAS ACR GAG GA -3'	rev

continued over...

Original species & gene	Primer Name	5' Posn	Original opsin gene template and Sense strand sequence of primer	Fwd/ Rev
			MWS OPSIN GENE	
chicken green	g 34+	34	5'- CCT ATG TCC AAC AAG ACA GG -3'	fwd
chicken green	g 43+	43	5'- AAC AAG ACA GGG GTG GTG CG -3'	fwd
chicken, budgerigar green	Bg 295+	295	5'-TGG ATT GGC TAC TTC GTC TTC GG-3'	fwd
chicken green	g338-	338	5'- TCC ACA GCA CAG CCA ACG GG -3'	rev
			ROD OPSIN GENE	
chicken rod	ρ 337+	337	5'- GAG GGC TTC TTT GCT ACG -3'	fwd
chicken, goldfish human rod	ρ 538+	538	5'- CCC GAG GGC ATG CAG TGC TC -3'	fwd
chicken rod	ρ 764-	764	5'- ATC ACC ATG CGG GTC ACT T -3'	rev
chicken & human rod	ρ 985-	985	5'- CCA GCG GGT TCT TGC CGC AGC-3'	rev
chicken rod	ρ 1052-	1052	5'-AGG GGA CAC CTG GCT GGT GGA GAC-3'	rev
			LWS OPSIN GENE	
chicken & canary red	r 544+	544	5'- CCV CCC ATC TTY GGB TGG AGC -3'	fwd
chicken red	r 978-	978	5'- RAA CTG YCG GTT CAT RAA GAC -3'	rev

Primers were designed by Dr. L.A. Heath and Dr. S.E. Wilkie with the exception of ρ538+(Rho Ex 3+) and ρ985- (Rho Ex 5-) which were supplied by Dr. J. Bellingham.

Denaturation: a 10 second initial denaturation at 94°C was used before cycling commenced followed by a 1 second denaturation at 94°C at the beginning of each cycle.
Number of cycles: 40 cycles of denaturation, annealing and extension was found to be adequate for most PCR reactions.
Hot Start: Hot starts were routinely performed in order to eliminate non-specific priming in PCR reactions. During a 'hot start' all the reaction components were combined in a tube, bar one essential component (usually the *Taq* polymerase), which was added following the first denaturation step.

6.15. Visualizing PCR products on an agarose gel

After cycling, 10 µl PCR product was mixed with 1µl 'orange g' loading dye before loading alongside Hae III digested ϕX174 DNA size marker on an agarose gel. 2.5 % agarose gels were made by pouring a pre-weighed amount of agarose powder into a flask containing an appropriate volume of 1x TAE buffer. The flask was microwaved until the agarose was fully melted and the gel solution allowed to cool to below 60°C before pouring into a casting tray on a levelled bench. Casting trays were prepared beforehand by sealing the two exposed sides with masking tape and placing a comb at one end to form the wells. Most gels were set within 20 minutes of pouring. If low melting point agarose had been used gels were allowed at least 1 hour to set.

Gels were electrophoresed in 1X TAE running buffer at 100 - 125 v until the dye front had migrated an appropriate distance. Following electrophoresis, gels were stained for 5 minutes in a tank containing a solution of the intercalating dye, ethidium bromide, to enable the subsequent visualization of the PCR product under UV light. The gel was briefly destained by rinsing under tap water to remove excess buffer salts known to effect cloning efficiency, before placing on the UV-transilluminator. To reduce the UV exposure of the DNA the gel was placed on a partially UV filtering tray while viewing on the UV-transilluminator.

6.16. Purification of PCR products and preparation for ligation

Using scalpel blades, any bands approximating the expected size of product were excised and the resulting gel slice trimmed of excess agarose before placing in a 0.5 ml eppendorf tube containing 50 µl deionised water. The DNA was allowed to elute out for 6 hours at room temperature or overnight at 4°C. 7 µl of the resulting eluate was used for ligation. Alternatively, if a single distinct band of PCR product was observed the remainder of the PCR reaction was spun through a MicroSpin Sephacryl S-200 HR column to remove primer-dimers prior to ligation. Due to the greater product recovery of this method, only 3 µl of column purified product was required in a ligation reaction.

6.17. Ligating the fragments

The LigATor kit was used to perform ligations and subsequent cloning.

For a ligation reaction, the following reagents were combined in a 0.5 ml eppendorf tube on ice:

1 µl	10x ligase buffer
0.5 µl	100 mM DTT
0.5 µl	10 mM ATP
1 µl	50 ng/µl vector (pTAg)
0.5 µl	T4 DNA ligase (2-3 Weiss units)

The eluate from the excised PCR band or the MicroSpin column purified PCR reaction was then added to a final volume of 10 µl. The ligation reaction was incubated overnight at 16°C.

6.18. Cloning the fragments

6.19. Transformation reaction

The competent cells provided were used with the LigATor kit. Competent cells were removed from the -80°C freezer and left on ice to thaw. The complete ligation reaction was added to 20 µl of competent cells and the tube left to incubate on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds and allowed to recover on ice for 2 minutes before adding 80 µl of SOC medium. The transformation culture was shaken at 200-250 rpm in a rotary shaking incubator for 1 hour at 37°C before plating.

6.20. Plating the transformants

The transformation culture was spread on prepared LB agar plates containing the appropriate antibiotic(s) plus IPTG and X-Gal (see appendix C). The plates were inverted and incubated overnight at 37°C. Following overnight incubation, plates were often kept for a further 2 hours at 4°C to intensify the colour of blue colonies. Transformant colonies containing putative recombinant plasmids (identified by their white colour (see appendix B) were circled and assigned an arbitrary number for later screening (see below). Very small white colonies, or whose which were at risk from cross contamination due to dense colony growth, were often streaked out onto fresh agar plates and regrown overnight.

6.21. PCR screening for recombinants

Transformant colonies were screened for the presence or absence of insert of the correct size by performing colony PCR. A bulk PCR mix (10 µl x number of colonies to be screened) containing the vector arm primers (pTAg SEQ 5' and pTAg SEQ 3') was made as follows: (values given for a 25 µl PCR reaction)

Reaction component	µl *
NH_4 buffer	2.5
dNTP	1
vector primer 5'	0.5
vector primer 3'	0.5
Taq polymerase	1
$MgCl_2$	1.5
H_20	18
colony picking -	-

* the exact volume of each reactant used in a bulk PCR mix was given by multiplying the above values by an appropriate common factor

10 µl PCR mix was dispensed into previously numbered 0.5 ml eppendorf tubes. A sterile toothpick was used to touch the edge of the colony to be screened before inoculating the correspondingly numbered PCR tube.

6.22. Parameters for colony PCR

An initial 1 minute denaturation step at 94°C was employed to ensure complete lysis of the bacterial cell wall, followed by 35 cycles of 1 second at 94°C, 1 second at 50°C and 20 seconds at 72°C (extension times were varied according to the size of fragment) and a final 1 minute extension at 72°C.

Following PCR, the entire sample was electrophoresed on an agarose gel. Since the vector primers anneal approximately 100 base pairs either side of the cloning site, non-recombinant transformants were identified as a band of 200 base pairs whilst recombinants were identified as larger bands (200 bp plus the size of insert). Recombinants containing an insert of the expected size were picked and inoculated into 7-10 ml of LB broth containing 50 µg/ml kanamycin and 15 µg/ml tetracycline. The recombinant culture was shaken overnight in a rotary shaking incubator (at 200-250 rpm) at 37°C.

6.23. Purification of plasmid DNA

A standard protocol for plasmid minipreps was used. 1-3 ml overnight culture was pelleted by centrifugation and resuspended in 200 µl of Cell Resuspension Solution (50 mM Tris-HCL, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A). Following transfer to a microcentrifuge tube, 200 µl of Cell Lysis Solution (0.2 M NaOH; 1% of SDS) was added and mixed by inverting the tube several times until a clear solution was obtained. 200 µl of Neutralization Solution (2.55 M potassium acetate, pH 4.8) was added and the tube mixed before spinning at top speed in a microcentrifuge for 5 minutes. The supernatant was decanted into a fresh tube. 1 ml of thoroughly mixed DNA Purification Resin was added to the supernatant and the tube inverted several times. The plasmid was purified using a vacuum manifold: An empty syringe barrel (minus the plunger) was attached to the top of a Minicolumn. The Minicolumn/syringe barrel assembly was inserted into the vacuum manifold. The resin/DNA mix was decanted into the syringe barrel and a vacuum applied to pull the resin/DNA mix into the Minicolumn. The column was washed by adding 2 ml of Column Wash (200 mM NaCl; 20 mM Tris-HCL, pH 7.5; 5 mM EDTA; diluted 1:1 with 95% EtOH) under vacuum. The resin was dried by continuing to draw a vacuum for an additional 1-2 minutes. The syringe barrel was removed and the Minicolumn transferred to a 1.5 ml microcentrifuge tube before being spun at top speed for 20 seconds to remove any residual Column Wash solution. The Minicolumn was transferred to a new microcentrifuge tube. To elute the DNA, 50 µl of deionized water pre-heated at 65-70°C was applied to the Minicolumn and allowed to soak in for at least 1 minute. The tube was then spun at top speed in a microcentrifuge for 20 seconds. The Minicolumn was removed and discarded and the plasmid DNA stored at -20°C. A typical yield of plasmid DNA was in the region of 1-10 µl although before sequencing, diluted plasmid was often run on an agarose gel to assess its quality and quantity.

6.24. Glycerol Stocks

1 ml overnight culture was added to 0.5 ml of 50% glycerol and the vial inverted several times before placing at - 80°C.

6.25. Sequencing reactions

Once cloned individual fragments were sequenced on both strands. Sequencing reactions were carried out using the T7 Sequencing Kit # 27-1682-01: Pharmacia Biotech.

6.26. Standard annealing of primer to double-stranded template

The plasmid DNA was diluted with sterile, deionized water to a concentration of 1.2 - 2.0 µg in 32 µl. To denature the double-stranded plasmid, 8 µl of 2M NaOH was

added and the tube briefly vortexed and centrifuged before incubating at room temperature for 10 minutes. 11 µl of 2M sodium acetate (pH 4.8) was added and mixed. 120 µl of 100% ice-cold ethanol was added and the tube vortexed before placing at - 80°C for 30 minutes. The precipitated DNA was pelleted by spinning the tube in a microfuge for 15 minutes. The supernatant was removed and the pellet carefully washed with 120 µl 70% ice-cold ethanol. The pellet was re-spun for 10 minutes and the supernatant removed and discarded. The pellet was dried under vacuum and redissolved in 10 µl sterile, deionized water. 2 µl of sequencing primer (pTAg SEQ 5' and pTAg SEQ 3', 5 pmol/µl) and 2 µl annealing buffer were added followed by briefly vortexing. The tube was incubated at 65°C for 5 minutes on a PCR block before being immediately transferred to a water bath (or PCR block) at 37°C for 10 minutes. The tube was placed at room temperature for at least 5 minutes before centrifuging briefly.

6.27. Sequencing reactions

Four 0.5 ml microcentrifuge tubes were labelled 'A', 'C', 'G', 'T' respectively. 2.5 µl of the corresponding short mix was pipetted into each of the tubes.

6.28. Labelling (incorporation) reaction

The following was added to the tube containing annealed template and primer:

(Annealed template/primer)	14 µl
T7 DNA Polymerase	0.5 µl
T7 Dilution buffer	2 µl
Label Mix dATP	3 µl
^{35}S dATP	0.5 µl
Total volume	20 µl

The components were mixed by gentle pipetting and briefly spinning before incubating at room temperature for 5 minutes. During this incubation the four sequencing mixes were warmed at 37°C for at least 1 minute.

6.29. Termination Reactions

4.5 µl of the labelling reaction was transferred into each of the four pre-warmed sequencing mixes using a fresh pipette tip for each transfer. The reaction was mixed by gentle pipetting and incubated at 37°C for 5-10 minutes. 6 µl of Stop Solution was added to each tube and mixed gently. Samples were heated to 90°C for two minutes and placed on ice prior to loading the appropriate well of a sequencing gel. The remaining

sample was stored at - 20°C.

6.30. Electrophoresis

6.31. Preparation of a 5% polyacrylamide gel

A horizontal gel rig was dismantled and the front and back glass plates were cleaned with soap, rinsed thoroughly with water, and dried. Both plates were rinsed with ethanol and dried before treating the back plate (attached to the buffer tank) with Repel-Silane. Two spacers of uniform thickness (approximately 0.25 mm) were inserted between the long edges of the plates which were then clamped together. The bottom of the rig was sealed with the 'plug tray' provided with the rig. For a 5% polyacrylamide gel, 16 ml SequaGel Concentrate, 74 ml SequaGel Diluent and 10 ml SequaGel Buffer were mixed in a flask. To accelerate the polymerisation of acrylamide 40 µl of TEMED plus 0.8 ml of 10% (w/v) ammonium persulphate were added. The solution was swirled to mix and immediately drawn up into a 150 ml syringe ejecting any air. With the rig in a horizontal position the solution was injected between the plates via the tubing attached to the bottom of the 'plug tray'. Any bubbles were which formed were dislodged by tapping the glass gently. After pouring the gel, the comb or surface former (if a sharks tooth comb was used) was inserted at the top of the gel and clamped with three bull-dog clips. The gel was left to polymerise for 45-60 minutes at room temperature. Once polymerised the gel was placed in the electrophoresis apparatus and the reservoirs filled with 1x TBE buffer.

6.32. Loading sequencing reactions onto the polyacrilamide gel

The sample wells were flushed out with 1x TBE to remove any accumulated urea. 3-5 µl aliquots of denatured sequencing reaction were loaded into adjacent wells in sets of four, corresponding to the A, C, G and T reactions. The gel was connected to the power supply and electrophoresed at 100 W constant power until the bromophenol blue was 4-5 cm from the bottom of the gel (approximately 2 hours for a 40 cm gel). The plates were separated and the gel/plate was soaked in fixer (10% acetic acid/10% methanol in distilled water) for 10 minutes before being transferred to a supporting sheet of filter paper (Whatman Number 1), covered with cling-film and dried using a vacuum gel dryer.

6.33. Autoradiography

Once dry the cling-film was removed, the gel was transferred to a film cassette with a sheet of X-ray film. Following overnight exposure at room temperature, the film was developed.

6.34. Analysis of sequence

The nucleotide sequence of the cloned cDNA fragment was read from the pattern of bands on the autoradiograph and stored on the computer using the Geneworks™ gene analysis package. The identity of the fragment was inferred from the published (normally avian) opsin cDNA sequence to which it showed the greatest degree of sequence homology, following a series of cDNA/protein sequence alignments in Geneworks™.

When creating phylogenetic trees, protein sequences were first aligned in Clustal V (Higgins *et al.,* 1992) using the multiple alignment option. Using the resulting alignment as a guide, gaps were manually inserted into the equivalent positions of the corresponding nucleotide sequence, where necessary, to ensure that sequences were all of equal length. Subsequent manipulations were carried out in the MEGA (Molecular Evolutionary Genetics Analysis) computer package, version 1.01 (Kumar *et al.,* 1993) which was used to determine pairwise divergence values for amino acids and total nucleotides. Phylogenetic trees were generated by the neighbour-joining method (Saitou and Nei, 1987) from the frequency of nucleotide substitutions between sequences using the *D. melanogaster RH1* opsin (O'Tousa *et al.,* 1985) as an out-group. Support for internal branching was assessed by bootstrapping using 500 replicates. Kyte-Doolittle hydrophobicity plots (Kyte and Doolittle, 1982) of the deduced amino acid sequence of the putative opsin genes were generated in Geneworks™ using a window of 11 residues.

7. RESULTS AND DISCUSSION

7.0. MICROSPECTROPHOTOMETRY - General summary of results

A detailed microspectrophotometric analysis of the retina of the canary, *Serinus canaria*, has established the following.

- The canary has a duplex retina, containing both rods, and single and double cones.

- The canary appears to be typical of passerine birds and possesses a LWS cone, combining the P569 with an R-type droplet, a MWS cone combining the P506 with a Y-type droplet, a SWS cone associating the P442 with a C-type droplet and a UV cone linking the P366 with a T-type droplet. The P568 is also found in the Principal member of double cones associated with a P-type droplet.

- The rods contain a P506, spectrally similar to the MWS cone pigment.

- With at least four spectrally distinct cone visual pigments the canary has the potential for tetrachromatic colour vision.

- The R-type droplets vary considerably between birds. Some individuals have typically highly pigmented droplets, whereas others have 'reduced' R-type droplets.

- No correlation was found between feather colouration and oil droplet complement.

7.1. VISUAL PIGMENTS

7.2. Cones

Absorbance spectra that satisfied the criteria were analysed from a total of 103 intact cones from twenty-four canaries. An intact cone was one in which the outer segment was still attached to the inner segment and both visual pigment and oil droplet were measureable (in terms of an adjacent cell-free area in which to place the measuring beam). Approximately 50% of records from intact cones were abandoned after application of the selection criteria outlined in the Methods section.

Spectral sensitivity determination

During measurements the beams were passed transversely through outer segments whereas in the eye light normally passes axially through outer segments. Therefore, when calculating the effective spectral sensitivities of the cones it was necessary to assume an effective end on visual pigment density. This was achieved by assuming a specific visual pigment absorbance of about 0.015 per μm at the λ_{max}, and a typical outer segment length of 10 μm to give a final end on density of about 0.15 (values are from Maier and Bowmaker, 1993) . These values were used instead of the actual pigment absorbance values obtained for each class of pigment because outer segment diameters were not recorded in the present study.

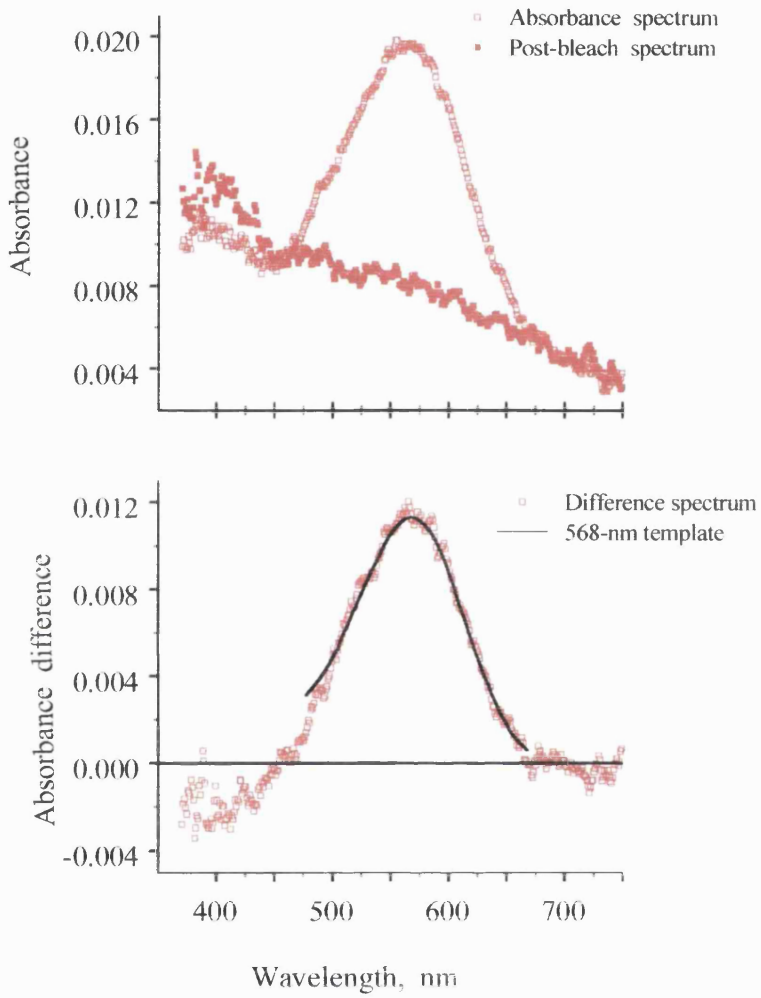
Calculating the sensitivity of the outer segment: Wavelength and absorbance values from the mean difference spectrum of a particular class of cones were imported into the Axum™ graphics package and smoothed using the "lowess" smoothing function (a noise reduction smoothing algorhythm, which uses locally weighted least squares for curve fitting). The absorbance values were adjusted to 'end on' absorbance before being converted to end on absorptance and normalized. The outer segment sensitivity was obtained by taking the log of the normalized absorptance values.

Another factor to be taken into account when calculating the effective spectral sensitivity of a cone is the filtering effect of the oil droplet which interposes between in coming light and the visual pigment in the outer segment. However, before subtracting the oil droplet absorbance values from the outer segment sensitivity, it was necessary to manipulate the oil droplet data. The averaged oil droplet absorbance values were converted into transmission values, normalized (by dividing by the total transmission range) and converted back to absorbance. The resulting normalized oil droplet absorbance values were then subtracted from the outer segment sensitivity to give the final cone sensitivity.

A LWS pigment (λ_{max} = 569 nm) was found in single cones paired with an R-type droplet and in the Principal and Accessory member of the double cones paired with a P-type and an A-type 'droplet' respectively (see later). The mean absorbance and difference spectra for the LWS cones in *Serinus canaria* are presented in fig. 7.3a. The absorbance spectra shown are the mean of 53 LWS cells and include both single and double cones. In the mean post-bleach absorbance spectrum there is evidence of a photoproduct peak at about 380-390 nm which is possibly all-*trans*- retinal (λ_{max} = 381 nm, Knowles and Dartnall, 1977). This is also seen in the mean difference spectrum as negative absorbance. In the canaries which possess them (see later), the R-type droplet will filter out all wavelengths of light below about 580 nm shifting the effective λ_{max} of the LWS pigment to about 600 nm as shown in fig. 7.3b. In contrast, the relative spectral sensitivity of the Principal member of double cones will not be altered significantly by the presence of a P-type oil droplet (see fig. 7.3c). A similar situation to that seen in the Principal member of double cones will occur in LWS single cones containing 'reduced' R-type oil droplets (see later) affording the birds possessing them greater sensitivity to wavelengths between 450-570 nm than birds possessing normal R-type oil droplets.

Fig 7.3a

The mean absorbance and difference spectra for the LWS pigment from *Serinus canaria*



The absorbance spectra are the mean of 53 individual cells, see Table 7.7c

Fig 7.3b
The relative spectral sensitivity of LWS single cones from *Serinus canaria*

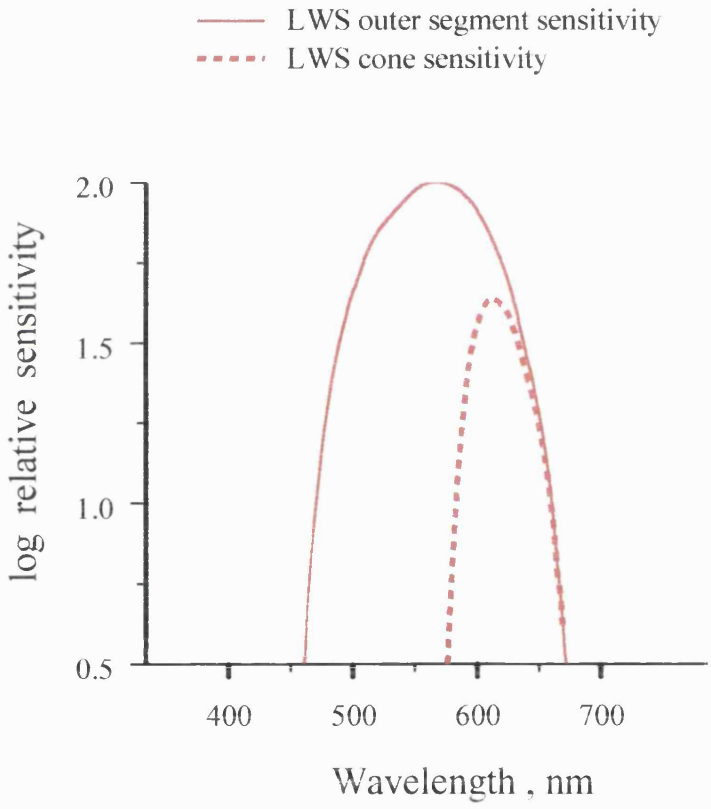
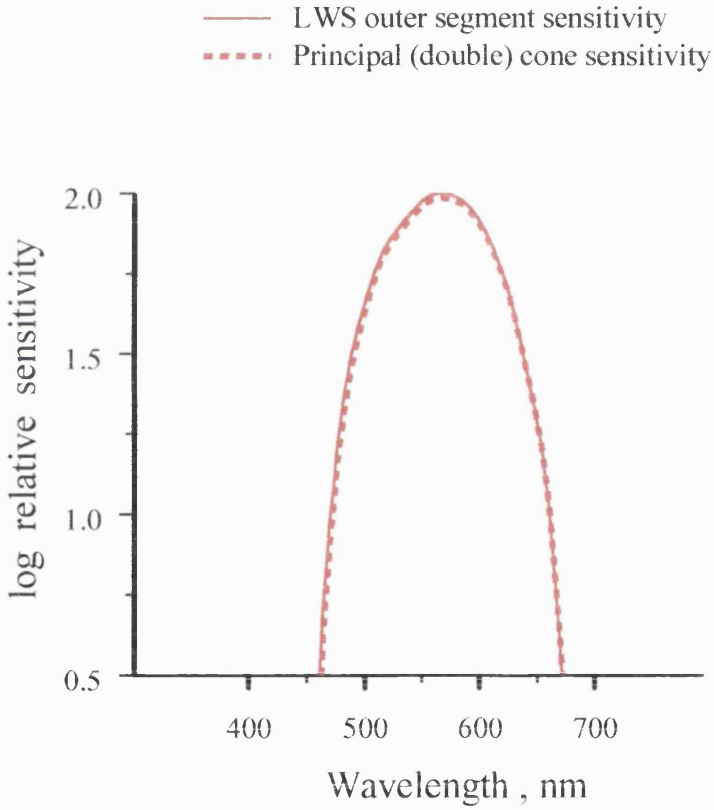


Fig 7.3c
The relative spectral sensitivity of LWS double cones from *Serinus canaria*

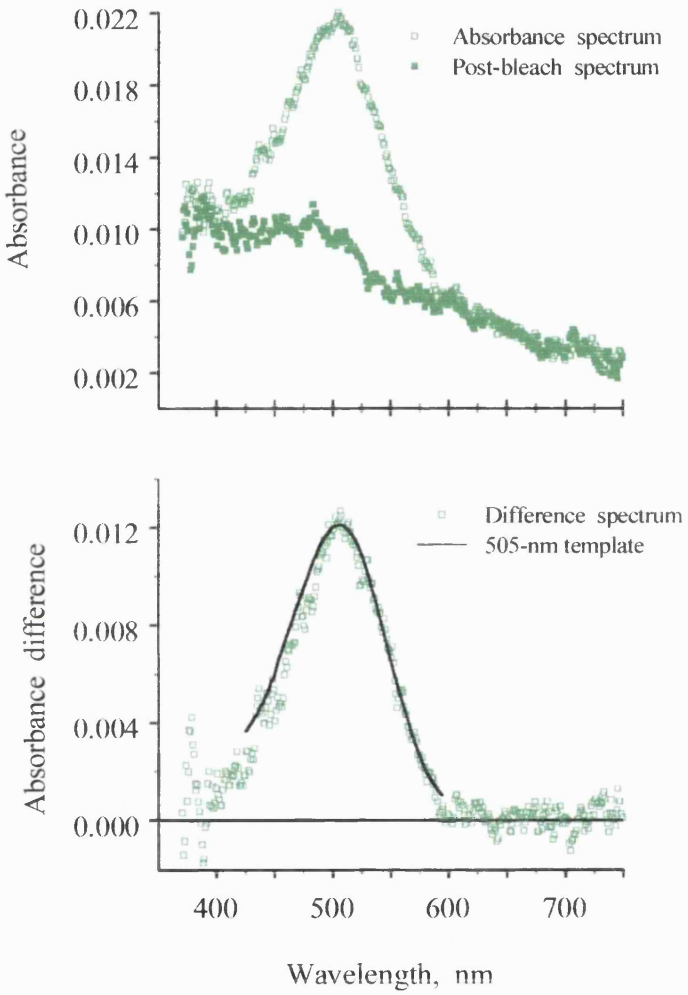


The canary possesses a population of single MWS cones containing a pigment which absorbs maximally at about 506 nm. The absorbance and difference spectra presented in fig. 7.4a are the mean of 14 individual MWS cones which were all found with Y-type oil droplets. Close inspection of fig. 7.4a reveals a slight peak at 380-390 in the post-bleach absorbance spectra. It is possible that this is all-*trans*- retinal (λ_{max} = 381 nm, Knowles and Dartnall, 1977). There is also evidence of photoproduct at about 480 nm in the post-bleach absorbance spectrum. The spectral location of the peak suggests that it may be due to slight contamination from the Y-type oil droplet carotenoid. This may have occurred due to a slight misalignment of the measuring beam, placing it too close to the oil droplet, for example. Alternatively, it is possible that some oil droplets may have lysed during the course of experiments resulting in the leakage of carotenoid into the outer segment. However since the post-bleach spectrum is the mean of 14 cells it would be expected that any chance contamination from the oil droplet carotenoid would have been lost in the averaging. Another possibility is that the peak is due to one of the intermediates in the photolysis of rhodopsin such as Metarhodopsin III which has an approximate λ_{max} at around 460 nm (Imai *et al.,* 1995). However, the time constant in chicken MWS cones for the transition of Metarhodopsin III (λ_{max} = 460) to opsin + all-*trans*- retinal has been estimated to be in the order of just 40 seconds at room temperature (Imai *et al.,* 1995). In contrast, the MWS cones in *Serinus canaria* were bleached for at least 3 minutes before the post-bleach absorbance spectrum was recorded, suggesting that the transition from Metarhodopsin III to opsin + all-*trans*-retinal would have been complete. The relatively high absorbance of the Y-type oil droplets (approximately 0.3) will ensure that all wavelengths of light below about 500 nm are filtered out before reaching the outer segment, shifting the effective λ_{max} of the MWS pigment to about 550 nm as shown in fig. 7.4b.

The SWS sensitive cones in the canary contain a pigment which absorbs maximally at about 442 nm. The absorbance and difference spectra presented in fig. 7.5a are the mean of 26 individual cells which were all found with C-type oil droplets. There is a noticeable peak in the mean post-beach absorbance spectrum at about 415-420 nm which is too long to be attributable to all-*trans*- retinal. The λ_{Tcut} of the C-type droplets in *Serinus canaria* is at about 415 nm (see later) which suggests that the peak may be due to contamination from the C-type oil droplet carotenoids. It is likely that the relatively small dimensions of the SWS cones may have made it slightly more difficult to place the measuring beam in the outer segments without accidentally touching the oil droplet. Interestingly, there is also evidence of an identical peak at about 415-420 nm in the mean absorbance spectrum which suggests the presence of a photostable substance. It is possible that photostable substances do occur within outer segments. Alternatively there may have been some leakage of photostable structures from the inner

Fig 7.4a

The mean absorbance and difference spectra for the MWS pigment from *Serinus canaria*



The absorbance spectra are the mean of 14 individual cells, see Table 7.7c

Fig 7.4b
The relative spectral sensitivity of MWS cones from *Serinus canaria*

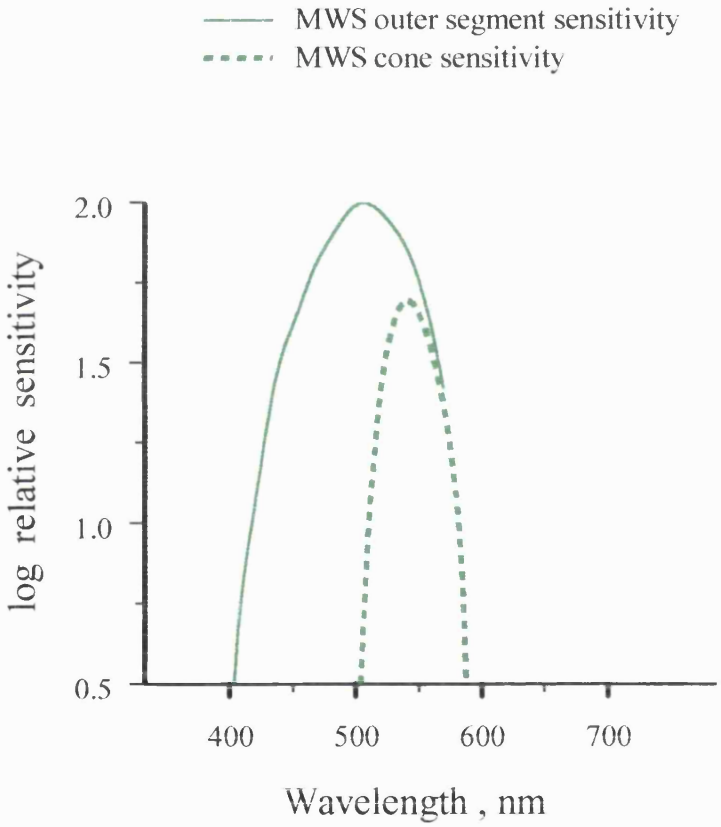
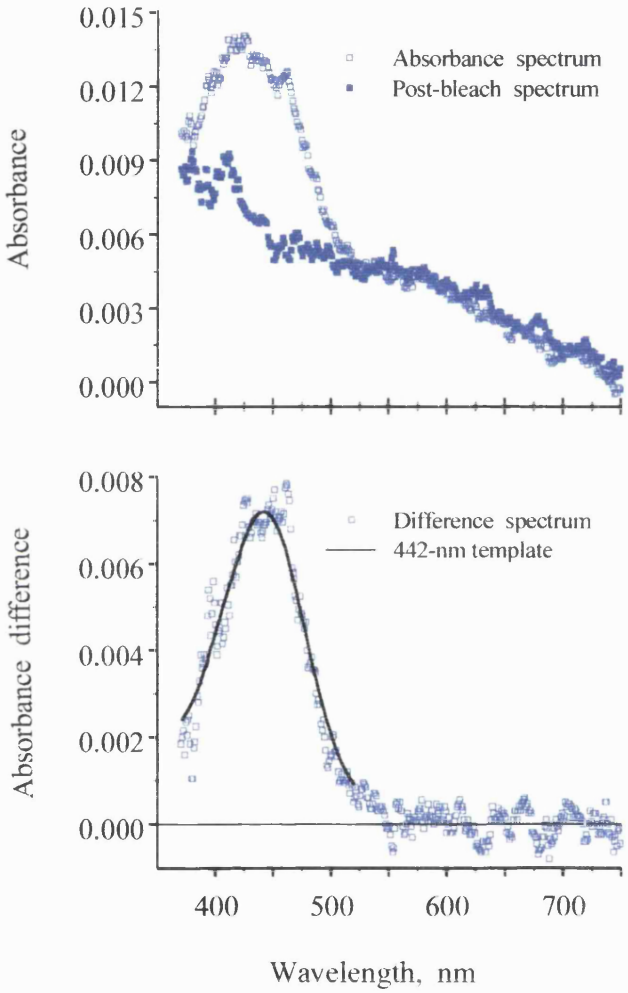


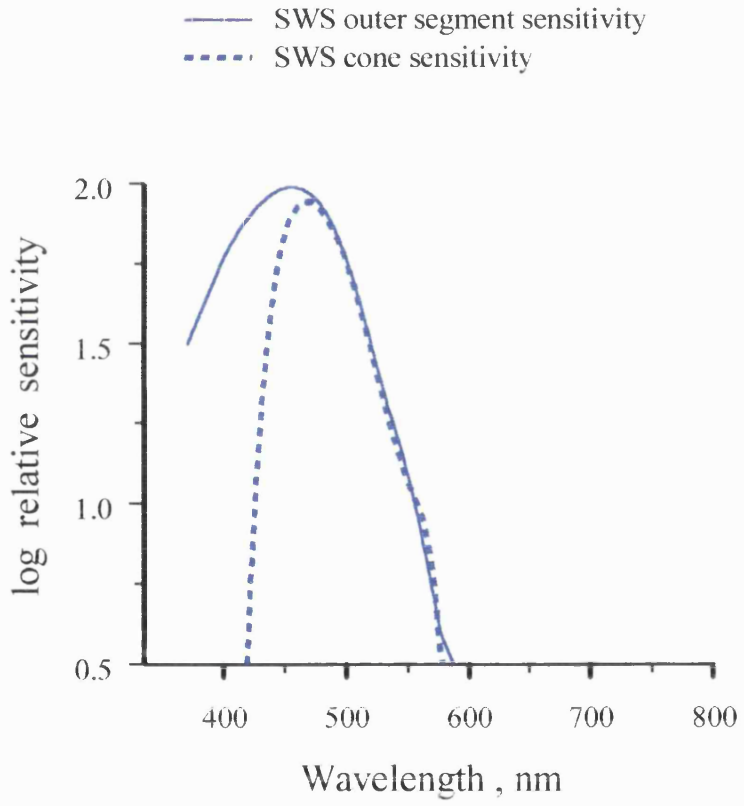
Fig 7.5a

The mean absorbance and difference spectra for the SWS pigment from *Serinus canaria*



The absorbance spectra are the mean of 26 individual cells, see Table 7.7c

Fig 7.5b
The relative spectral sensitivity of SWS cones from *Serinus canaria*

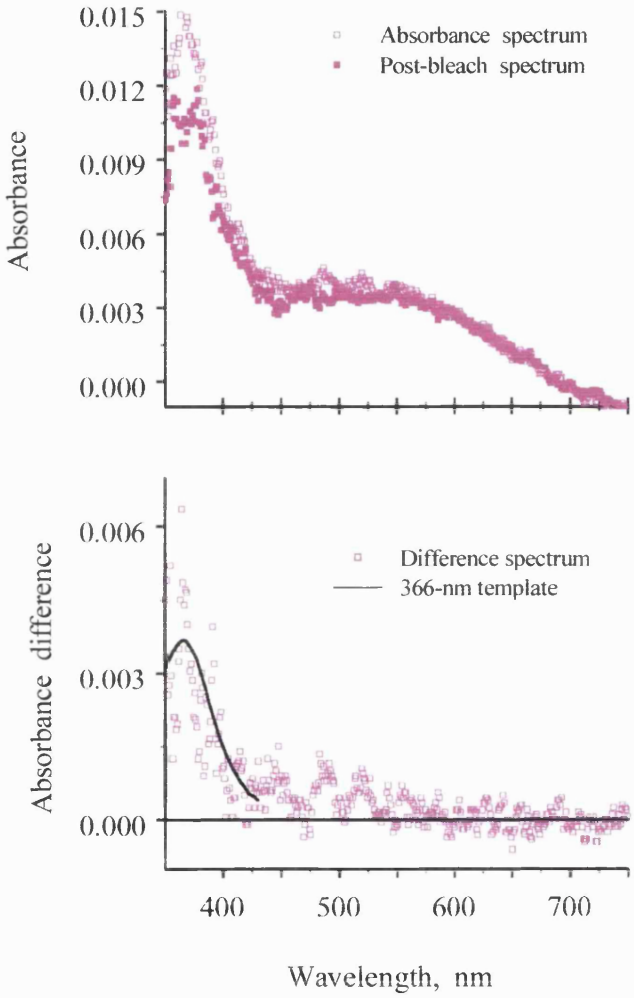


segment into the outer segment, such as mitochondrial cytochromes which absorb in this region (Bowmaker, 1991). The C-type oil droplets in the canary have a relatively low absorbance (≤ 0.15) and have little effect on the spectral sensitivity of the SWS cones other than a slight narrowing of the shortwave limb of the SWS pigment as illustrated in fig. 7.5b.

The canary possesses a population of single cones which are maximally sensitive at about 366 nm, in the near ultraviolet region of the spectrum. The absorbance and difference spectra presented in fig. 7.6a are the mean of 10 individual UVS cones which were all found with T-type oil droplets. What is apparent from fig. 7.6a is the very small absorbance difference (0.004) between the pre- and post-bleach absorbance spectra. Of all the classes of cone in the canary retina the UVS cones were the most difficult to bleach, despite the 10 minute exposure to white light. However, the apparent lack of bleach in UVS cones may be due to photoproduct formation. For example, it is possible that the peak at around 370 nm in the mean post-bleach absorbance spectrum is caused by some from of retinal which is still partially bound to opsin, since free retinal absorbs at slightly longer wavelengths (380 nm, Knowles and Dartnall, 1977). Another possibility which may account for the apparent poor bleach is that there are (previously unidentified) photostable elements in UVS cones which absorb weakly in the ultraviolet region. These elements may normally be obscured in the pre-bleach absorbance spectra due the absorbance of the UVS visual pigment, but are revealed following bleaching of the UVS outer segment. Having no appreciable absorbance anywhere in the spectrum, the T-type droplets will have no significant effect on the spectral sensitivity of the UVS cones. However, the transparency of the ocular media in the canary are crucial to the functioning of the P366. The spectral absorbance of the lens from *Serinus canaria* is shown in fig. 7.6c. The absorbance spectrum shown is the average of three lens measurements (two from the right lens and one from the left). As can be seen, the canary lens is very transparent down to about 380 nm with an absorbance of just 0.2 at about 350 nm. Below this, the lens begins to absorb strongly. The effective relative spectral sensitivity curve for the UVS cones (taking the lens absorbance into account) is shown in fig 7.6b. As can be seen, even after correcting for the lens the relative spectral sensitivity of UVS cones changes very little. In contrast to the relatively UV-transparent canary lens, the ocular media in the duck, *Anas platyrhynchos,* show significant shortwave absorbance below 400 with absorbances approaching 2.0 at 350 nm. As a result the sensitivity of the violet sensitive cones (λ_{max} = 420 nm) in this bird will be reduced below about 400 nm, making it relatively insensitive to UV light (Jane and Bowmaker, 1988).

Fig 7.6a

The mean absorbance and difference spectra for the UVS pigment from *Serinus canaria*



The absorbance spectra are the mean of 10 individual cells, see Table 7.7c

Fig 7.6b
The relative spectral sensitivity of UVS cones from *Serinus canaria*

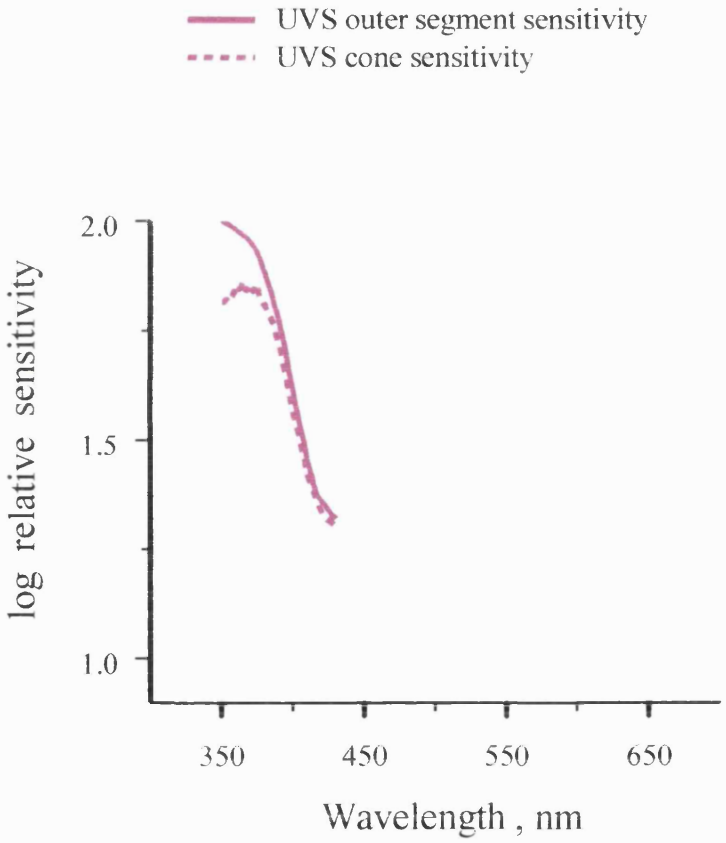
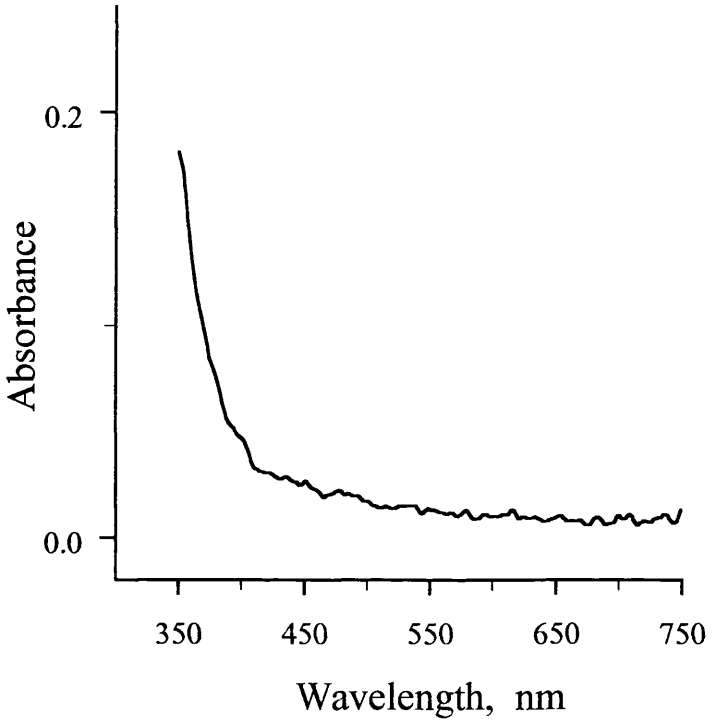


Fig 7.6c

The spectral absorbance of the lens from *Serinus canaria*



The Absorbance spectrum shown is the average of three measurements

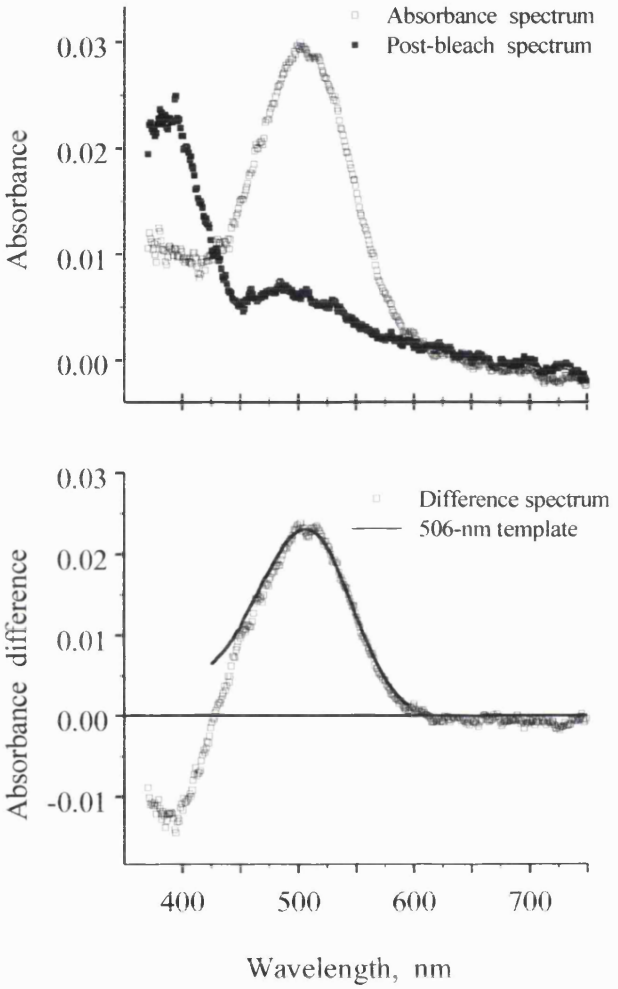
7.7 Rods

Rods were identified by their rod-shaped outer segment and lack of an oil droplet or traces of carotenoid in the inner segment. Interestingly, relatively few rods were observed in the canary retina reflecting its highly diurnal lifestyle (Walker and Avon, 1993). The outer segments of rods contained a typical rod pigment maximally sensitive at about 506 nm with a transverse absorbance of about 0.025 to 0.030 (see table 7.7c). The mean absorbance and difference spectra from rods in the canary, are shown in fig. 7.7a. The large peak in the post-bleach spectrum at around 380 nm is probably due to the presence of all-*trans*- retinal. It is interesting that the all-*trans*- retinal peak appears to be more prominent in the post-bleach absorbance spectrum of rods than in cones perhaps reflecting a more complete dissociation of all-*trans*- retinal from Meta III rhodopsin. There is a smaller peak at around 480 nm in the post-bleach absorbance spectrum which may be due Metarhodopsin III which absorbs maximally at around 480 nm and is thought to be very stable at room temperature in rods (Imai *et al.,* 1995).

Figure 7.7b shows the spectral distribution of individual cones and rods used in the analysis. The mean λ_{max} values for each class of cells are given beside each histogram. Table 7.7c summarizes the results from the three different estimates of λ_{max} (as determined from the mean absorbance spectra, difference spectra and distribution histogram) for each class of cone and the rods. The mean transverse absorbances of cells was between 0.012-0.016 for LWS and MWS cones, 0.007-0.010 for SWS cones and 0.003-0.010 for UVS cones. The lower transverse absorbance of the SWS and UVS cones is presumably a reflection of their smaller dimensions and the low signal to noise ratio at short wavelengths associated with a tungsten light source (see methods). Rod outer segments had a considerably higher transverse absorbance (0.024-0.030) than cones reflecting their larger size, higher specific absorbance and dichroic ratio. Figure 7.7d shows the relative spectral sensitivity of each class of single cone present in the retina of *Serinus canaria.* The effective λ_{max} of cones in the retina of *Serinus canaria* (taking the effect of the associated oil droplet into consideration) are summarized in table 7.7e.

Fig 7.7a

The mean absorbance and difference spectra for the rod pigment from *Serinus canaria*



The absorbance spectra are the mean of 17 individual cells, see Table 7.7c

Fig 7.7b
Spectral distribution of visual pigments from *Serinus canaria*

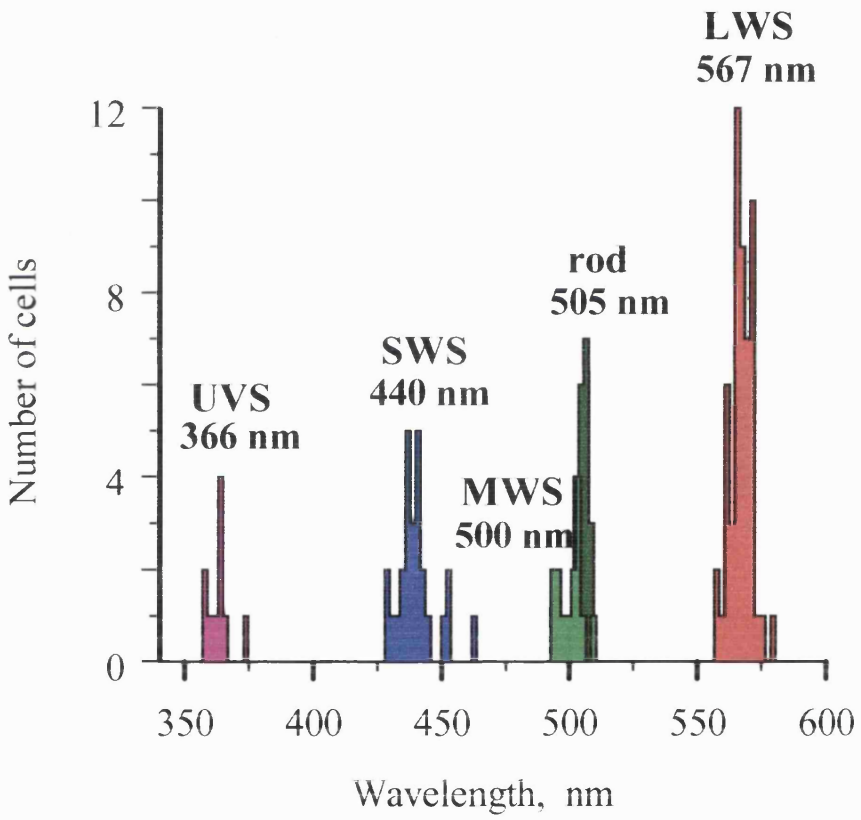


Table 7.7c

The λ_{max} of visual pigments in the retina of *Serinus canaria.*

Pigment found in the outer segment	*N*	λ_{max} of mean **absorbance** curve (nm)	λ_{max} of mean **difference** curve (nm)	mean λ_{max} from distribution **histogram** of individual cells (nm)	true absorbance from **absorbance** and **difference** spectrum
LWS 'red' cones	53	571.1 ± 1	568.6 ± 2	567.2 ± 4	[a] 0.016 [d] 0.012
MWS 'green' cones	14	500.2 ± 2	505.5 ± 2	500.5 ± 5	[a] 0.016 [d] 0.012
SWS 'blue' cones	26	444.2 ± 2	441.7 ± 3	440.4 ± 8	[a] 0.009 [d] 0.007
UVS 'ultraviolet' cones	10	369.1 ± 3	366.1 ± 8	362.9 ± 5	[a] 0.010 [d] 0.003
rods	17	505.7 ± 1	506.1 ± 1	505.5 ± 2	[a] 0.030 [d] 0.024

Values given are ± SD. *N*, the number of cells that passed the criteria (see Methods). [a] , the maximum absorbance value derived from the mean absorbance spectrum. [d] , the maximum absorbance value derived from the mean difference spectrum. The three estimates of λ_{max} of each visual pigment are derived from the mean absorbance spectrum, the mean difference spectrum and the mean of the individual λ_{max}s. The maximum visual pigment absorbance values, derived from the absorbance spectrum and difference spectrum, are also shown.

Fig 7.7d

Relative spectral sensitivity of cones from *Serinus canaria*

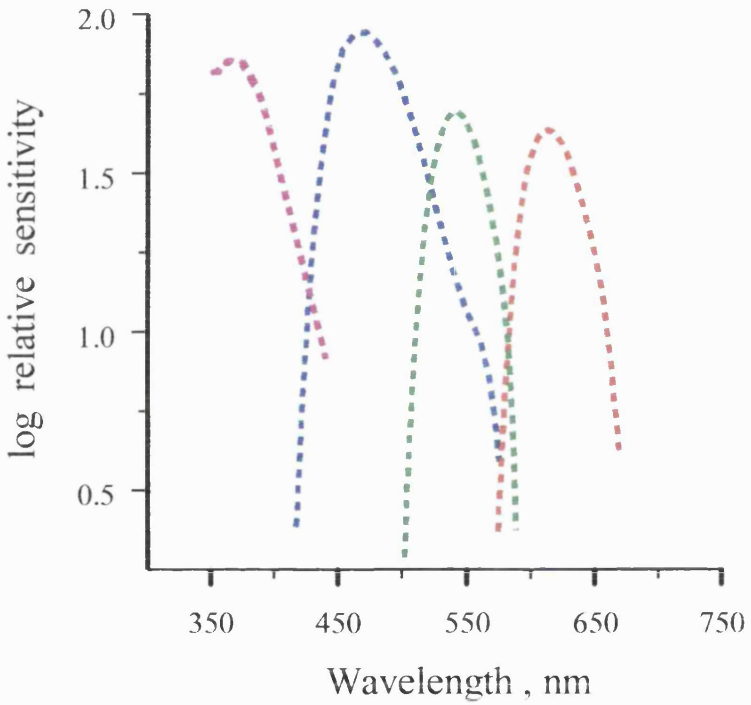


Table 7.7e

The effective λ_{max} of cones in the retina of *Serinus canaria.*

cone class	λ_{max} of visual pigment (nm)	oil droplet type	λ_{Tcut} of oil droplet (nm)	Effective λ_{max} of combination (nm)
LWS	568	R-type	578	600
MWS	506	Y-type	504	540
SWS	442	C-type	415	450
UVS	366	T-type	-	366

When calculating the outer segment sensitivity, a mean specific absorbance value of 0.015 per μm and outer segment length of 10 μm were assumed to give an end on visual pigment density of 0.15. The filtering effect of the oil droplet was subtracted from the outer segment sensitivity to give the estimated relative sensitivity of each class of cones (see text).

The visual pigments in *Serinus canaria* in relation to other avian species.

Table 7.7d is a summary of visual pigment data obtained microspectrophotometrically from other avian species. As can be seen, the canary is similar to other passerine species such as the zebra finch, *Taeniopygia guttata,* (Bowmaker *et al.,* 1997) and the Pekin robin, *Leiothrix lutea,* (Maier and Bowmaker, 1993) in possessing four cone pigments including an ultraviolet sensitive pigment. The LWS pigment in the canary absorbs at similar wavelengths to that found in most of the birds which have been examined to date (565-570 nm). In contrast, the tawny owl, *Strix aluco,* (Bowmaker and Martin, 1978) and the penguin, *Spheniscus humboldti,* (Bowmaker and Martin, 1985) have a LWS pigment which absorbs maximally at shorter wavelengths (λ_{max} = 555 nm, and 543 nm, respectively). A cone mechanism maximally sensitive at about 555 nm has also been measured in the Great Horned Owl, suggesting that this may be a common feature amongst the *Strigiformes* (Jacobs *et al.,* 1987).

Interestingly, the spectral similarity of the pigment in rods and MWS cones appears to be a common feature in birds, a fact which is also reflected at the DNA and amino acid level (Okano *et al.,* 1992; Heath *et al.,* 1997; Bowmaker *et al.,* 1997) (see Molecular Genetics results section). In contrast, the λ_{maxs} of the MWS- and rod pigments of mammals and many teleosts are usually separated by as much as 40 nm (Jacobs, 1993; Bowmaker, 1995). However, a similar separation is achieved in birds via the filtering action of the Y-type droplet. In the canary, for example, the Y-type droplet (λ_{Tcut} = 504 nm) will shift the effective λ_{max} of the MWS pigment to about 540 nm. See fig. 7.4b for relative spectral sensitivity curve.

Inspection of table 7.7d suggests that the SWS pigment λ_{max}s appear to vary considerably in birds. For example, in the two other members of the Order Passeriformes which have been examined to date, the zebra finch, *Taeniopygia guttata,* and the Pekin robin, *Leothrix lutea,* the λ_{maxs} of the SWS pigment are at about 430 nm and 453 nm, respectively (Maier and Bowmaker, 1993; Bowmaker *et al.,* 1997). In birds and turtles, the spectral sensitivity of SWS cones will depend, to an extent, on the absorbance of the C-type oil droplet. A recent comparative study of the retinas of birds (Bowmaker *et al.,* 1997) suggests that Passeriform and Psittaciform species possess dilute C-type oil droplet carotenoids (absorbance $\leq$ 0.15) when compared to Galliformes and Columbiformes (absorbance $\geq$ 0.35). This can be linked to whether the species in question possesses 'violet' or 'ultraviolet' sensitive cones (Bowmaker *et al.,* 1997) . For example, in species of the Orders Galliformes, Columbiformes and Anseriformes which possess 'violet' sensitive cones with λ_{maxs} between 400-420 nm,

the C-type droplet in SWS cones is relatively dense (absorbances ≥ 0.35) and probably acts as a cut-off filter. As a result, the SWS pigment ($\lambda_{max} \approx 450$ nm) will be displaced to longer wavelengths in the functional cone (Bowmaker *et al.,* 1997). On the other hand, in species of the Orders *Passeriformes* and *Psittaciformes,* which possess 'ultraviolet' sensitive cones with λ_{maxs} below 380 nm, the C-type droplet in SWS cones is relatively non-dense (absorbances ≤ 0.15), and probably does not act as a cut-off filter. The SWS cone λ_{max} will thus not be affected. The data obtained from the canary study also agree with this trend.

Table 7.7d
Summary of avian pigments obtained from MSP

Avian species	pigment λ max (nm)					
NEOGNATHUS BIRDS	ultra-violet	violet	blue	green	red	rod
[a] ***Sphenisciformes:*** Humboldt penguin *Spheniscus humboldti*	-	403	450		543	504
[b] ***Procellariformes:*** Manx shearwater *Puffinus puffinus*	-	402	452	P	P	505
[c] ***Anseriformes:*** Mallard duck *Anas platyrhynchos domesticus*	-	420	452	502	570	505
[d] ***Galliformes:*** domestic chicken *Gallus gallus*	-	418	455	507	569	509
[e] Japanese quail *Coturnix Japonica*	-	419	456	505	569	505
[f] ***Columbiformes:*** pigeon *Columba livia*	(366)	409 (410)	453	507	568	506
[g] ***Psittaciformes:*** budgerigar *Melopsittacus undulatus*	371	-	444	508	564	509
[h] ***Passeriformes:*** Pekin robin *Leiothrix lutea*	355	-	453	501	567	500
[i] DOMESTIC CANARY ***Serinus canaria***	366	-	442	501	567	500
[j] zebra finch *Taeniopygia guttata*	*ca* 360 - 380	-	430	506	568	507
[k] ***Strigiformes:*** tawny owl *Strix aluco*	-	-	463	503	555	503

References for table 3.12a

a Bowmaker and Martin, 1978

b Bowmaker, unpublished

c Jane and Bowmaker, 1988

d Bowmaker and Knowles, 1977; Bowmaker *et al.,* 1997

e Bowmaker *et al.,* 1993

f Bowmaker, 1977; Bowmaker *et al.,* 1997; (366), Vos Hzn *et al.,* (410), Graf and Norren, 1974

g Bowmaker *et al.,* 1997

h Maier and Bowmaker, 1993

i Bowmaker *et al.,* 1997

j Bowmaker and Martin, 1978

7.8. Oil droplets

Absorbance spectra were recorded microspectrophotometrically from oil droplets over the spectral range 370 nm to 750 nm. Five types of oil droplet were identified in three strains of canary (clear yellow, variegated and heavily variegated). These were termed R-type (red), Y-type (yellow), P-type (pale/principal), C-type (clear/colourless) and T-type (transparent), loosely based on the classification suggested by Goldsmith *et al.,* (1984) (see Bowmaker, 1991b). The R-, Y-, C- and T-type droplets were located in single cones whereas the P-type droplet was found in double cones.

Figure 7.8a is a photograph of a section of canary retina taken under standard illumination with visible light at times 100 magnification. This photograph was taken from a bird which possessed normal R-type oil droplets (see later). The R- and Y-type oil droplets can easily be distinguished on the basis of their colour and respective sizes, the R-type oil droplets appearing slightly larger. However, since the R- and Y-type oil droplets occur at slightly different depths in the retina, they may not come into focus together and it is possible that the difference in their respective sizes may have been slightly exaggerated, making the Y-type droplets look smaller than they actually are. The similar absorbance characteristics of P- and C-type oil droplets make them difficult to distinguish from each other and from the T-type droplets, all of which look transparent or pale-green in colour.

Figure 7.8b is a photograph of the same retinal region taken under ultraviolet illumination. Under these conditions the P- and C-type oil droplet carotenoids are thought to fluoresce brightly (Ohtsuka, 1984; Oishi *et al.,* 1990). Inspection of fig. 7.8b shows that some oil droplets fluoresce strongly whereas others fluoresce weakly and are slightly smaller. It is possible that the larger, brightly fluorescing oil droplets are P-types and the smaller ones are the C-types. This would partly agree with the results from a study by Ohtsuka (1984) on the red eared turtle, *Pseudemys scripta elegans,* in which the P-type oil droplets were found to have a larger diameter than C-type oil droplets and showed a strong fluorescence under UV illumination. However in the study by Ohtsuka (1984) the C-type droplets showed no fluorescence. In contrast, a study on the retina of the chicken and Japanese quail by Oishi *et al.* (1990) found that the C-type oil droplets had an intense fluorescence under UV illumination whereas the P-types fluoresced weakly. The possibility exists that the difference in fluorescence is partly due to the focusing problem associated with the oil droplets lying at differing depths in the retina. From a comparison of both pictures it can be seen that the R-type oil droplets do not fluoresce whereas the Y-type oil droplets show a slight fluorescence. T-type oil droplets can normally be identified by their complete lack of fluorescence although it was not possible to clearly identify them in the retinal region photographed.

The mean absorbance spectra of each type of oil droplet found in the canary retina are presented in figure 7.8c. As can be seen, the absorbance spectra of R- and Y-type droplets were typically flat-topped with maximum absorbances of about 0.4 (R-type) and 0.3 (Y-type).The lack of spectral detail is a result of the high optical densities of the concentrated carotenoid pigments within these droplets and the associated problems of light leakage around the droplet (Liebman and Granda, 1975). In contrast, more complete spectra were measured from the C-type droplets due to the relatively low maximum absorbances (close to 0.1). The average λ_{Tcut} of the C-type droplets was at 415 nm, but showed some variation (± 10 nm). The T-type droplets showed no significant absorbance over the measured range.

The P-type droplets were found with LWS outer segments in what were assumed to be the Principal members of double cones. Only very rarely were intact double cones seen, presumably due to the Principal and Accessory members becoming separated during tissue preparation for microspectrophotometry. Although the λ_{Tcut} of the P-type droplets (about 420 nm) was similar to that of C-type droplets, in general they had higher absorbances (about 0.3), and occasionally displayed a shoulder at about 480 nm, which was approximately 5% of the peak absorbance. The absorbance of P-type droplets varied within an individual retina although there was no obvious correlation with retinal region. In some instances the droplet pigment was relatively dense with a maximum absorbance at 415 nm of ≥ 0.25 and a 5 % shoulder at about 480 nm. In other cases the pigment was much less concentrated with a maximum absorbance of ≤ 0.1 and displayed no shoulder. In some birds the P-type droplets were generally rather dilute throughout the retina. For example, the P-type droplets from canary no. 16 had maximal absorbances which were consistently in the range 0.08 - 0.1. (See table 7.9a). The Accessory member of double cones did not contain a visible oil droplet although low concentrations of carotenoid (with a triple-peaked absorbance spectrum) were recorded in the distal tip of the inner segment as is seen in several other avian species (Bowmaker, 1977; Jane and Bowmaker, 1988; Maier and Bowmaker, 1993; Braekevelt *et al.,* '1996). In contrast a minute A-type droplet or 'spot' can occasionally be distinguished in the Accessory member of double cones in chicken, *Gallus gallus,* and the Japanese quail, *Coturnix Japonica* , (both members of the Order *Galliformes)* (Morris and Shorey, 1967; Meyer and May, 1973; Bowmaker and Knowles, 1977; Bowmaker *et al.,* 1993).

Unusual photopigment-oil droplet combinations were found in some retinal preparations. For example, six intact cones were measured in which a LWS outer segment was combined with a T-type oil droplet. Interestingly, these cells were all found in the retinas of birds which possessed 'reduced' R-type oil droplets.

Fig 7.8a
Section of canary retina taken under standard illumination with visible light (see text).

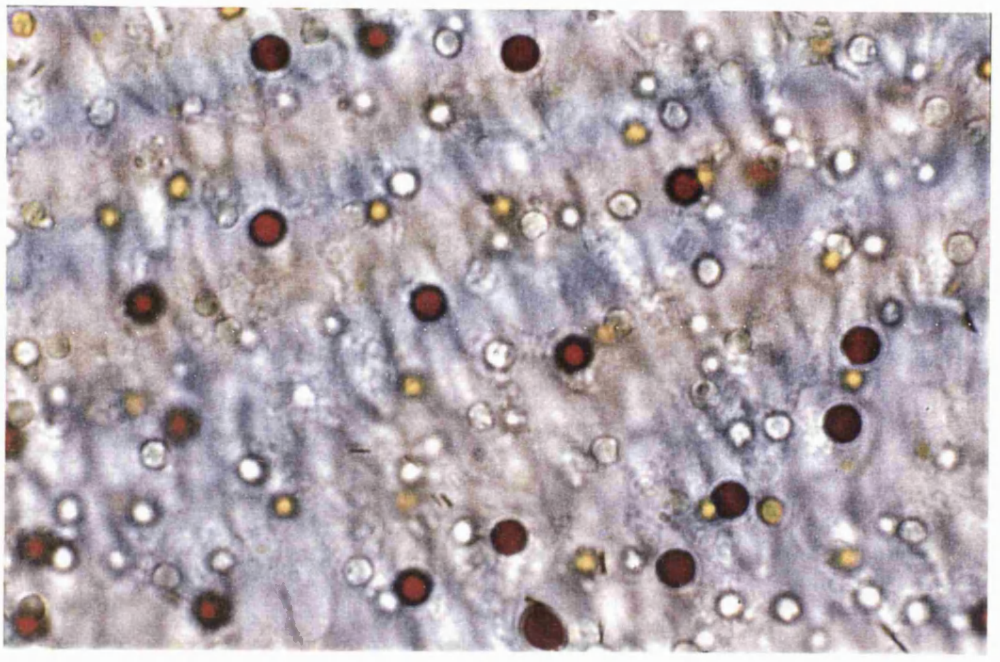


Fig 7.8b
The same section of canary retina as shown in Fig 7.8a taken under UV illumination (see text).

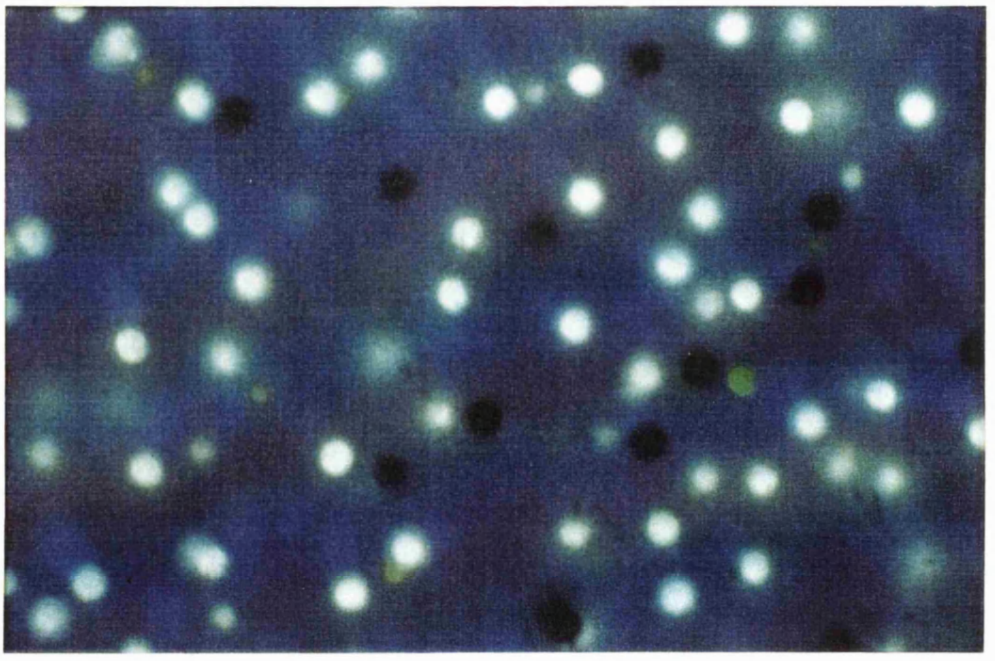
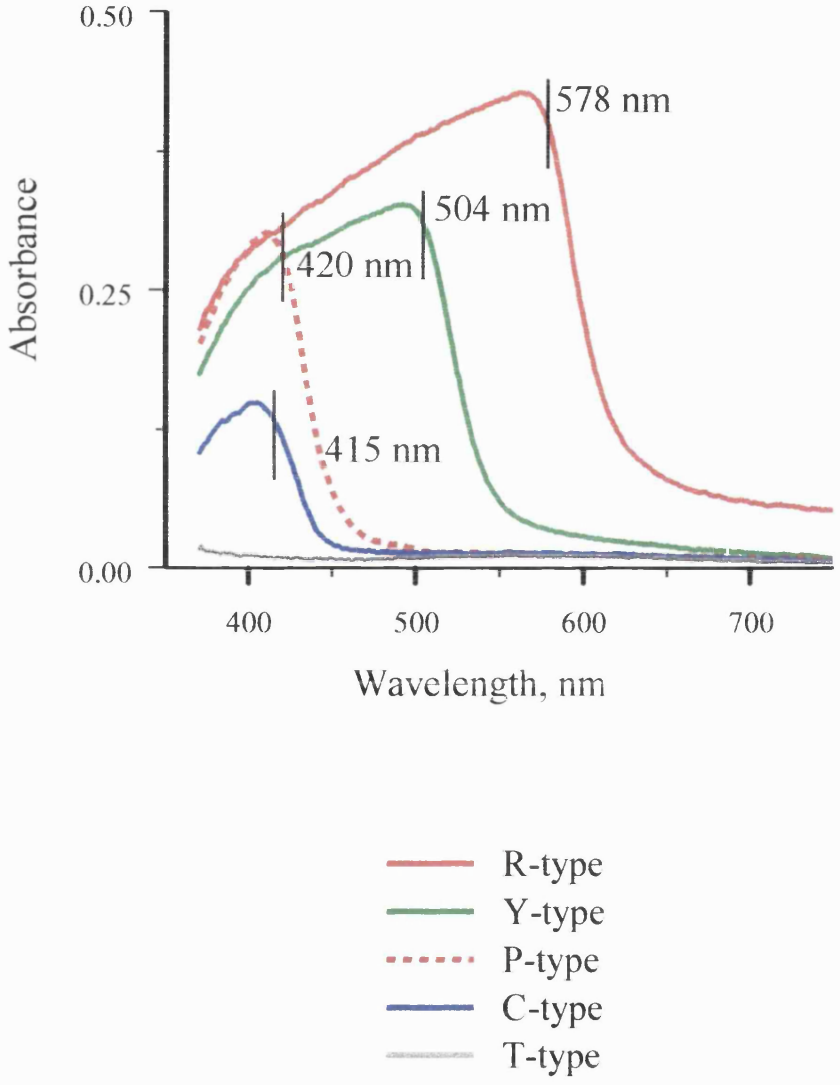


Fig 7.8c

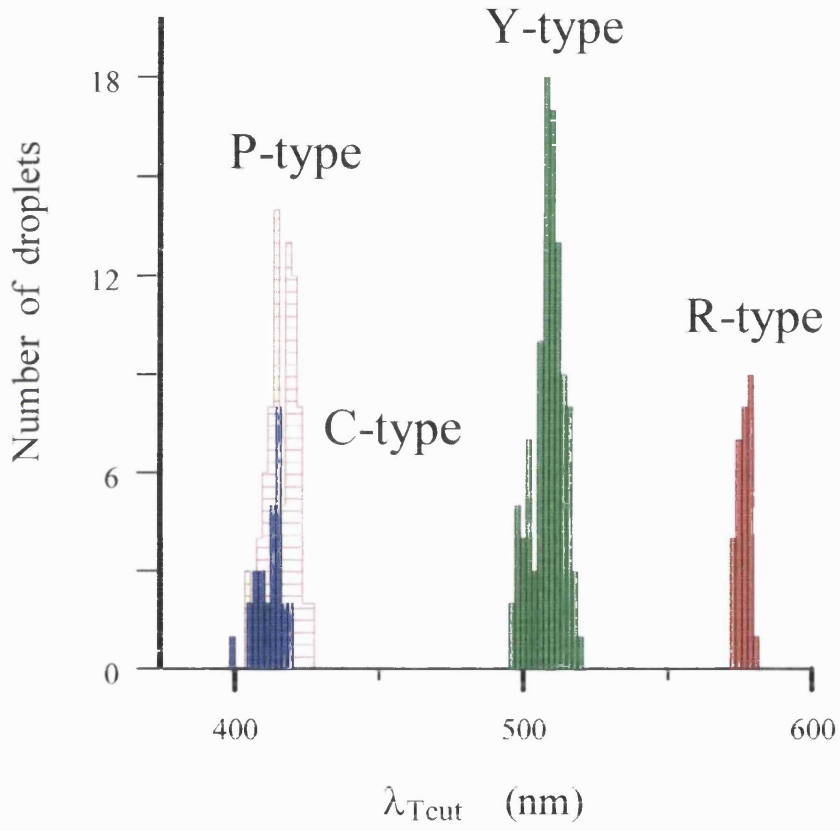
The mean oil droplet spectra of the R-, Y-, C-, P- and T-type oil droplets from *Serinus canaria*



See table 7.8e for details of numbers of oil droplets used.

Fig 7.8d

The distribution of λ_{Tcuts} from individual oil droplets from *Serinus canaria*



See table 7.8e for details of mean droplet λ_{Tcut} values.

Figure 7.8d is a histogram showing the spectral distribution of cut-off wavelengths (λ_{Tcut}) from individual oil droplets found in the retina of *Serinus canaria.* Details of the mean λ_{Tcut} and absorbance values for each droplet type are tabulated in table 7.8e.

7.9. Correlating feather and Oil droplet carotenoids

The normal yellow ground colour of canaries depends partly on genetic make up and carotenoids such as xanthophyll taken in the diet. One of the aims of this study was to ascertain whether there is any correlation between the carotenoid content of the feathers of canaries (as determined by their external phenotype) and the carotenoid content of the retinal oil droplets. Table 7.9a is a record of individual canary phenotypes together with a brief description of the appearance of the retina in the light. The three strains of canary used in experiments were 'clear yellow birds' (clear meaning feathers totally devoid of any melanistic pigmentation), 'variegated birds' (birds with feathers showing light melanistic pigmentation) and 'heavily variegated birds' (birds with feathers showing heavy melanin pigmentation in all feathers). From table 7.9a it appears that the oil droplet complement in the retina of individual canaries can vary, the most striking difference being, the apparent absence of typical R-type oil droplets in the majority of birds. However, this could not be correlated with canary phenotype.

Only five out of a total of twenty-five birds studied possessed typical R-type oil droplets (λ_{Tcut} = 578 nm) as identified by direct microspectrophotometric measurement. When viewed in the light, red (R-type) and orange (Y-type) oil droplets were clearly visible in these retinas. The P-, C- and T-type droplets were indistinguishable, all appearing pale-green in colour. In the majority of canary retinae (twenty birds) only orange (Y-type) and pale-green (P-, C- and T-type) oil droplets were observable in the light and there was a notable absence of typical red oil droplets (i.e. droplets which were red in colour when viewed in the light with λ_{Tcut} at about 578 nm).

Table 7.8e

Mean λ_{Tcut} and absorbance values of oil droplets from *Serinus canaria.*

Droplet	*N*	Found in single or double cone ?	Pigment in adjacent outer segment	λ_{Tcut} from mean droplet **absorbance** spectrum (nm)	Mean λ_{Tcut} from **distribution histogram** of individual droplets (nm)	Average droplet absorbance at λ_{Tcut}
R-type	29	single	**LWS**	578	576.2 ± 2	0.43
Y-type	101	single	**MWS**	504	508.3 ± 5	0.33
C-type	29	single	**SWS**	415	410.5 ± 10	0.14
T-type	10	single	**UVS**	-	-	-
P-type						
birds with 'reduced' R-type droplets	78	double (single ?)	**LWS**	420	416.2 ± 5	0.29
birds with normal R-type droplets	7	double	**LWS**	420	413.3 ± 4	0.13

N , number of oil droplets. Mean droplet λ_{Tcut} values from distribution histogram are ± SD. LWS = longwave sensitive 'red' cones; MWS = middlewave sensitive 'green' cones; SWS = shortwave sensitive 'blue' cones; UVS = ultraviolet sensitive 'UV' cones.

Table 7.9a
Record of the phenotypes of canaries used in the present study.

NO. OF CANARY & DATE		STRAIN 'clear', 'variegated' or 'heavily variegated'	CANARY PLUMAGE COLOUR	APPEARANCE OF RETINA IN THE LIGHT	COMMENTS
1	19-10-93	heavily variegated	cinnamon brown	red droplets not observed	some dilute P-types
2	27-10-93	clear yellow	yellow	red droplets not observed	a T-type droplet found with LWS OS; some dense P-types have a shoulder
3	08-12-93	variegated	yellow flanks, rust underside	red droplets not observed	two T-type droplets found with LWS OS
4	16-12-93	heavily variegated	cinnamon brown	red droplets not observed	P-type with a 5% shoulder
5	10-01-94	heavily variegated	cinnamon brown	red droplets not observed	
6	01-02-94	clear yellow	pale yellow	**red droplets present**	
7	28-02-94	heavily variegated	cinnamon brown	red droplets not observed	a T-type droplet found with LWS OS
8	12-04-94	variegated	cinnamon flanks, yellow underside	**red droplets present**	
9	01-06-94	variegated	cinnamon brown flanks yellow & white underside	red droplets not observed	
10	14-06-94	heavily variegated	cinnamon brown	**red droplets present**	
11	28-07-94	clear yellow	bright yellow	red droplets not observed	
12	03-08-94	clear yellow	bright yellow	red droplets not observed	

NO. OF CANARY & DATE		STRAIN 'clear', 'variegated' or 'heavily variegated'	CANARY PLUMAGE COLOUR	APPEARANCE OF RETINA IN THE LIGHT	COMMENTS
13	11-08-94	heavily variegated	cinnamon brown flanks, white underside	red droplets not observed	T-type droplet found with red OS
14	06-09-94	heavily variegated	cinnamon brown flanks, white underside	red droplets not observed	T-type droplet found with red OS, P-type droplet found with green OS
15	27-09-94	clear yellow	yellow	red droplets not observed	
16	27-10-94	variegated	yellow with some cinnamon brown	**red droplets present**	particularly dilute P-type droplets [0.08; cf. 0.1] found with presence of slight shoulder (10-30 %)
17	04-11-94	variegated	yellow, some cinnamon brown on underside	**red droplets present**	*tissue used for frozen sectioning*
18	08-11-94	variegated	grey-brown flanks, white head & underside	red droplets not observed	P-types +shoulders also some triple peaked P-types
19	30-01-95	variegated	cinnamon brown, yellow-green underside	red droplets not observed	Y-type droplets found with red OS, P-type droplets + shoulder 90% at 390 nm
20	13-02-95	variegated	cinnamon brown flanks, yellow-green underside	red droplets not observed	some P-types with shoulders
21	21-02-95	clear yellow	bright yellow with some white	red droplets not observed	very dilute C-type droplet with blue OS found [similar to random noise]
22	30-01-96	clear yellow	yellow	red droplets not observed	
23	04-02-96	clear yellow	yellow and white	red droplets not observed	P-types vary in density from, < 0.1- 0.3
24	21-02-96	heavily variegated	cinnamon brown	red droplets not observed	
25	18-03-96	clear yellow	yellow	red droplets not observed	

Of the relatively few avian species which have been examined microspectrophotometrically, the only other bird which appears to display a similar apparent absence of Red oil droplets is the penguin, *Spheniscus humboldti* (Bowmaker and Martin, 1985). In this study, the retinas of just two penguins were examined microspectrophotometrically and under the light microscope. Both were found to be dominated by yellow and pale droplets. The penguin possesses a 'longwave sensitive pigment' which absorbs maximally at about 543 nm - i.e. at intermediate wavelengths between the MWS (λ_{max} = 500 nm) and LWS (λ_{max} = 560 nm) pigments of most birds (see bird MSP chapter, table 3.12a). Clearly, the P543 cannot be paired with an R-type droplet, (λ_{Tcut} at about 560 nm) since virtually all of the useable wavelengths of light will be absorbed by the droplet before reaching the outer segment, drastically attenuating the sensitivity of the cone. Not surprisingly, 20% of the cones in *Spheniscus* are reported to consist of the P543 in combination with a typical Y-type droplet cutting off all light below about 500 nm. In contrast to the situation in *Spheniscus,* the LWS pigment in the canary, *Serinus canaria,* has a λ_{max} at about 568 nm which does not warrant the substitution of a Y-type droplet for the R-type droplet in LWS cones. The calculated spectral sensitivities derived from cones identified in the penguin suggest that it would be incapable of any wavelength discrimination beyond about 550 nm. This has been correlated its aquatic environment, which consists of 'blue-water' bodies such as deep coastal and oceanic waters in which there is relatively little light beyond about 575 nm (Bowmaker and Martin, 1985). In contrast to the penguins' aquatic habitat, longwave incident light is not attenuated in the terrestrial environment of the wild canary. It should be remembered, however, that the birds used in this study were domestic canaries (see below); studying the oil droplet complement in the retina of wild canaries would be informative; if wild birds also show an apparent absence of R-type droplets, then perhaps this condition has a functional significance. However, based on the evidence available from other *Passerine* species (Maier and Bowmaker, 1993; Goldsmith *et al.*, 1984; Bowmaker *et al.,* 1997) it is more likely that the retina of wild-type canaries contains the full set of five oil droplets, including the R-type droplet.

An obvious explanation for the pattern of occurrence of R-type oil droplets in these canaries is that it is a consequence of domestication. The canaries used in these experiments are all descendants of the wild canary which inhabits woods, scrubland and savanna. The wild canary has been removed from its natural habitat, confined to a cage or aviary, and had its food restricted to whatever breeders have chosen to provide (Walker and Avon, 1993). Such changes in diet and levels of exercise in birds, have been suggested to cause significant alterations to various body organs, possibly leading to degeneration and disease (Wood, 1917). Perhaps this theory can be extended to the

retinal oil droplets.

Since birds are unable to synthesize carotenoids (the precursors of oil droplet pigments) *de novo,* and must obtain them from ingested food (Fox and Vevers, 1960), one could argue that the apparent absence of typical red oil droplets in some canaries is due to a reduction in the level of dietary carotenoids. However all the birds were maintained on the same standard canary diet, containing recommended levels of carotenoid. Furthermore oil droplet carotenoids are very stable and experiments on the Japanese quail, *Coturnix coturnix japonica,* have shown that birds subjected to several months of carotenoid deprivation, still retain the pigment in their oil droplets (Wallman, 1979). Moreover, in cases where there is a genuine lack of dietary carotenoids, all droplets are affected - not just the R-types. For example, quail, maintained on carotenoid free rations over at least two generations possess oil droplets which are all carotenoid-free and colourless (Wallman, 1979; Meyer *et al.,* 1971; Bowmaker *et al.,* 1993).

Pézard (1931; 1957 a,b ; 1964 as cited by Meyer, 1977) suggested that the frequency of different types of coloured oil droplet in the chicken retina could be altered by maturity, season of the year, castration, sexual and spermatogenic activity. Table 7.9a catalogues all the birds used in the present study, showing the day month and year of sacrifice. Canaries 10 to 25 were used in experiments over a period of 21 months and, being of the same batch, were identical in age and sex (male - as inferred from their singing ability). However, in contrast to Pézards' findings, there is no change in the incidence of R-type droplets in the retina of young canaries (i.e. those sacrificed early on in the study) compared to older, maturer canaries (i.e. those sacrificed later on), nor is there any change during a particular season of the year, e.g. in the Summer months. If the frequency of oil droplets in a retina do change with the hormonal state of a bird, then the overall spectral sensitivity of the cones is also likely to change. For example, changes in the mixture of oil droplet carotenoids, will alter the spectral quality of the light reaching the adjacent outer segment, and, possibly the spectral sensitivity of the constituent cone (depending on the extent of the changes). This could lead to alterations in the photopic sensitivity of the bird, similar to the seasonal spectral shifts which occur in fish, such as cyprinids and migratory salmonids, due to variations in the ratios of A1 and A2 pigments in the photoreceptors (see Bowmaker, 1991a). Pézard suggested that capons (castrated male cocks) had fewer red droplets, thereby being less sensitive to the red combs of other chickens, and are thus less aggressive. However, Dücker (1970) and Mayr (1970) [as cited by Meyer, 1977], in testing Pézards results, were unable to show any correlation between colour preference and oil droplet frequency distribution in chickens. A separate study on selected strains of quail showing differences in their early colour preferences also failed to find any statistically significant difference

between the relative proportions of droplet types in control and test birds (Bowmaker *et al.,* 1993). Subtle differences in the proportion of droplets probably occur naturally between individual birds, and it is possible that this is what Pézard observed.

A genetic mutation

The pattern of occurrence of typical R-type oil droplets in the present study can best be described as being 'all or nothing' - in the sense that the canaries either contain red oil droplets or do not. This suggests that the mechanism responsible, is similarly 'on' or 'off' in nature rather than being gradual in onset. Since the characteristic red colour of R-type droplets is due to the carotenoid, astaxanthin (Wald & Zussman, 1938; Strother and Wolken, 1960; Meyer *et al.,* 1965) the most probable explanation for the apparent absence of red oil droplets in some canaries is that a mutation has occurred in one of the genes coding for a critical enzyme in the astaxanthin synthesis pathway. This would render all R-type droplets astaxanthin-free and reduce their content to any remaining secondary carotenoids. These droplets will be referred to as 'reduced' R-type droplets.

In any population, a mutation may arise in a gene and go unnoticed if it is not deleterious and does not change the outward appearance of the animal. In the wild, there is a strong chance that the faulty gene will be 'corrected' through successive out-breeding (i.e. through natural selection). However, this might not occur in domestic breeds of canary due to subsequent intensive inbreeding (i.e. consanguineous crosses) in order to enhance a certain unrelated aspect of the canary phenotype, such as feather colour or posture. In theory, the faulty gene could rapidly pass on to subsequent generations, eventually becoming established within the genome of that particular breed.

If the mutation theory is correct, identification of the exact step in the astaxanthin synthesis pathway would be an interesting subject for future research. An alternative possibility is that there has been an interruption to the astaxanthin transportation mechanism into oil droplets. However, disruption of the astaxanthin synthesis pathway seems the more likely explanation since this is specific to the R-type droplet carotenoid whereas it is probable that a common carotenoid transportation mechanism exists, disruption of which would affect all droplet-types. Although the molecular basis underlying the production and segregation of specific carotenoids into particular cone types has not been investigated, several theories have been suggested. For example, Meyer *et al.,* (1971a) postulate that astaxanthin is either biosynthesized from precursors within the retina or is produced elsewhere (e.g. in the liver) and selectively transported into the eye. In contrast, Johnson and Hudson (1976) suggest the possibility that the exact composition of the mixed lipids may be important in determining the carotenoid

which will eventually 'fill' a droplet, due to the varying solubilities of carotenoids in different lipid mixtures.

One question remaining is which carotenoid, if any, replaces astaxanthin in 'reduced' R-type droplets; in other words, what pigment-oil droplet combination constitutes a single red cone? Astaxanthin is usually found to be the sole carotenoid in R-type droplets (Goldsmith *et al.,* 1984). However some birds (and turtles) posses O-type or 'orange' droplets which can either contain low concentrations of astaxanthin, as in the common tern, *Sterna hirundo,* or else a mixture of two carotenoids, as is found in the mourning dove, *Zenaida macroura,* (Goldsmith *et al.,* 1984; Liebman and Granda, 1975; Lipetz, 1984b; Partridge, 1989). In a study looking at the development of oil droplet carotenoids from carotenoid-deprived Japanese quail chicks, *Coturnix coturnix japonica,* (following the introduction of a normal diet) Bowmaker *et al.,* (1993), noticed a secondary peak at about 440 nm in R-type droplets suggesting the presence of a mixture of two carotenoids. Hence, R-type droplets in the retina of the canary may contain a mixture of carotenoids and in the absence of astaxanthin in 'reduced' R-type droplets only a secondary pigment would remain, possessing a peak at about 440 nm similar in structure to the P-type droplets. In support of this in canaries possessing 'reduced' R-type oil droplets, LWS outer segments were only ever found with P-type oil droplets in what were assumed to be the Principal member of double cones. A proportion of these 'Principal cones' may actually have been 'single red cones' containing astaxanthin-free oil droplets. In birds with 'reduced' R-type droplets it may be possible to distinguish two populations of 'P-type' droplets; authentic P-type droplets and pseudo P-type droplets which are the R-type droplets minus astaxanthin.

Figure 7.9b is a scatterplot of the λ_{Tcut}s of all oil droplet types found in the canary. The P-type droplets appear to show a normal distribution, with no suggestion of a bimodality. Figure 7.9c looks at P-type droplets in detail, correlating the individual droplet λ_{Tcut}s, with maximal droplet absorbance. The P-type droplets from birds possessing normal R-type droplets, are also plotted, since we can be sure that these are genuine P-type droplets. At first glance the genuine P-type droplets [filled red squares] appear to form a cluster of points around a λ_{Tcut} at about 415 nm, and a relatively low droplet absorbance, at about 0.1. However, there is one conspicuous outlier which has a longer λ_{Tcut} (about 420 nm) and a higher absorbance (0.3). Thus, there are no uniform characteristics with which to clearly define genuine P-type droplets. Nevertheless, figure 7.9c confirms the absence of two groups of P-type droplets from birds with 'reduced' R-type droplets. Evidently, the spectral signature of the putative secondary carotenoid in the 'reduced' R-type droplets is very similar (or identical) to the P-type droplet carotenoid(s).

The presence of a secondary carotenoid in R-type droplets besides astaxanthin, may be more common than results suggest. Its extremely low concentration may preclude detection since it is completely masked by the astaxanthin spectrum. Goldsmith *et al.,* (1984) expanded all oil droplets and diluted them with mineral oil before taking measurements, yet it was only in the orange 'O-type' droplets that a secondary pigment was detectable, presumably due to the particularly low concentrations of astaxanthin. Bowmaker *et al.,* (1993), looked at the development of oil droplet carotenoids in the retinas of carotenoid-free quail chicks [see their figure 2]. Following the introduction of a normal diet, a secondary pigment in R-type droplets was detectable for just three days, when the concentration of astaxanthin was sufficiently low, (peak absorbance $\leq$ 0.1.), but by seven days on a normal diet, the steady accumulation of astaxanthin (peak absorbance of $\leq$ 0.36) was high enough to completely obscure it. For a comparison R-type droplet carotenoids from the domestic chicken, *Gallus domesticus* , a close relative of the quail, have also been examined by Goldsmith *et al.,* (1984) using the method of oil droplet dilution with mineral oil. However, a secondary carotenoid was not observed using this method. Alternatively the R-type oil droplet carotenoid(s) may not have been diluted sufficiently to allow the detection of secondary carotenoid.

If there is a secondary pigment in R-type droplets, its concentration seems to vary considerably; in some droplets it is found in high concentration whereas in others in minuscule quantities. In the latter case only neutral lipids would occupy the droplet which would consequently have an absorbance spectrum resembling that of T-type droplets. In support of this six cones consisting of a LWS outer segment associated with a T-type oil droplet (see table 7.9a under 'comments') were found in the retinas of canaries with the 'reduced' R-type droplets.

Fig 7.9b

Correlation of λ_{Tcut} with maximal absorbance of oil droplets from *Serinus canaria*

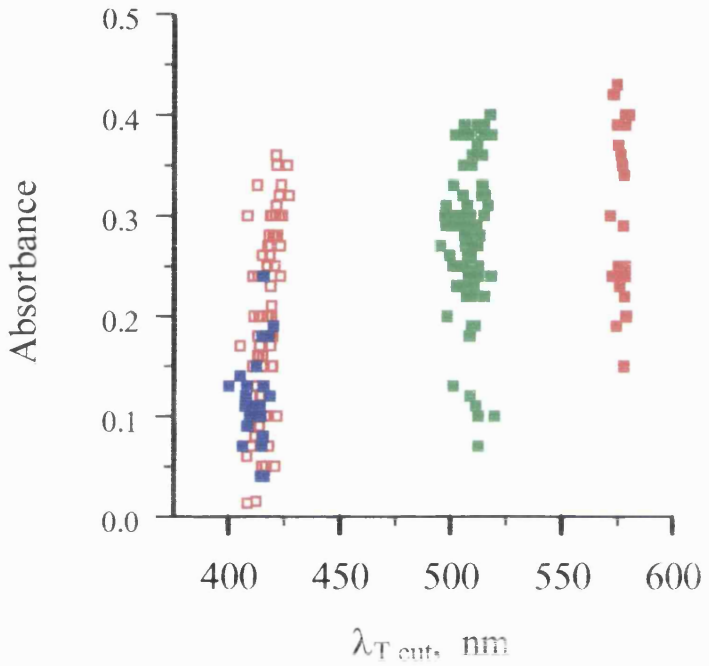
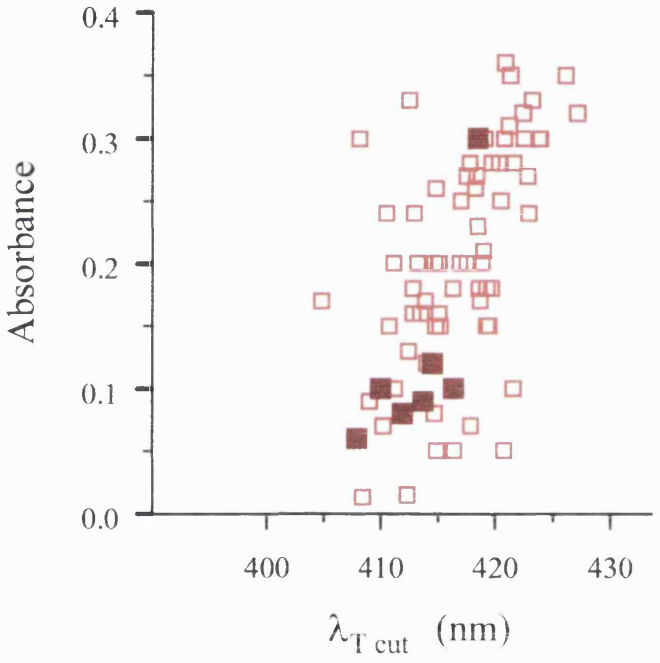


Fig 7.9c

Correlation of maximal absorbance of P-type droplets with λ_{Tcut} from birds possessing typical R-type oil droplets or 'reduced' R-type oil droplets



□ Birds possessing reduced R-type oil droplets
■ Birds possessing typical R-type oil droplets

RESULTS AND DISCUSSION

7.10. MOLECULAR GENETICS - General summary of results

- The complete coding region of the ultraviolet sensitive opsin gene from *Serinus canaria* including the 5' and 3' untranslated regions was cloned and sequenced from retinal cDNA.

- Approximately 1 Kb of partial cDNA sequence, encompassing exons 1- 5 of the canary SWS-, MWS- and rod opsins was cloned and sequenced, together with 620 bp of the LWS opsin.

The following pages describe the individual cloning strategies used to isolate each opsin gene from *Serinus canaria.* The final opsin cDNA sequence together with the predicted amino acid translation is presented for each gene together with subsequent manipulations of the data. These include drawing of hydrophobicity plots, comparisons of percentage nucleotide and amino acid sequence identity with other opsins, classification in an opsin phylogenetic tree and finally drawing a two dimensional model of each canary opsin. Putative spectral tuning sites will be discussed in the Discussion **section 7.16.**

7.11. Isolation of the ultraviolet sensitive opsin cDNA of *Serinus canaria*

Table 7.11a depicts the cloning strategy used to isolate fragments of the putative UVS sensitive opsin gene from *Serinus canaria* . Details are given of each combination of primers used and specific PCR (polymerase chain reaction) conditions employed. For convenience, the approximate position of each primer has been drawn schematically on the gene in fig. 7.11b (refer to table 6.14a for actual primer sequences).

Table 7.11a

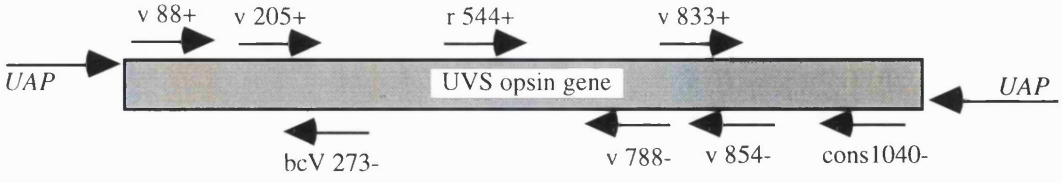
Primer pairs and specific reaction conditions used to amplify fragments of the putative UVS opsin gene from canary cDNA.

PRIMER PAIR	T_m	PCR cycle used	$[Mg^{2+}]$ (mM)	approx. size of product (bp)	Probable identity of amplified fragment *a*
v 88+ v 854-	60° 61°	T_d 94° @ 1s T_a 59° @ 2s T_e 72° @ 15s	3.0	800	UVS opsin gene
v 88+ cons 1040-	60° 65°	T_d 94° @ 1s T_a 59° @ 2s T_e 72° @ 20s	3.0	950	UVS opsin gene
r 544+ cons 1040-	69° 64°	T_d 94° @ 1s T_a 58° @ 1s T_e 72° @ 2s	3.0	500	UVS opsin gene
bcV 833+ UAP	68° 93°	T_d 94° @ 1s T_a 64° @ 1s *no* T_e	2.5	400	5' end of UVS opsin gene
bcV 273- UAP	64° 93°	T_d 94° @ 1s T_a 62° @ 1s *no* T_e	2.5	400	3' end of UVS opsin gene

* For each PCR, the DNA was subjected to an initial 10 second denaturation at 94°C before cycling commenced, and a final 1 minute extension at 72°C. T_d, T_a, T_e, T_m, denaturation, annealing, extension and calculated primer annealing temperatures (see methods section); $[Mg^{2+}]$=magnesium ion concentration. UAP=universal adaptor primer (supplied with RACE kit).

Figure 7.11b

Schematic diagram showing the approximate position of primers used in PCRs to amplify fragments of the putative canary UVS opsin gene (represented schematically by the shaded bar).



UAP=universal adaptor primer (supplied with RACE kit). See table 6.14a for actual primer sequences.

```
CGC ATGGACGAGGAAGAGTTCTACCTGTTCAAGAACCAGTCGTCGGTGGGGCCCTGGGATGGG      60
    M  D  E  E  E  F  Y  L  F  K  N  Q  S  S  V  G  P  W  D  G       20

    CCCCAGTACCACATCGCGCCCATGTGGGCCTTCTACCTRCAGACCATCTTCATGGGCCTG     120
    P  Q  Y  H  I  A  P  M  W  A  F  Y  L  Q  T  I  F  M  G  L       40

    GTGTTTGTGGCGGGCACCCCCCTGAACGCCATCGTCCTCATCGTCACCGTCAAGTACAAG     180
    V  F  V  A  G  T  P  L  N  A  I  V  L  I  V  T  V  K  Y  K       60

    AAGCTGCGGCAGCCGCTCAACTACATCCTGGTGAACATCTCCGTCAGCGGCCTCATGTGC     240
    K  L  R  Q  P  L  N  Y  I  L  V  N  I  S  V  S  G  L  M  C       80

    TGCGTCTTCTGCATCTTCACCGTCTTCGTCGCCAGCTCCCAGGGATACTTTGTCTTCGGG     300
    C  V  F  C  I  F  T  V  F  V  A  S  S  Q  G  Y  F  V  F  G      100

    AAGCACATGTGTCGCTTTGAGGGCTTTGCGGGGGCCACCGGAGGGATGGTGACAGGGTGG     360
    K  H  M  C  R  F  E  G  F  A  G  A  T  G  G  M  V  T  G  W      120

    TCCTTGGCCTTCCTGGCCTTTGAGCGCTACATCGTCATCTGCAAACCCTTCGGCAACTTC     420
    S  L  A  F  L  A  F  E  R  Y  I  V  I  C  K  P  F  G  N  F      140

    CGCTTCAACTCCCGCCACGCGCTGCTGGTGGTGGCGGCCACCTGGATCATCGGCGTCGGC     480
    R  F  N  S  R  H  A  L  L  V  V  A  A  T  W  I  I  G  V  G      160

    GTCGCCATCCCGCCCTTCTTCGGCTGGAGCAGGTACATCCCCGAGGGGCTGCAGTGCTCC     540
    V  A  I  P  P  F  F  G  W  S  R  Y  I  P  E  G  L  Q  C  S      180

    TGCGGCCCTGACTGGTACACGGTGGGCACCAAGTACAAGAGCGAGTACTACACCTGGTTC     600
    C  G  P  D  W  Y  T  V  G  T  K  Y  K  S  E  Y  Y  T  W  F      200

    CTCTTCATCTTCTGCTTCATCATCCCCCTCTCCCTCATCATCTTCTCCTACTCCCAGCTG     660
    L  F  I  F  C  F  I  I  P  L  S  L  I  I  F  S  Y  S  Q  L      220

    CTCAGTGCCCTGCGCGCCGTGGCGGCGCAGCAGCAGGAGTCGGCCACGACGCAGAAGGCG     720
    L  S  A  L  R  A  V  A  A  Q  Q  Q  E  S  A  T  T  Q  K  A      240

    GAGCGGGAGGTGTCGCGCATGGTCGTGGTCATGGTGGGTTCCTTCTGCATGTGCTACGTG     780
    E  R  E  V  S  R  M  V  V  V  M  V  G  S  F  C  M  C  Y  V      260

    CCCTACGCGGCGCTGGCCATGTACATGGTGAACAACCGCGAGCACGGCATCGACCTGCGC     840
    P  Y  A  A  L  A  M  Y  M  V  N  N  R  E  H  G  I  D  L  R      280

    CTCGTCACCATCCCTGCCTTCTTCTCCAAGAGCTCCTGCGTCTACAACCCCATCATCTAC     900
    L  V  T  I  P  A  F  F  S  K  S  S  C  V  Y  N  P  I  I  Y      300

    TGCTTCATGAACAAACAGTTCCGCGCCTGCATCATGGAGACGGTGTGCGGGCGGCCCATG     960
    C  F  M  N  K  Q  F  R  A  C  I  M  E  T  V  C  G  R  P  M      320

    TCCGACGACTCCGACGTGTCCAGCTCGGCCCAGCGCACCGAGGTCTCCTCCGTGTCCTCC    1020
    S  D  D  S  D  V  S  S  S  A  Q  R  T  E  V  S  S  V  S  S      340

    AGCCAGGTCGGCCCCGGCCAGCCTAGGATGTAG CGCGGGTGGGACAATAAACCA         1053
    S  Q  V  G  P  G  Q  P  R  M  stop                               350

    TGGCCGCGGTTTGAGCGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 7.11c
Complete cDNA sequence and predicted amino acid translation of the UVS opsin gene from *Serinus canaria*

The complete nucleotide sequence (obtained by extracting consensus sequence from overlapping fragments) as well as the predicted amino acid translation product of the putative canary UVS opsin gene is shown in fig. 7.11c. From fig. 7.11c it can be seen that the ATG start codon is in agreement with the Kozak criteria for eukaryotic initiation sites (Kozak, 1981), and is followed by an open reading frame of 1053 bases ending at the termination codon TAG. Taking the ATG as the translation initiation site, the canary UVS cDNA encodes an opsin of 350 amino acid residues. A poly (A) addition signal (AATAAA) is present in the 3' untranslated region, 13 bp downstream from the stop codon, and 21 bp upstream of the poly (A) tail. The Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence of the UVS opsin (fig. 7.11d) indicates the presence of seven potential transmembrane domains, separated by hydrophillic regions, typical of the family of G-protein coupled receptors.

Comparison of the deduced amino acid sequence of the putative canary UVS opsin with that of 28 other vertebrate opsins reveals that it shares the highest percentage identity with the budgerigar UVS opsin (87%) followed by the putative chameleon violet- (85%) and chicken violet- (84%) opsins (See table 7.11e) Comparisons at the nucleotide level yield very similar results (not shown). When compared to 28 published vertebrate opsin sequences, the 3' end of the coding sequence of the canary UVS opsin appears to be fractionally longer by 12 bp with the result that the C-terminal segment of the opsin has four extra amino acids. Since this only appears to occur in the canary UVS opsin, a tentative explanation would be that the 12 extra bases arose as a recent insertion event. However, since DNA from only one canary was used in this study, data from other individuals would be required to verify this.

Fig 7.11d

Kyte-Doolittle Hydrophobicity plot of the UVS opsin isolated from *Serinus canaria*

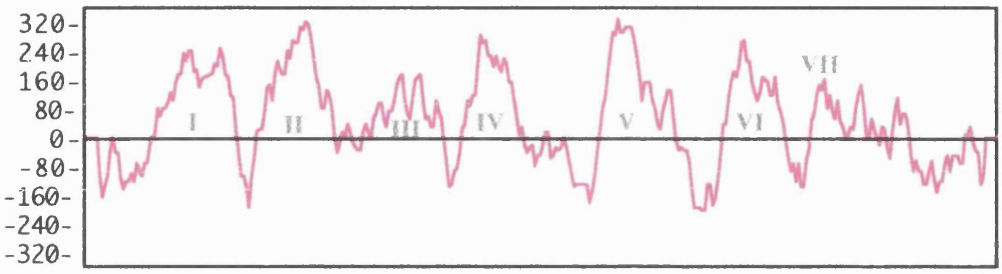


Table 7.11e

Percentage of identical amino acid residues per site between the putative canary UVS opsin and 28 vertebrate opsins.

Vertebrate opsin	% similarity (± S.E.) to putative UVS opsin from *Serinus canaria*
mou 360	**79.6 ± 2**
gfsh 365	62.8 ± 3
cmln viol	**85.3 ± 2**
can 366	**100**
bud 371	**86.7 ± 2**
rat blu	**79.0 ± 2**
bov blu	**73.7 ± 2**
xen viol	**78.1 ± 2**
chk 418	**83.5 ± 2**
hum420	**79.0 ± 2**
mar 423	**80.2 ± 2**
tal 429	**79.2 ± 2**
can 442	53.7 ± 3
gfsh 441	48.8 ± 3
bud 444	36.6 ± 3
cmln blu	52.3 ± 3
chk 455	51.9 ± 3
cfsh blu	52.2 ± 3
can 506g	49.8 ± 3
duc 502g	50.2 ± 3
chk 507g	50.4 ± 3
bud 508g	49.1 ± 3
can 506r	49.1 ± 3
duc 505r	47.9 ± 3
chk 506r	48.5 ± 3
bud 509r	47.6 ± 3
can 569	46.0 ± 4
chk 571	42.8 ± 3
dmRh1 (invertebrate out group)	25.3 ± 2

Percentage identity values greater than 70 % are indicated in bold lettering. Colours represent the opsin class (e.g. red lettering represents opsins belonging to the LWS class). (see page 166 for list of abbreviations used)

List of abbreviations and references used in alignment tables and phylogenetic trees

Abbreviation	Full name	λ_{max} (nm)	Reference
duc 502g	duck MWS opsin	502	Heath *et al.*, in prep; Bowmaker *et al.*, 1997
chk 507g	chicken MWS opsin	507	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
bud 508g	budgerigar MWS opsin	508	Heath *et al.*, in prep; Bowmaker *et al.*, 1997
can 506g	canary MWS opsin	506	present thesis
bud 509r	budgerigar rod opsin	509	Heath *et al.*, in prep
chk 506r	chicken rod opsin	506	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
can 506r	canary rod opsin	506	present thesis
duc 505r	duck rod opsin	505	Heath *et al.*, in prep
gfsh 441	goldfish SWS opsin	441	Johnson *et al.*, 1993
cfsh blu	cavefish SWS opsin	unknown	Yokoyama & Yokoyama, 1993
bud 444	budgerigar SWS opsin	444	Wilkie *et al.*, in prep; Bowmaker *et al.*, 1997
cmln 447	chameleon SWS opsin	(A1) 447	Kawamura & Yokoyama, 1996
can 440	canary SWS opsin	440	present thesis
chk 455	chicken SWS opsin	455	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
gfsh 365	goldfish UV opsin	365	Hisatomi *et al.*, 1996; Bowmaker *et al.*, 1991c
can 366	canary UV opsin	366	present thesis
bud 371	budgerigar UV opsin	371	Wilkie *et al.*, 1997; Bowmaker *et al.*, 1997
mou 360	mouse UV opsin	360	Jacobs *et al.*, 1991; Chiu *et al.*, 1994
chk 418	chicken violet opsin	418	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
mar 423	marmoset 'blue' opsin	423	Hunt *et al.*, 1995 a & b
hum 420	human 'blue' opsin	420	Nathans *et al.*, 1986; Bowmaker & Dartnall, 1980
tal 429	talapoin 'blue' opsin	429	Hunt *et al.*, 1995 a & b
xen viol	xenopus violet opsin	unknown	Knox, 1995
cmln viol	chameleon violet opsin	unknown	Kawamura & Yokoyama, 1996
rat blu	rat'blue like' opsin	unknown	Zhao *et al.*, 1996
bov blu	bovine 'blue' opsin	unknown	Chiu *et al.*, 1994
can 567	canary LWS opsin	567	present thesis
chk 571	chicken LWS opsin	571	Kuwata *et al.*, 1990; Bowmaker *et al.*, 1997
dm Rh1	*Drosophila melanogaster* Rh1 opsin		O'Tousa *et al.*, *1985*

Further confirmation of the identity of this gene as the canary UVS opsin comes from the phylogenetic analyses, in which it is grouped into the same clade as budgerigar UV and chicken violet opsin (see fig. 7.11f). The tree shown in fig. 7.11f is based on the frequency of amino acid substitutions between sequences, using the *Drosophila melanogaster Rh1* opsin as an outgroup (O'Tousa *et al.,* 1985). The tree topology seen in fig. 7.11f is almost exactly replicated in the phylogenetic tree drawn from calculations based on the frequency of nucleotide substitutions shown in fig. 7.11g. (Some opsins including the putative chameleon violet-, chameleon blue- and cave fish blue opsins have been omitted from the tree in fig. 7.11g since their nucleotide sequences were not available on the genome database at the time of tree construction).

The phylogenetic tree based on nucleotide substitutions (fig. 7.11g) shows that the node at which the canary and budgerigar UVS opsins branch off within the avian violet sensitive opsin clade is well supported, as indicated by the high bootstrap confidence value (BCV) of 99. However the equivalent node in the tree based on amino acid substitutions is not as well supported (BCV=69) perhaps reflecting the smaller number of amino acid than nucleotide substitutions between the genes in these two species. In both phylogenetic trees the opsins seem to group on the basis of species similarity (i.e. phylogenetic/taxonomic similarity) as well as opsin class. This is seen most clearly in the violet sensitive opsin clade in fig. 7.11f which contains 12 different species. After the initial branching of the tree which separates the LWS opsins from the other opsins, the violet sensitive lineage is the next to branch off with the goldfish ultraviolet and xenopus violet opsins (representatives of the older, non- or semi- terrestrial vertebrate groups, fish and amphibia, respectively) forming separate branches. The violet/ultraviolet sensitive opsins from avian and reptilian species (as represented by the putative chameleon violet opsin) cluster in a separate clade, distinct form the mammalian violet/ultraviolet sensitive opsin clade.

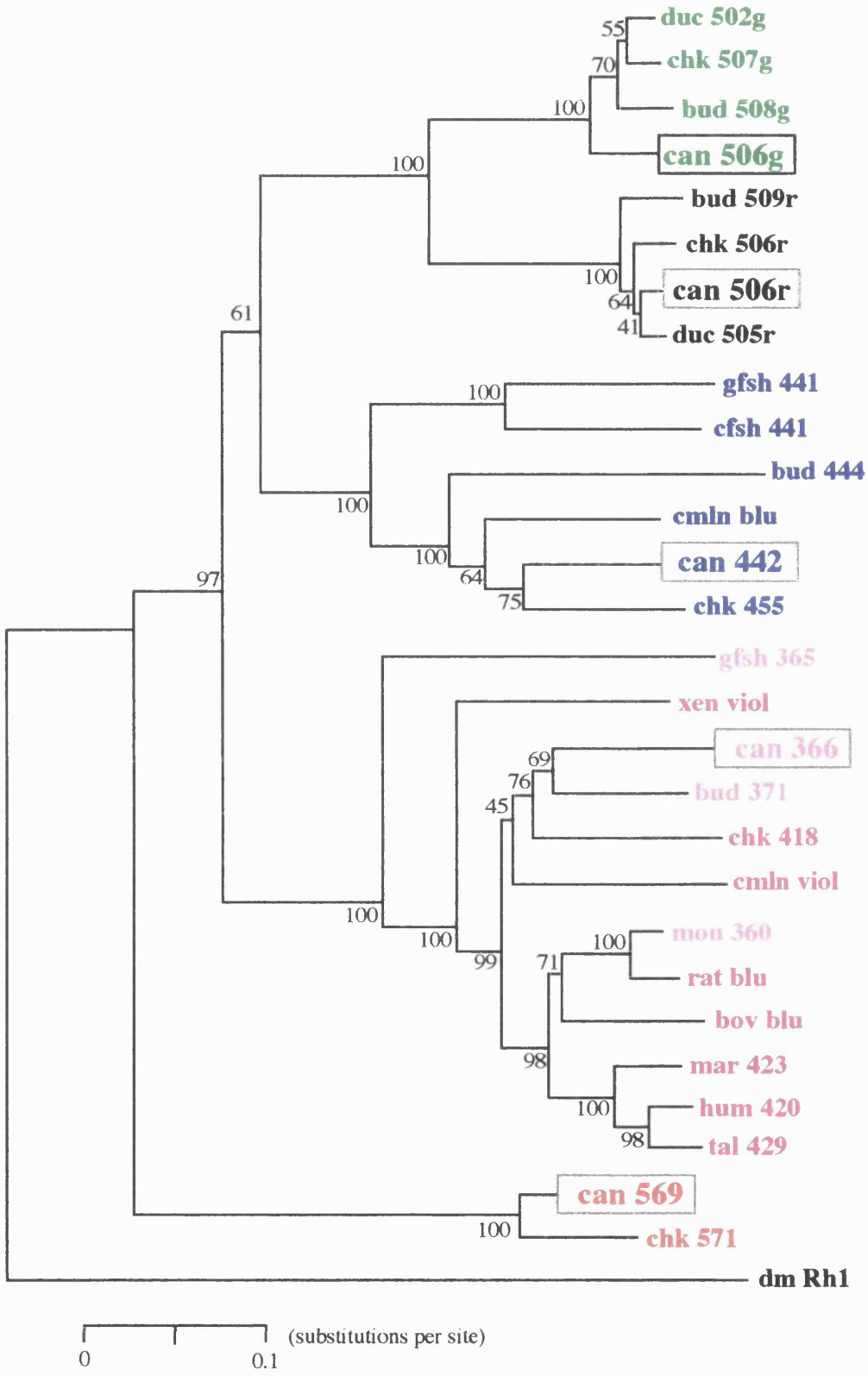


Fig 7.11f
Phylogenetic tree of vertebrate opsins based on amino acid similarity.

The tree was generated by the neighbour-joining method (Saitou and Nei, 1987) from the frequency of amino acid substitutions between sequences, using the *Drosophila melanogaster* Rh1 opsin (O'Tousa *et al.*, 1985) as an out group. The bootstrap confidence values based on 500 replicates are shown.

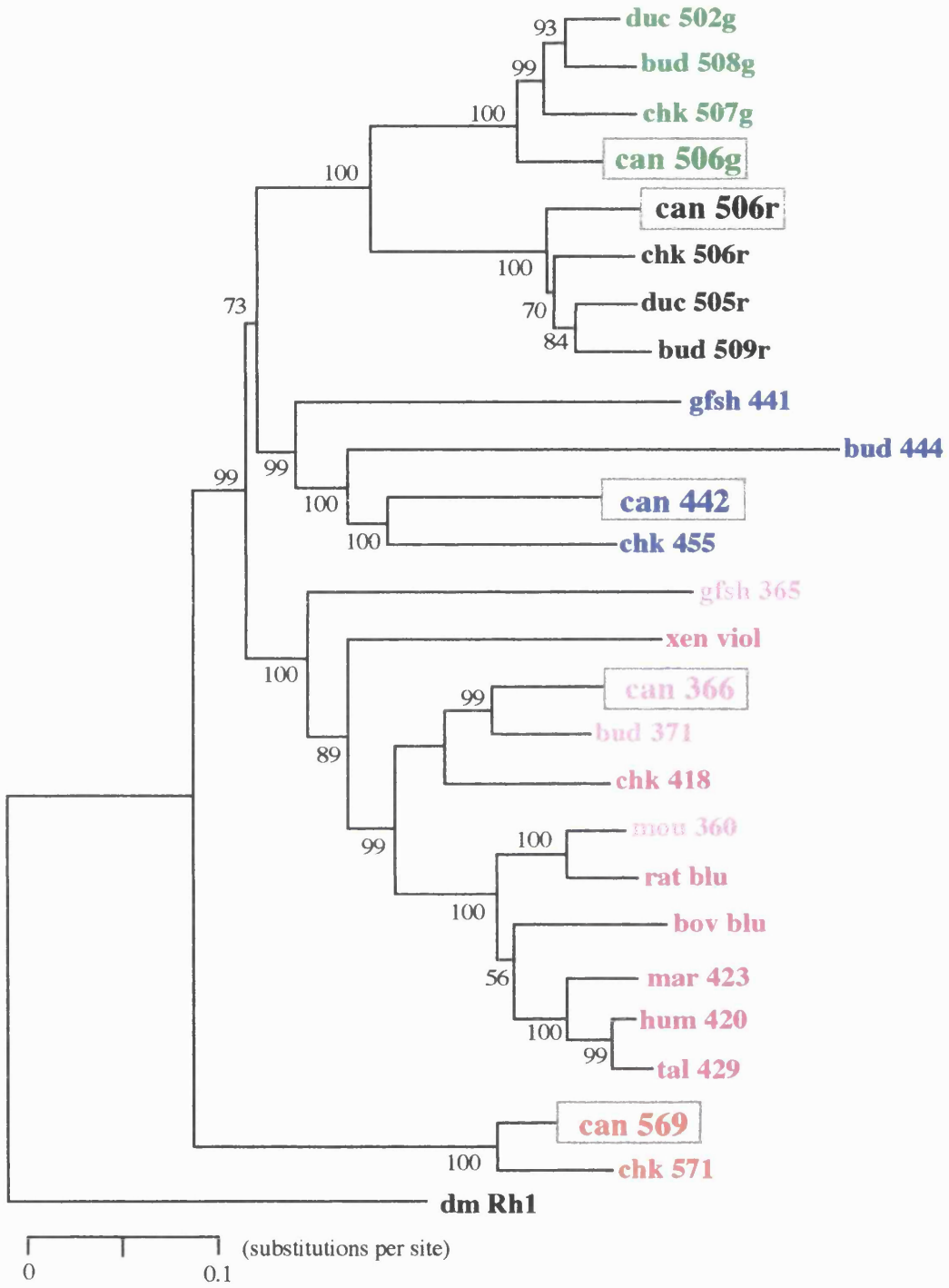


Fig 7.11g
Phylogenetic tree of vertebrate opsins based nucleotide similarity.

The tree was generated by the neighbour-joining method (Saitou and Nei, 1987) from the frequency of nucleotide substitutions between sequences, using the *Drosophila melanogaster* Rh1 opsin (O'Tousa *et al.*, 1985) as an out group. The bootstrap confidence values based on 500 replicates are shown.

Figure 7.11h is a two dimensional representation of the UVS opsin from *Serinus canaria.* Each α-helical region is shown as 26 residues in length, with only 18 core residues embedded in the membrane. All residue numbers in parenthesis refer to the numbering system of bovine rod opsin. A Lys residue for the attachment of retinal, via a Schiff's base linkage is present at position 290 (Lys-296) in helix VII (Bownds, 1967; Wang *et al.,* 1980). Glu-107 (Glu-113) located towards the end of helix III close to the extracellular surface, is the Schiff's base counterion (Sakmar *et al.,* 1989; Zhukovsky and Oprian, 1989; Nathans, 1990a). The residues Cys-104 and Cys-181 (Cys-110 and Cys-187), located in the first and second extracellular loops, respectively, are capable of forming a disulphide bridge, essential for opsin stability (Karnik and Khorana, 1990). There is a conserved Glu-128 - Arg-129 - Tyr-130 (ERY) motif (Glu-134 - Arg-135 - Tyr-136) at the junction of helix III and the second intracellular loop, which is thought to interact directly with the G-protein transducin (Franke *et al.,* 1990). A single potential glycosylation site, Asp-11 -X- Ser-13 (Asp-15 -X-Ser/Thr-17), is present in the amino terminal domain which is important for anchoring the polypeptide into the membrane. Multiple serines and threonines are present near the carboxy-terminal which are potential sites of rhodopsin kinase phosphorylation (Hargrave, 1982). One potential palmitoylation site Cys-316 (Cys-322) exists in the C-terminal region of the canary UVS opsin and may anchor it to the membrane to form an extra cytoplasmic loop (Ovchinnikov *et al.,* 1988). The three amino acid residues Gly-77, Ser-211 and Pro-261 (Gly-82, Ser-217 and Pro-267) which are sites of mutations in the human blue pigment (which clusters in the ultraviolet lineage in phylogenetic trees - see figs. 7.11f and 7.11g causing tritanopia (Weitz *et al.,* 1992a; 1992b), are all conserved in the canary UVS opsin indicating its functional significance.

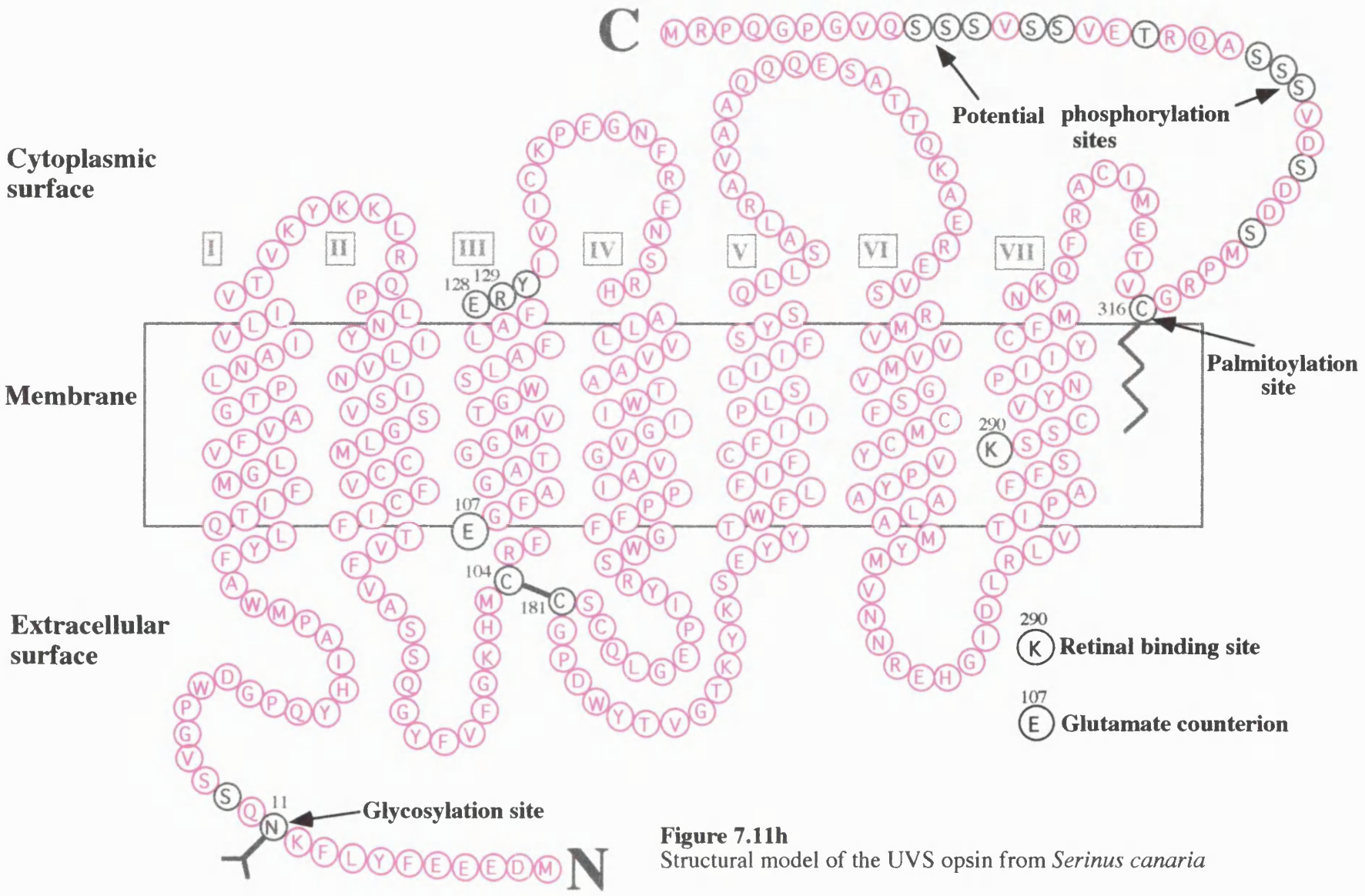


Figure 7.11h
Structural model of the UVS opsin from *Serinus canaria*

7.12. Isolation of the SWS opsin cDNA of *Serinus canaria*

The cloning strategy used to isolate fragments of the putative SWS opsin gene from *Serinus canaria* is depicted in table 7.12a which gives details of each combination of primers used and specific PCR conditions employed. For convenience, the approximate position of each primer has been drawn schematically in fig. 7.12b

Table 7.12a

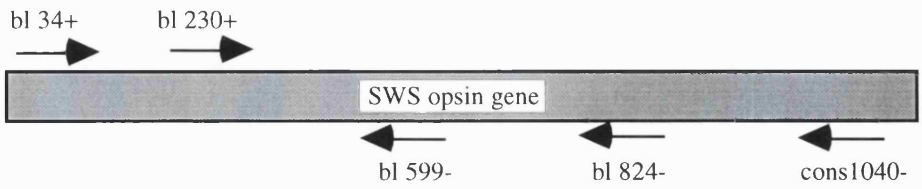
Details of primer pairs and specific reaction conditions used to amplify fragments of the putative SWS opsin gene from canary cDNA.

PRIMER PAIR	T_m	PCR cycle used	$[Mg^{2+}]$ (mM)	approx. size of product (bp)	Probable identity of amplified fragment
bl 34+ bl 599-	59° 68°	T_d 94° @ 1s T_a 59° @ 2s T_e 72° @ 15s	3.0	550	SWS opsin gene
bl 34+ cons1040-	59° 65°	T_d 94° @ 1s T_a 59° @ 2s T_e 72° @ 20s	3.0	1000	SWS opsin gene
bl 230+ bl 824-	68° 64°	T_d 94° @ 1s T_a 60° @ 2s T_e 72° @ 15s	3.0	600	SWS opsin gene

For each PCR, the DNA was subjected to an initial 10 second denaturation at 94°C before cycling commenced, and a final 1 minute extension at 72°C. T_d, T_a, T_e, T_m, denaturation, annealing, extension and calculated primer annealing temperatures (see methods section); $[Mg^{2+}]$=magnesium ion concentration.

Figure 7.12b

Schematic diagram showing the approximate position of primers used in PCRs to amplify the putative canary SWS opsin gene, represented schematically by the shaded bar.



(See table 6.14a for actual primer sequences.)

Figure 7.12c shows the partial cDNA sequence obtained from the putative canary SWS opsin, together with the predicted amino acid translation. Approximately 1 Kb of sequence was obtained, roughly corresponding to the region between exons 1 through to 5 (it is not possible to state precisely where the intron/exon boundaries are since the genomic structure of the canary SWS gene is unknown). The 3' and 5' untranslated regions of the gene have yet to be cloned. The Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence of the putative canary SWS opsin indicates the presence of seven hydrophobic transmembrane domains separated by hydrophillic regions, typical of a member of the family of G-protein coupled receptors (see fig. 7.12d).

When the deduced amino acid sequence of the putative canary SWS opsin is compared with that of 28 vertebrate opsins, it displays the greatest homology to opsins belonging to the SWS group, sharing the highest percentage identity with the chicken SWS opsin (84 %) and the putative chameleon SWS opsin (83%) (see table 7.12e). Surprisingly, the putative canary SWS opsin shows a lower homology to the budgerigar SWS opsin (58 %). Further confirmation of the identity of this gene as the canary SWS opsin comes from the phylogenetic analyses in which it groups in the same clade as opsins from the SWS lineage, distinct from other VS, LWS, MWS and rod opsin classes described in vertebrates (see figures 7.11f and 7.11g). Branching within the SWS opsin clade in both trees is well supported, as indicated by the high bootstrap confidence values shown for each branch. In the phylogenetic tree based on amino acid sequence identity (fig. 7.11f) the goldfish SWS opsin and putative cavefish SWS opsin branch off before the avian SWS subclade, perhaps providing evidence for the older ancestry of fish, whereas the avian and reptilian SWS opsins form a separate clade. Interestingly, in both phylogenetic trees (figs 7.11f and 7.11g) the budgerigar SWS opsin branches off before the other opsins in the avian/reptilian clade, possibly indicating an earlier divergence from the ancestral gene than the canary, chicken and (putative) chameleon SWS opsins.

```
AACCTGGACACGCCCAACGTGACGGCGCTGAGCCCCTTCCTGGTACCCCAAACCCACCTG     60
N  L  D  T  P  N  V  T  A  L  S  P  F  L  V  P  Q  T  H  L      20

GGCAGCCCTGGCATCTTCAAGGCCATGGCGGCCTTCATGTTCCTGCTGGTGGTGCTGGGT    120
G  S  P  G  I  F  K  A  M  A  A  F  M  F  L  L  V  V  L  G      40

GTCCCCATCAACGCGCTGACCGTGGTGTGCACGGCCAAGTACAGAAAGCTGAGGTCGCAC    180
V  P  I  N  A  L  T  V  V  C  T  A  K  Y  R  K  L  R  S  H      60

CTGAACTACATCCTGGTCAACCTGGCGGTGGCCAACCTGCTGGTGGTGTGCGTGGGATCC    240
L  N  Y  I  L  V  N  L  A  V  A  N  L  L  V  V  C  V  G  S      80

ACCACGGCCTTCTACAGCTTCTCCCAGATGTACTTTGCCCTGGGCCGGCTGGCCTGCAAA    300
T  T  A  F  Y  S  F  S  Q  M  Y  F  A  L  G  R  L  A  C  K     100

ATCGAGGGGTTCACGGCCACGCTGGGCGGGATGGTGTCCCTGTGGTCACTGGCCGTGGTG    360
I  E  G  F  T  A  T  L  G  G  M  V  S  L  W  S  L  A  V  V     120

GCCTTCGAGCGGTTCCTGGTCATCTGCAAGCCCCTGGGCAACTTCACCTTCCGGGGCAGC    420
A  F  E  R  F  L  V  I  C  K  P  L  G  N  F  T  F  R  G  S     140

CACGCCGTGCTGGGCTGTGCCATCACCTGGATCTTCGGCCTCATCGCCTCCGTGCCCCCC    480
H  A  V  L  G  C  A  I  T  W  I  F  G  L  I  A  S  V  P  P     160

CTCTTCGGCTGGAGCAGGTACATCCCGGAGGGGCTGCAGTGCTCGTGTCGGCCGGACTGG    540
L  F  G  W  S  R  Y  I  P  E  G  L  Q  C  S  C  R  P  D  W     180

TACACGACGGACAACAAATGGAACAACGAGTCATCGTTGATTTTCTCTTCCTGCTTCTGC    600
Y  T  T  D  N  K  W  N  N  E  S  S  L  I  F  S  S  C  F  C     200

TTCGGCTTCCCCCTGAGCGTCATCGTCTTCTCCTACGGGCGGCTGCTGCTCACCCTGCGC    660
F  G  F  P  L  S  V  I  V  F  S  Y  G  R  L  L  L  T  L  R     220

GCGGTGGCCAGCCAGCAGGAGCAGTCGGCCACCACGCAGAAGGCGGAGCGCGAGGTGACC    720
A  V  A  S  Q  Q  E  Q  S  A  T  T  Q  K  A  E  R  E  V  T     240

AAGATGGTGGTGGTGATGGTGCTGGGCTTCCTGGTGTGCTGGCTGCCCTACTGCTCCTTC    780
K  M  V  V  V  M  V  L  G  F  L  V  C  W  L  P  Y  C  S  F     260

GCGCTCTGGGTGGTGACACACCGGGGACACCCCTTCGACCTGGGGCTGGCCTCCATCCCC    840
A  L  W  V  V  T  H  R  G  H  P  F  D  L  G  L  A  S  I  P     280

TCCGTCTTCTCCAAGGCCTCCACCGTCTACAACCCCATCATCTACGTCTTCATGAACAAG    900
S  V  F  S  K  A  S  T  V  Y  N  P  I  I  Y  V  F  M  N  K     300

CAGTTCCGCTCCTGCATGCTCAAGCTCGTGTTCTGTGGCCGGAGCCCCTTCGGGGATGAC    960
Q  F  R  S  C  M  L  K  L  V  F  C  G  R  S  P  F  G  D  D     320

GACGACGTGTCCGGCTCCTCCCAGGCCACCCAGGTGTCC                         999
D  D  V  S  G  S  S  Q  A  T  Q  V  S                          333
```

Figure 7.12c
Partial nucleotide sequence and deduced amino acid translation of the coding region of the SWS opsin gene from *Serinus canaria*

Figure 7.12d

Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence of the putative SWS opsin from *Serinus canaria*

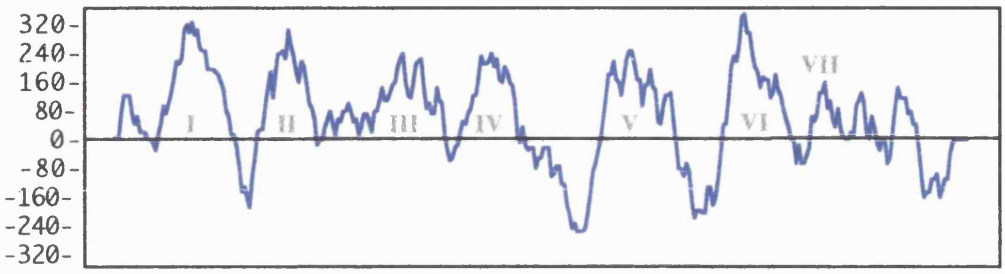


Table 7.12e

Percentage of identical amino acid residues per site between the putative canary SWS opsin and 28 vertebrate opsins.

Vertebrate opsin	% similarity (± S.E.) to putative SWS opsin from *Serinus canaria*
mou 360	51.9 ± 3
gfsh 365	46.3 ± 3
cmln viol	52.8 ± 3
can 366	53.7 ± 3
bud 371	52.6 ± 3
rat blu	51.5 ± 3
bov blu	46.5 ± 3
xen viol	54.2 ± 3
chk 418	49.9 ± 3
hum420	50.9 ± 3
mar 423	49.9 ± 3
tal 429	50.3 ± 3
can 442	**100**
gfsh 441	67.8 ± 3
bud 444	58.0 ± 3
cmln blu	**83.1 ± 2**
chk 455	**84.3 ± 2**
cfsh blu	**73.0 ± 2**
can 506g	51.1 ± 3
duc 502g	52.5 ± 3
chk 507g	51.5 ± 3
bud 508g	51.4 ± 3
can 506r	52.1 ± 3
duc 505r	51.9 ± 3
chk 506r	50.6 ± 3
bud 509r	50.9 ± 3
can 569	46.7 ± 4
chk 571	42.2 ± 3
dmRh1 (invertebrate out group)	24.8 ± 2

Percentage identity values greater than 70 % are indicated in bold lettering. Colours represent the opsin class (e.g. red lettering represents opsins belonging to the LWS class). (see page 166 for list of abbreviations used).

Figure 7.12h is a two dimensional representation of the SWS opsin from *Serinus canaria.* Each α-helical region is shown as 26 residues in length, with only 18 core residues embedded in the membrane. Since partial coding sequence was obtained from the canary SWS opsin, references to amino acid residues will be based on the numbering system of bovine rod opsin. From the 333 amino acid residues deduced from the cDNA translation, it can be seen that the structural features common to other opsins are also conserved in the canary SWS opsin. These are labelled in fig. 7.12h and include;

- Lys-296, the site of the Schiff's base linkage to retinal (Bownds, 1967; Wang *et al.,* 1980),
- Glu-113, the Schiff's base counterion (Zhukovsky and Oprian, 1989; Sakmar *et al.,* 1989, 1991; Nathans, 1990a),
- two Cys residues at positions 110 and 187 which form a disulphide bond (Karnik and Khorana, 1990)
- Glu-134 and Arg-135, thought to interact directly with the G-protein transducin (Franke *et al.,* 1990)
- numerous Ser and Thr residues in the C-terminal region which are potential phosphorylation sites (Hargrave, 1982; Thompson and Findlay, 1984).
- one potential palmitoylation site, Cys-322 which may anchor the C-terminus of the opsin to the membrane, forming a fourth cytoplasmic loop (Ovchinnikov *et al.,* 1988)

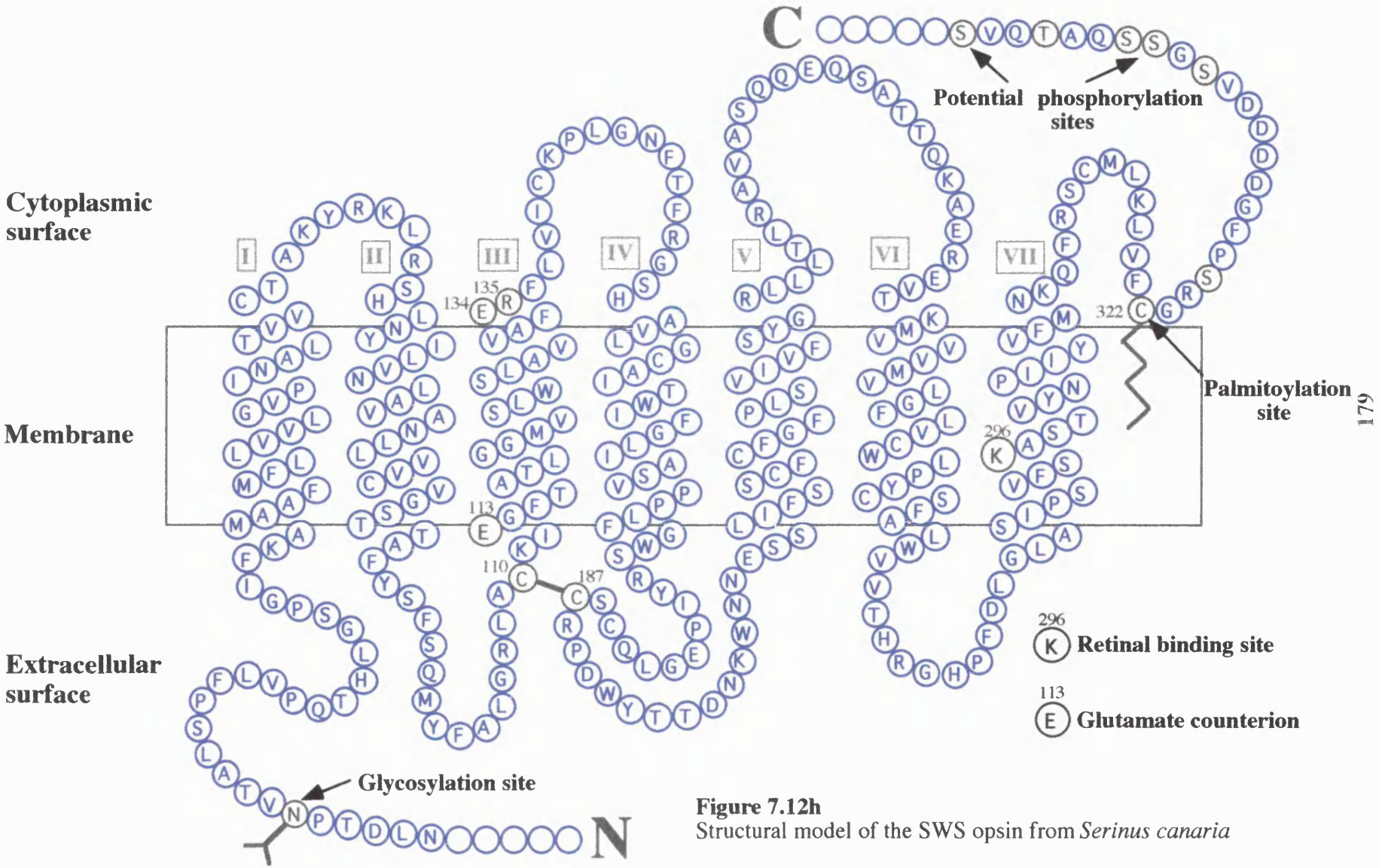


Figure 7.12h
Structural model of the SWS opsin from *Serinus canaria*

7.13. Isolation of the MWS opsin gene from *Serinus canaria*

The cloning strategy used to isolate fragments of the putative MWS opsin gene from *Serinus canaria* is depicted in table 7.13a. Details are given of combinations of primers and specific PCR conditions employed. For convenience, the approximate position of each primer has been drawn schematically in fig. 7.13b.

Table 7.13a

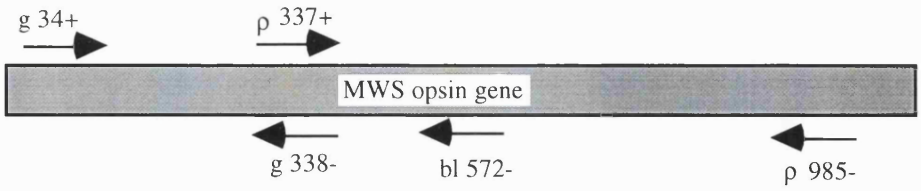
Details of primer pairs and specific reaction conditions used to amplify fragments of the putative MWS opsin gene from canary cDNA.

PRIMER PAIR	T_m	PCR cycle used	$[Mg^{2+}]$ (mM)	approx. size of product (bp)	Probable identity of amplified fragment
ρ 337+ ρ 985-	62° 72°	T_d 94° @ 1s T_a 62° @ 2s T_e 72° @ 30s	2.0	650	MWS opsin gene
g 34+ g 338-	60° 66°	T_d 94° @ 1s T_a 60° @ 1s *no* T_e	1.5	300	MWS opsin gene
ρ 337+ bl 572-	62° 68°	T_d 94° @ 1s T_a 60° @ 1s *no* T_e	2.0	200	MWS opsin gene

For each PCR, the DNA was subjected to an initial 10 second denaturation at 94°C before cycling commenced, and a final 1 minute extension at 72°C. T_d, T_a, T_e, T_m, denaturation, annealing, extension and calculated primer annealing temperatures (see methods section); $[Mg^{2+}]$=magnesium ion concentration.

Figure 7.13b

Schematic diagram showing the approximate position of primers used in PCRs to amplify fragments the putative canary MWS opsin gene, represented schematically by the shaded bar.



(See table 6.14a for actual primer sequences.)

Figure 7.13c shows the partial cDNA sequence obtained from the putative canary MWS opsin, together with the predicted amino acid translation. A total of 931 base pairs of sequence was obtained, roughly corresponding to the region between exons 1 through to 5. The 3' and 5' untranslated regions of the gene have yet to be cloned. The Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence from the MWS opsin gene indicates the presence of seven hydrophobic transmembrane regions separated by hydrophillic segments, typical of members of the family of G-protein coupled receptors (see fig. 7.13d)

When compared with 28 vertebrate opsins, the canary MWS opsin appears to share the highest degree of homology (92-96 % identity) with avian MWS opsins compared to 70% identity with avian rod opsins and 40-50 % identity with other opsin classes at the amino acid level (see table 7.13e). In both phylogenetic trees (Figures 7.11f and 7.11g) based on amino acid and nucleotide sequence identity, the canary MWS opsin groups in the same clade as the duck, budgerigar and chicken MWS opsins, providing evidence for its identity as the canary MWS opsin. Bootstrap confidence values within the MWS opsin clade in the tree based on amino acids are lower than those seen in the tree based on nucleotide identity reflecting the smaller number of amino acid than nucleotide substitutions between these opsins.

```
CCTATGTCCAACAAGACAGGGGTGGTGCGGAGCCCCTTCGAGTACCCGCAGTACTACCTG     60
P  M  S  N  K  T  G  V  V  R  S  P  F  E  Y  P  Q  Y  Y  L      20

GCCGAGCCCTGGAAATACCGCCTCGTGTGCTGCTACATCTTCTTCCTCATCTCCACCGGC    120
A  E  P  W  K  Y  R  L  V  C  C  Y  I  F  F  L  I  S  T  G      40

TTCCCCATCAACTTCCTCACCCTCCTGGTCACCTTCAAGCACAAGAAGCTTCGGCAGCCT    180
F  P  I  N  F  L  T  L  L  V  T  F  K  H  K  K  L  R  Q  P      60

CTCAACTACATCCTGGTCAACCTGGCGGTGGCTGACCTGTGCATGGCCTGCTTTGGTTTC    240
L  N  Y  I  L  V  N  L  A  V  A  D  L  C  M  A  C  F  G  F      80

ACCGTCACCTTCTACACCGCCTGGAACGGCTACTTTGTGTTTGGCCCCGTTGGCTGTGCT    300
T  V  T  F  Y  T  A  W  N  G  Y  F  V  F  G  P  V  G  C  A     100

GTGGAGGGCTTCTTTGCTACGCTGGGAGGCCAGGTTGCCCTGTGGTCCCTGGTTGTCCTG    360
V  E  G  F  F  A  T  L  G  G  Q  V  A  L  W  S  L  V  V  L     120

GCCATCGAGCGCTACATTGTCATCTGCAAGCCCATGGGCAACTTCCGCTTCTCCGCCAGC    420
A  I  E  R  Y  I  V  I  C  K  P  M  G  N  F  R  F  S  A  S     140

CATGCCATGATGGGCATCGCTTTCACCTGGGTCATGGCCATCTCCTGCGCCGCCCCGCCG    480
H  A  M  M  G  I  A  F  T  W  V  M  A  I  S  C  A  A  P  P     160

CTCTTCGGCTGGTCCAGGTACATCCCGGAGGGGATGCAGTGCTCCTGCGGCCCGGACTAC    540
L  F  G  W  S  R  Y  I  P  E  G  M  Q  C  S  C  G  P  D  Y     180

TACACCCACAACCCCGACTTCCACAACGAGTCCTACGTGCTCTACATGTTCGTCATCCAC    600
Y  T  H  N  P  D  F  H  N  E  S  Y  V  L  Y  M  F  V  I  H     200

TTCATCATCCCTGTCGTCATCATCTTCTTCTCCTACGGGCGCCTCGTCTGCAAAGTCCCC    660
F  I  I  P  V  V  I  I  F  F  S  Y  G  R  L  V  C  K  V  P     220

GAGGCAGCTGCCCAGCAGCAGGAATCAGCCACGACCCAGAAGGCGGAGAAGGAGGTGACG    720
E  A  A  A  Q  Q  Q  E  S  A  T  T  Q  K  A  E  K  E  V  T     240

CGGATGGTGATCCTCATGGTGCTGGGCTTCATGCTGGCCTGGACGCCCTACGCCGTGGTG    780
R  M  V  I  L  M  V  L  G  F  M  L  A  W  T  P  Y  A  V  V     260

GCGTTCTGGATCTTCACCAACAAGGGCGCCGACTTCACGGCCACGCTGATGGCAGTGCCT    840
A  F  W  I  F  T  N  K  G  A  D  F  T  A  T  L  M  A  V  P     280

GCCTTCTTCTCCAAGAGCTCCTCCCTCTACAACCCCATCATCTACGTGCTCATGAACAAA    900
A  F  F  S  K  S  S  S  L  Y  N  P  I  I  Y  V  L  M  N  K     300

CAGTTCCGTAACTGCATGATCACCACAATCT                                 931
Q  F  R  N  C  M  I  T  T  I                                    310
```

Figure 7.13c
Partial nucleotide sequence and deduced amino acid translation of the coding region of the MWS opsin gene from *Serinus canaria*

Figure 7.13d

Kyte-Doolittle Hydrophobicity plot of the deduced amino acid sequence of the putative MWS opsin from Serinus canaria

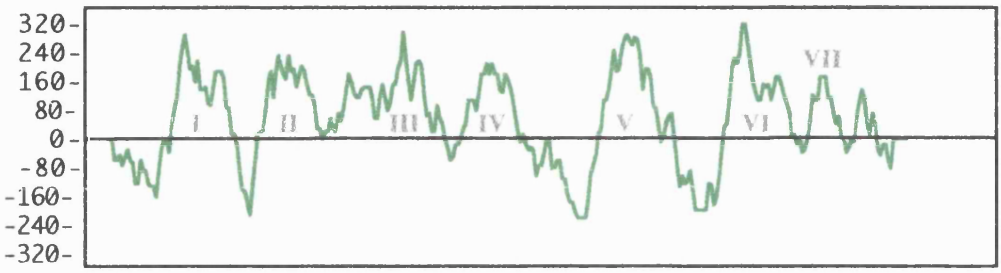


Table 7.13e

Percentage of identical amino acid residues per site between the putative canary MWS opsin and 28 vertebrate opsins.

Vertebrate opsin	% similarity (± S.E.) to putative MWS opsin from *Serinus canaria*
mou 360	47.4 ± 3
gfsh 365	47.0 ± 3
cmln viol	49.0 ± 3
can 366	49.8 ± 3
bud 371	49.0 ± 3
rat blu	46.7 ± 3
bov blu	47.4 ± 3
xen viol	51.0 ± 3
chk 418	47.7± 3
hum420	48.0 ± 3
mar 423	48.4 ± 3
tal 429	47.0 ± 3
can 442	51.1 ± 3
gfsh 441	49.4 ± 3
bud 444	37.3 ± 3
cmln blu	52.0 ± 3
chk 455	52.9 ± 3
cfsh blu	49.7 ± 3
can 506g	**100**
duc 502g	**93.7 ± 1**
chk 507g	**96.1 ± 1**
bud 508g	**92.7 ± 1**
can 506r	**72.6 ± 3**
duc 505r	**72.6 ± 3**
chk 506r	**72.3 ± 3**
bud 509r	**73.0 ± 3**
can 569	43.4 ± 4
chk 571	42.4 ± 3
dmRh1 (invertebrate out group)	26.0 ± 3

Percentage identity values greater than 70 % are indicated in bold lettering. Colours represent the opsin class (e.g. red lettering represents opsins belonging to the LWS class). (see page 166 for list of abbreviations used)

Figure 7.13h is a two dimensional representation of the MWS opsin from *Serinus canaria.* Each α-helical region is shown as 26 residues in length, with only 18 core residues embedded in the membrane. Since partial coding sequence was obtained from the canary MWS opsin, references to amino acid residues will be based on the numbering system of bovine rod opsin. From the 310 amino acid residues deduced from the predicted cDNA translation product, it can be seen that the structural features common to other opsins are also conserved in the canary MWS opsin. These are labelled in fig. 7.13h and include;

- Lys-296, the site of the Schiff's base linkage to retinal (Bownds, 1967; Wang *et al.,* 1980),
- Glu-113, the Schiff's base counterion (Zhukovsky and Oprian, 1989; Sakmar *et al.,* 1989, 1991; Nathans, 1990a),
- two Cys residues at positions 110 and 187 which form a disulphide bond (Karnik and Khorana, 1990)
- Glu-134 and Arg-135, thought to interact directly with the G-protein transducin (Franke *et al.,* 1990)

- Additionally, His-211 which is present in all members of the M2 branch of opsins (Okano *et al.,* 1992) is also present in the MWS opsin of *Serinus canaria* (Weitz and Nathans, 1992c).

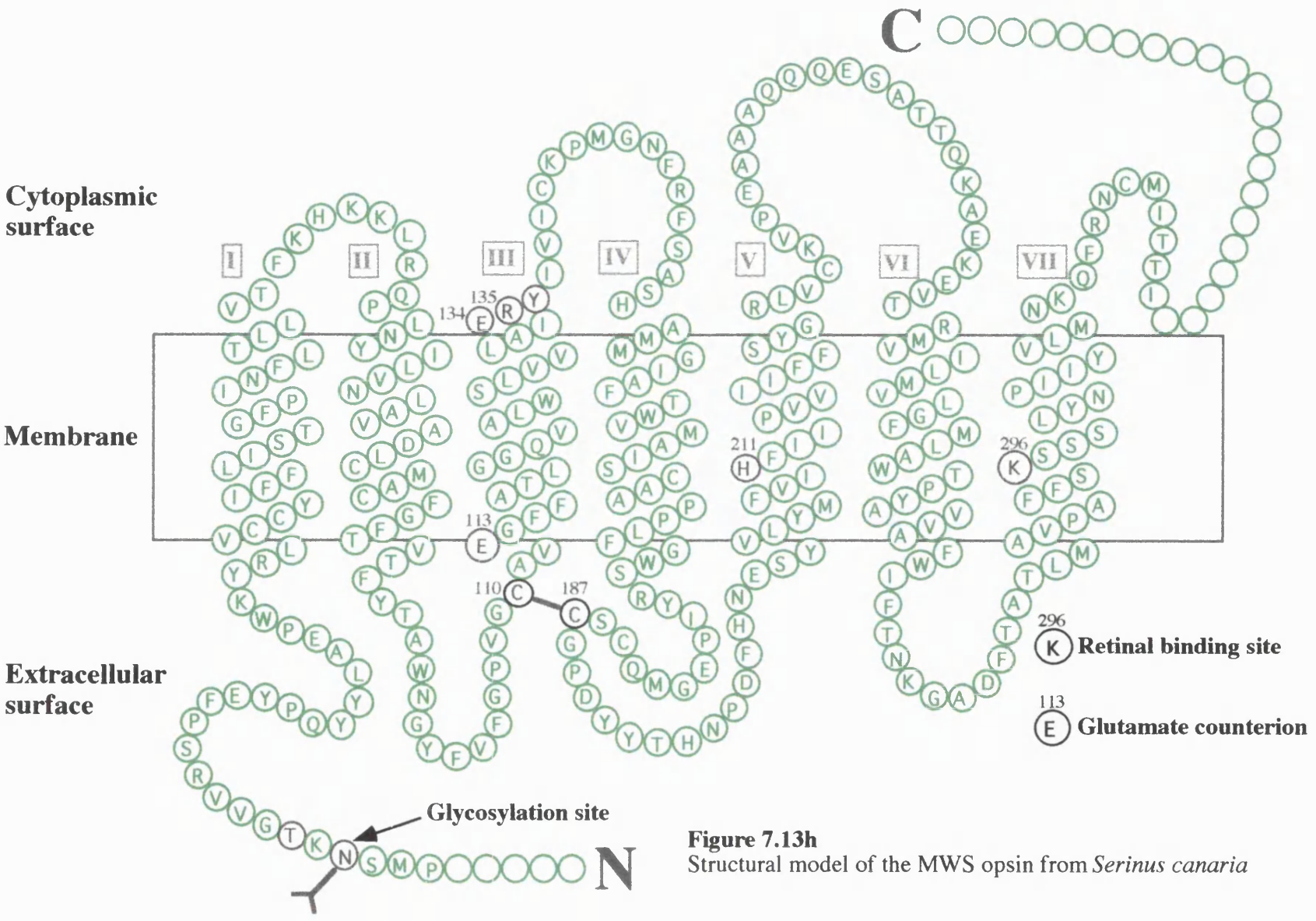


Figure 7.13h
Structural model of the MWS opsin from *Serinus canaria*

7.14. Isolation of cDNA from the rod opsin gene of *Serinus canaria*

The cloning strategy used to isolate fragments of the putative rod opsin gene from *Serinus canaria* is depicted in table 7.14a Details are given of combinations of primers and specific PCR conditions employed. For convenience, the approximate position of each primer has been drawn schematically in fig. 7.14b.

Table 7.14a

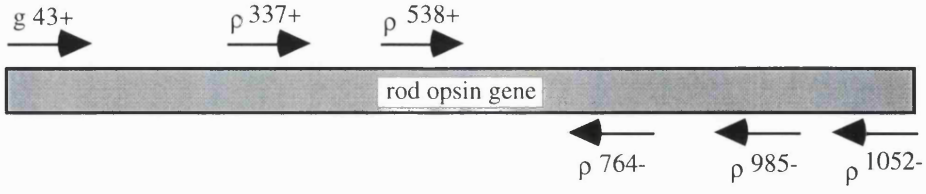
Details of primer pairs and specific reaction conditions used to amplify fragments of the putative rod opsin gene from canary cDNA.

PRIMER PAIR	T_m	PCR cycle used	$[Mg^{2+}]$ (mM)	approx. size of product (bp)	Probable identity of amplified fragment
g 43+ ρ 1052-	64° 66°	T_d 94° @ 1s T_a 63° @ 2s T_e 72° @ 10s	3.0	1000	rod opsin gene
ρ 337+ ρ 764-	62° 63°	T_d 94° @ 1s T_a 62° @ 1s T_e 72° @ 2s	3.5	400	rod opsin gene
ρ 538+ ρ 985-	68° 72°	T_d 94° @ 1s T_a 67° @ 1s T_e 72° @ 30s	2.0	450	rod opsin gene
ρ 538+ ρ 1052-	68° 66°	T_d 94° @ 1s T_a 66° @ 1s T_e 72° @ 2s	2.0	500	rod opsin gene

For each PCR, the DNA was subjected to an initial 10 second denaturation at 94°C before cycling commenced, and a final 1 minute extension at 72°C. T_d, T_a, T_e, T_m, denaturation, annealing, extension and calculated primer annealing temperatures (see methods section); $[Mg^{2+}]$=magnesium ion concentration.

Figure 7.14b

Schematic diagram showing the approximate position of primers used in PCRs to amplify fragments of the putative canary rod opsin gene, represented schematically by the shaded bar.



(See table 6.14a for actual primer sequences.)

Figure 7.14c shows the partial cDNA sequence obtained from the putative canary rod opsin, together with the predicted amino acid translation. A total of 960 base pairs of sequence was obtained, roughly corresponding to the region between exons 1 through to 5. The 3' and 5' untranslated regions of the cDNA have yet to be cloned. The Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence from the rod opsin gene indicates the presence of seven hydrophobic transmembrane regions separated by hydrophillic segments, typical of members of the family of G-protein coupled receptors (see fig. 7.14d)

When compared with 28 vertebrate opsins, the canary rod opsin appears to share the highest degree of homology (96-98 % identity) with avian rod opsins compared to approximately 70% with avian MWS opsins and 40-50 % identity with other vertebrate opsin classes, at the amino acid level (see table 7.14e). A relatively high degree of homology is also seen between the MWS and rod opsins of other avian species and reflects the identical or very similar λ_{max} values of the MWS and rod visual pigments (that is, the MWS/rod opsin + 11 *-cis-* retinal), obtained from microspectrophotometric analysis (see above).

In both phylogenetic trees (Figures 7.11f and 7.11g) based on amino acid and nucleotide sequence identity, the canary rod opsin groups in the same clade as other avian rod opsins (duck, budgerigar and chicken), providing further evidence for its identity. Bootstrap confidence values within the rod opsin clade in the tree based on amino acids are lower than those seen in the tree based on nucleotide identity reflecting the smaller number of amino acid than nucleotide substitutions between these opsins.

```
CCCTTTGAGTACCCCCAGTATTACCTGGCTGAGCCTTGGAAGTTCTCGGCGCTGGCTGCC     60
P  F  E  Y  P  Q  Y  Y  L  A  E  P  W  K  F  S  A  L  A  A      20

TACATGTTCATGCTGATCCTGCTCGGCTTCCCCATCAACTTCCTCACGCTGTACGTCACC    120
Y  M  F  M  L  I  L  L  G  F  P  I  N  F  L  T  L  Y  V  T      40

ATCCAGCACAAGAAGCTCCGCACACCTCTGAACTACATCCTTCTGAACCTGGCTGTTGCC    180
I  Q  H  K  K  L  R  T  P  L  N  Y  I  L  L  N  L  A  V  A      60

GACCTCTTCATGGTCTTCGGAGGCTTCACAACCACCATGTACACATCCATGAACGGGTAC    240
D  L  F  M  V  F  G  G  F  T  T  T  M  Y  T  S  M  N  G  Y      80

TTTGTCTTTGGAGTAACAGGGTGCTACATCGAAGGCTTCTTTGCCACACTGGGCGGTGAA    300
F  V  F  G  V  T  G  C  Y  I  E  G  F  F  A  T  L  G  G  E     100

ATTGCTCTCTGGTCACTGGTGGTCCTGGCTATCGAAAGATACGTAGTGGTCTGCAAGCCC    360
I  A  L  W  S  L  V  V  L  A  I  E  R  Y  V  V  V  C  K  P     120

ATGAGCAACTTCCGCTTTGGAGAGAACCATGCCATCATGGGTGTTGCCTTCTCCTGGATC    420
M  S  N  F  R  F  G  E  N  H  A  I  M  G  V  A  F  S  W  I     140

ATGGCCTTGGCATGTGCAGCTCCCCCACTTTTCGGCTGGTCCAGGTACATCCCTGAGGGC    480
M  A  L  A  C  A  A  P  P  L  F  G  W  S  R  Y  I  P  E  G     160

ATGCAGTGCTCGTGCGGGATCGACTATTACACTCTGAAGCCAGAGGTCAACAATGAATCT    540
M  Q  C  S  C  G  I  D  Y  Y  T  L  K  P  E  V  N  N  E  S     180

TTTGTCATCTACATGTTTGTGGTTCACTTCATGATCCCGCTGTTGATCATTTTCTTCTGC    600
F  V  I  Y  M  F  V  V  H  F  M  I  P  L  L  I  I  F  F  C     200

TATGGGAACCTGGTTTGCACAGTCAAGGAGGCTGCCGCCCAGCAGCAAGAGTCTGCCACC    660
Y  G  N  L  V  C  T  V  K  E  A  A  A  Q  Q  Q  E  S  A  T     220

ACCCAGAAGGCAGAGAAAGAAGTGACTCGCATGGTCATCATCATGGTCATCTCCTTCCTC    720
T  Q  K  A  E  K  E  V  T  R  M  V  I  I  M  V  I  S  F  L     240

ATCTGCTGGGTCCCCTATGCCAGCGTCGCCTTCTACATCTTCACCAACCAGGGATCAGAC    780
I  C  W  V  P  Y  A  S  V  A  F  Y  I  F  T  N  Q  G  S  D     260

TTTGGGCCCATCTTCATGACCATCCCGGCATTCTTTGCCAAGAGCTCGGCCATCTACAAC    840
F  G  P  I  F  M  T  I  P  A  F  F  A  K  S  S  A  I  Y  N     280

CCTGTGATCTACATCGTAATGAACAAACAGTTCCGTAACTGCATGATCACAACCCTCTGC    900
P  V  I  Y  I  V  M  N  K  Q  F  R  N  C  M  I  T  T  L  C     300

TGTGGCAAGAACCCACTGGGTGACGAGGACACATCTGCTGGCAAGACAGAGACCTCCTCC    960
C  G  K  N  P  L  G  D  E  D  T  S  A  G  K  T  E  T  S  S     320
```

Figure 7.14c
Partial nucleotide sequence and deduced amino acid translation of the coding region of the rod opsin gene from *Serinus canaria*

Figure 7.14d

Kyte-Doolittle Hydrophobicity plot of the deduced amino acid sequence of the putative rod opsin from *Serinus canaria*

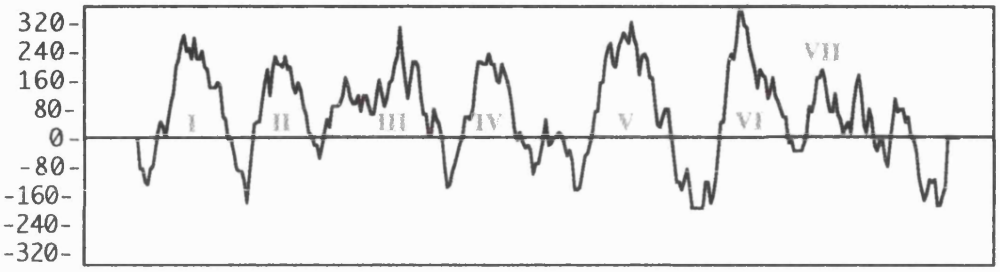


Table 7.14e

Percentage of identical amino acid residues per site between the putative canary rod opsin and 28 vertebrate opsins.

Vertebrate opsin	% similarity (± S.E.) to putative rod opsin from *Serinus canaria*
mou 360	47.3 ± 3
gfsh 365	46.0 ± 3
cmln viol	48.3 ± 3
can 366	49.1 ± 3
bud 371	47.6 ± 3
rat blu	46.4 ± 3
bov blu	42.8 ± 3
xen viol	49.2 ± 3
chk 418	47.0 ± 3
hum420	47.6 ± 3
mar 423	46.7 ± 3
tal 429	46.7 ± 3
can 442	52.1 ± 3
gfsh 441	52.5 ± 3
bud 444	36.6 ± 3
cmln blu	54.2 ± 3
chk 455	52.0 ± 3
cfsh blu	51.4 ± 3
can 506g	**72.6 ± 3**
duc 502g	**71.6 ± 3**
chk 507g	**72.2 ± 3**
bud 508g	**72.9 ± 3**
can 506r	**100**
duc 505r	**98.1 ± 1**
chk 506r	**97.5 ± 1**
bud 509r	**95.8 ± 1**
can 569	49.6 ± 4
chk 571	42.8 ± 3
dmRh1 (invertebrate out group)	27.6 ± 3

Percentage identity values greater than 70 % are indicated in bold lettering. Colours represent the opsin class (e.g. red lettering represents opsins belonging to the LWS class). (see page 166 for list of abbreviations used)

Figure 7.14h is a two dimensional representation of the rod opsin from *Serinus canaria.* Each α-helical region is shown as 26 residues in length, with only 18 core residues embedded in the membrane. Since partial coding sequence was obtained from the canary rod opsin, references to amino acid residues will be based on the numbering system of bovine rod opsin. From the 320 amino acid residues deduced from the predicted cDNA translation product, it can be seen that the structural features common to other opsins are also conserved in the canary rod opsin. These are labelled in fig. 7.14h and include;

- Lys-296, the site of the Schiff's base linkage to retinal (Bownds, 1967; Wang *et al.,* 1980),
- Glu-113, the Schiff's base counterion (Zhukovsky and Oprian, 1989; Sakmar *et al.,* 1989, 1991; Nathans, 1990a),
- two Cys residues at positions 110 and 187 which form a disulphide bond (Karnik and Khorana, 1990)
- Glu-134 and Arg-135, thought to interact directly with the G-protein transducin (Franke *et al.,* 1990)

- Additionally, His-211 which is present in all members of the M2 branch of opsins (Okano *et al.,* 1992) is also present in the rod opsin of *Serinus canaria* (Weitz and Nathans, 1992c).
- Two (rather than one) cysteine palmitoylation sites exist (Cys-322 and Cys-323), which may anchor the C-terminal segment into the membrane, forming a fourth cytoplasmic loop.

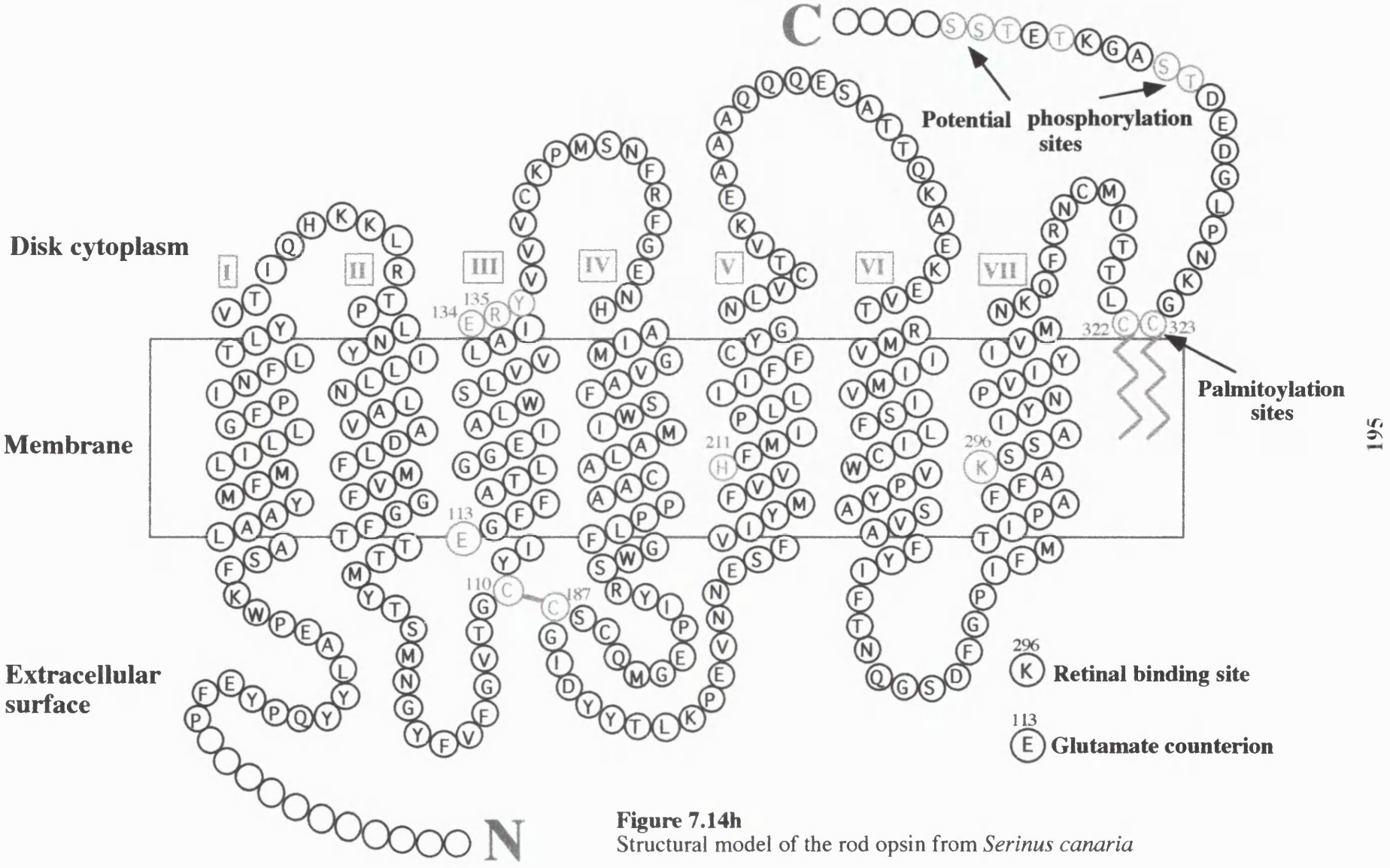


Figure 7.14h
Structural model of the rod opsin from *Serinus canaria*

7.15. Isolation of the LWS opsin cDNA of *Serinus canaria*

Table 7.15a depicts the cloning strategy used to isolate fragments of the putative LWS opsin gene from *Serinus canaria.* Details are given of each combination of primers used and specific PCR (polymerase chain reaction) conditions employed. For convenience, the approximate position of each primer has been drawn schematically on the gene in fig. 7.15b (refer to table 6.14a for actual primer sequences).

Table 7.15a

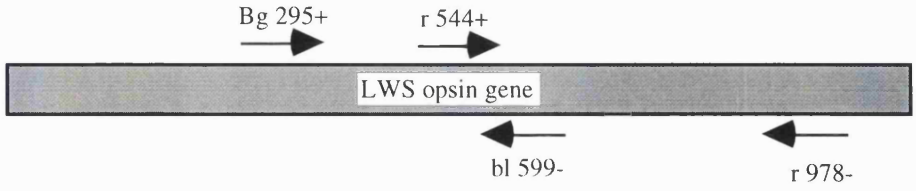
Details of primer pairs and specific reaction conditions used to amplify fragments of the putative LWS opsin gene from canary cDNA.

PRIMER PAIR	T_m	PCR cycle used	$[Mg^{2+}]$ (mM)	approx. size of product (bp)	Probable identity of amplified fragment
Bg 295+ bl 599-	66° 68°	T_d 94° @ 1s T_a 66° @ 1s T_e 72° @ 1s	4.0	300	LWS opsin gene
r 544+ r 978-	69° 61°	T_d 94° @ 1s T_a 60° @ 1s T_e 72° @ 1s	3.0	400	LWS opsin gene

For each PCR, the DNA was subjected to an initial 10 second denaturation at 94°C before cycling commenced, and a final 1 minute extension at 72°C. T_d, T_a, T_e, T_m, denaturation, annealing, extension and calculated primer annealing temperatures (see methods section); $[Mg^{2+}]$=magnesium ion concentration.

Figure 7.15b

Schematic diagram showing the approximate position of primers used in PCRs to amplify fragments of the putative canary LWS opsin gene (represented schematically by the shaded bar).



(See table 6.14a for actual primer sequences.)

Figure 7.15c shows the partial cDNA sequence obtained from the putative canary LWS opsin, together with the predicted amino acid translation. In total, 621 bp of DNA sequence was obtained. The Kyte-Doolittle hydrophobicity plot of the deduced amino acid translation of the putative canary LWS opsin shows the region from the third transmembrane helix through to the seventh helix of a typical G-protein coupled receptor. As is seen for the other opsins obtained in the present study, each hydrophobic (helical) domain is separated by hydrophillic regions (see fig. 7.15d).

When the deduced amino acid sequence of the putative canary LWS opsin is compared with that of 28 vertebrate opsins, it shares the highest percentage identity with the chicken LWS opsin (94 %) (see table 7.15e) compared with less than 45 % homology shared with other groups. In the phylogenetic analyses the putative canary LWS opsin groups in the same clade as the chicken LWS opsin, distinct from other VS, SWS, MWS and rod opsin classes described in vertebrates (see Figures 7.11f and 7.11g on pages 168 and 169).

```
GGCCACCCCATGTGCGTCATCGAGGGCTACACCGTCAGCGCCTGCGGCATCACGGCGCTC     60
G  H  P  M  C  V  I  E  G  Y  T  V  S  A  C  G  I  T  A  L      20

TGGTCCCTGGCCATCATCTCCTGGGAGCGCTGGTTCGTGGTCTGCAAACCCTTCGGCAAC    120
W  S  L  A  I  I  S  W  E  R  W  F  V  V  C  K  P  F  G  N      40

ATCAAGTTCGACGGGAAGCTGGCGGTGGCCGGGGTCCTCTTCTCCTGGATCTGGTCCTGC    180
I  K  F  D  G  K  L  A  V  A  G  V  L  F  S  W  I  W  S  C      60

GCCTGGACCGCGCCCCCCATCTTCGGCTGGAGCAGGTACTGGCCCCACGGGCTGAAGACC    240
A  W  T  A  P  P  I  F  G  W  S  R  Y  W  P  H  G  L  K  T      80

TCGTGCGGGCCGGACGTGTTCAGCGGCACGACGGACCCAGGGGTGCAGTCCTACATGGTG    300
S  C  G  P  D  V  F  S  G  T  T  D  P  G  V  Q  S  Y  M  V     100

GTGCTGATGGTCACCTGCTGCTTCTTCCCGCTGGCCGTCATCATCTTCTGCTACCTGCAA    360
V  L  M  V  T  C  C  F  F  P  L  A  V  I  I  F  C  Y  L  Q     120

GTCTGGCTGGCCATCCGTGCGGTGGCGGCCCAGCAGAAGGAGTCGGAGTCGACGCAGAAG    420
V  W  L  A  I  R  A  V  A  A  Q  Q  K  E  S  E  S  T  Q  K     140

GCGGAGAAGGAGGTGTCGCGCATGGTGGTGGTGATGATCCTGGCCTACTGCTTCTGCTGG    480
A  E  K  E  V  S  R  M  V  V  V  M  I  L  A  Y  C  F  C  W     160

GGGCCCTACACCATCTTCGCCTCGTTCGCCGCCGCCAACCCGTACGCCTTCCACCCGCTC    540
G  P  Y  T  I  F  A  S  F  A  A  A  N  P  Y  A  F  H  P  L     180

ACCGCCGCCCTGCCCGCCTTCTTCGCCAAGAGCGCCACCATCTACAACCCCATCATCTAC    600
T  A  A  L  P  A  F  F  A  K  S  A  T  I  Y  N  P  I  I  Y     200

GTCTTCATGAACCGGCAGTTT                                           621
V  F  M  N  R  Q  F                                             207
```

Figure 7.15c
Partial nucleotide sequence and deduced amino acid translation of the coding region of the LWS opsin gene from *Serinus canaria*

Fig 7.15d

Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence of the putative LWS opsin from Serinus canaria

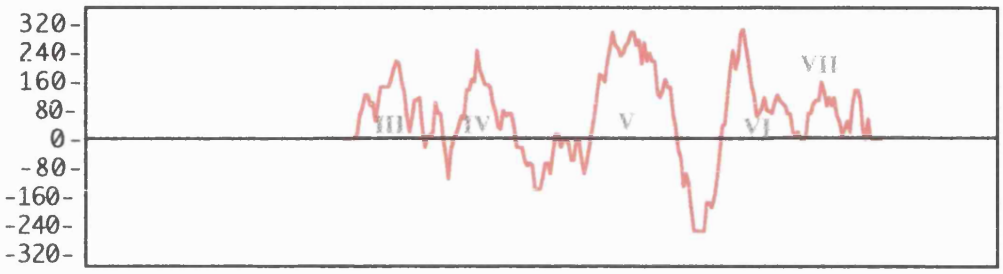


Table 7.15e

Percentage of identical amino acid residues per site between the putative canary LWS opsin and 28 vertebrate opsins.

Vertebrate opsin	% similarity (± S.E.) to putative LWS opsin from *Serinus canaria*
mou 360	45.5 ± 4
gfsh 365	45.0 ± 4
cmln viol	47.1 ± 3
can 366	46.0 ± 4
bud 371	46.0 ± 4
rat blu	45.5 ± 4
bov blu	44.4 ± 4
xen viol	43.9 ± 4
chk 418	46.9 ± 4
hum420	47.0 ± 4
mar 423	47.1 ± 3
tal 429	47.5 ± 4
can 442	46.7 ± 4
gfsh 441	42.9 ± 4
bud 444	28.4 ± 3
cmln blu	45.2 ± 3
chk 455	46.0 ± 4
cfsh blu	44.2 ± 3
can 506g	43.4 ± 4
duc 502g	45.5 ± 4
chk 507g	45.5 ± 4
bud 508g	44.4 ± 4
can 506r	49.6 ± 4
duc 505r	46.5 ± 4
chk 506r	47.5 ± 4
bud 509r	45.5 ± 4
can 569	**100**
chk 571	**93.5 ± 1**
dmRh1 (invertebrate out group)	31.6 ± 3

Percentage identity values greater than 70 % are indicated in bold lettering. Colours represent the opsin class (e.g. red lettering represents opsins belonging to the LWS class). (see page 166 for list of abbreviations used)

Figure 7.15h is a two dimensional representation of the LWS opsin from *Serinus canaria.* Each α-helical region is shown as 26 residues in length, with only 18 core residues embedded in the membrane. Since partial coding sequence was obtained from the canary LWS opsin, references to amino acid residues will be based on the numbering system of bovine rod opsin. From the 207 amino acid residues deduced from the predicted cDNA translation product, it can be seen that the structural features common to other opsins are also conserved in the canary LWS opsin. These are labelled in fig. 7.15h and include;

- Lys-296, the site of the Schiff's base linkage to retinal (Bownds, 1967; Wang *et al.,* 1980),
- Glu-113, the Schiff's base counterion (Zhukovsky and Oprian, 1989; Sakmar *et al.,* 1989, 1991; Nathans, 1990a),
- two Cys residues at positions 110 and 187 which form a disulphide bond (Karnik and Khorana, 1990)
- Glu-134 and Arg-135, thought to interact directly with the G-protein transducin (Franke *et al.,* 1990)

Additionally,

- His-213 and Lys-216 which form the chloride binding pocket in all LWS opsins (Wang *et al.,* 1993), are also found in the canary LWS opsin.

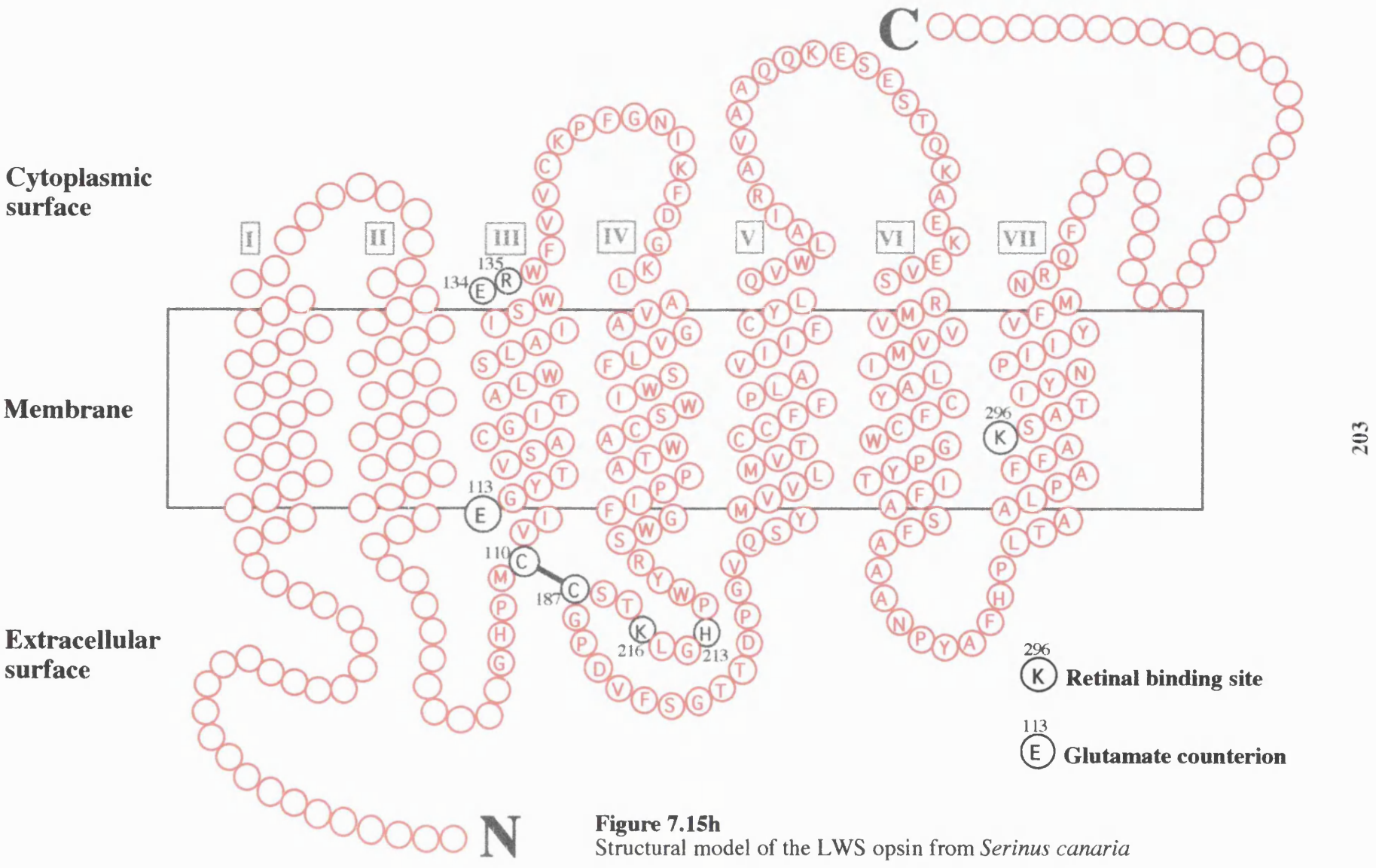


Figure 7.15h
Structural model of the LWS opsin from *Serinus canaria*

RESULTS AND DISCUSSION - Molecular genetics II

7.16. IDENTIFICATION OF PUTATIVE SPECTRAL TUNING SITES

In the following section putative violet sensitive spectral tuning sites will be discussed in detail, followed by brief discussions of SWS, LWS, MWS and rod spectral tuning sites.

> The acronym, 'VS' (violet sensitive) will be used to describe all opsins within the violet lineage (See phylogenetic trees) i.e. all visual pigments with λ_{maxs} between 370 - 420 nm. However, when distinguishing between visual pigments which absorb at 400-420 nm and those absorbing below 370 nm, the terms 'violet-' and 'ultraviolet-' opsin will be used, respectively. All residue numbers refer to the numbering system of bovine rod opsin.

In the present study, the main purpose for sequencing opsins from the canary has been to identify putative spectral tuning sites within each opsin. This can be achieved by aligning to several opsins from the same phylogenetic group and identifying highly conserved polar/charged residues. Table 7.16a (page 205) is the result of an alignment of 29 vertebrate opsins including those sequenced from *Serinus canaria.* Colours have been used to represent each opsin class, e.g. light / dark pink for ultraviolet- and violet-sensitive opsins, respectively. Only the helical residues are shown, since these are more likely to be in the vicinity of the chromophore (Baldwin, 1993). In order to limit the analysis, non-helical sites will not be discussed even though they may play an indirect role in spectral tuning (as was demonstrated in the site-directed mutagenesis studies of Asenjo *et al.,* 1994). Inspection of table 7.16a shows that residues at just 52 sites, out of a possible total of 340-500, face the retinal binding pocket in an opsin molecule as indicated by an asterisk in the left hand margin (also, see fig. 7.16b) (Baldwin, 1993). Of these residues, 17 are highly conserved across all opsin classes (shown in black, underlined lettering) and are likely to have important functions, common to all opsins. This is particularly so if residues are charged/polar, Glu-113, the Schiff's base counterion, being an example (Zhukovsky and Oprian, 1989; Sakmar *et al.,* 1989; Nathans, 1990a; Baldwin, 1993). Reference will be made back to table 7.16a when discussing putative spectral tuning sites in SWS, MWS, LWS and rod opsins.

Table 7.16a »

Alignment of 29 vertebrate opsin sequences from different opsin classes.

See page 166 for a list of abbreviations. Only residues located in helical portions of the opsin are shown. The position of residues within a helix are indicated on the left hand side of each table, together with highly conserved residues (Baldwin, 1993). Numbers in bold italics refer to the numbering according to bovine rod opsin (Wang *et al.,* 1980). An asterisk indicates residues which are predicted to point into the retinal binding groove (Baldwin, 1993). Colours represent the opsin class, red for example, indicating LWS opsins. Numbers in the top margin are the pigment λ_{max} if known. Residues which are highly conserved across all opsin classes are indicated in black, underlined lettering.

HELIX I

aa		aa	mou	gfsh	can	bud	bov	xen	chk	hum	can	gfsh	bud	chk	can	duc	chk	bud	can	duc	chk	bud	hum	can	chk
posn		no.	360	365	366	371	blu	viol	418	420	440	441	444	455	500	502	507	508	505	505	506	509	560	567	571
3		40	Q	Q	Q	Q	Q	Q	Q	Q	M	M	M	M	V	V	V	V	L	L	L	L	T		T
4		41	A	A	T	T	A	A	T	A	A	S	A	A	C	C	C	C	A	A	A	A	S		S
5		42	A	A	I	A	V	I	A	A	A	V	A	A	C	C	C	C	A	A	A	A	V		L
6		43	F	F	F	F	F	F	F	F	F	F	F	F	Y	Y	Y	Y	Y	Y	Y	Y	W		W
7	*	44	M	M	M	M	M	M	M	M	M	M	M	M	I	I	I	I	M	M	M	M	M		M
8		45	G	G	G	G	G	G	G	G	F	F	A	F	F	F	F	F	F	F	F	F	I		I
9		46	F	F	L	F	F	M	I	T	L	F	A	L	F	F	F	F	M	M	M	M	F		F
10	I	47	V	V	V	V	V	V	V	V	L	I	L	L	L	L	L	L	L	L	L	L	V		V
11		48	F	F	F	F	F	F	F	F	V	F	V	I	I	I	I	I	I	I	I	I	V		V
12		49	F	F	V	M	F	L	A	L	V	I	A	A	S	S	S	S	L	L	L	L	T		A
13		50	V	V	A	V	V	I	V	I	L	G	L	L	T	T	T	T	L	L	L	L	A		A
14	G *	51	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	S		S
15		52	T	T	T	T	T	T	T	F	V	A	V	V	F	L	L	L	F	F	F	F	V		V
16		53	P	P	P	P	P	P	P	P	P	S	P	P	P	P	P	P	P	P	P	P	F		F
17	G	54	L	L	L	L	L	L	L	L	I	I	V	I	I	I	I	I	I	I	V	I	T		T
18	N	55	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		N
19		56	A	A	A	A	A	F	A	A	A	I	A	T	F	L	L	L	F	F	F	F	G		G
20	L	57	I	I	I	I	T	I	V	M	L	L	L	L	L	L	L	L	L	L	L	L	L		L
21	V *	58	V	V	V	V	V	V	V	V	T	T	T	T	T	T	T	T	T	T	T	T	V		V
22	I	59	L	L	L	L	L	L	L	L	V	I	V	I	L	L	L	L	L	L	L	L	L		L
23		60	V	F	I	V	V	L	W	V	V	L	A	F	L	L	L	L	Y	Y	Y	Y	A		V
24		61	A	V	V	V	A	V	V	A	C	C	C	C	V	V	V	V	V	V	V	V	A		A
25		62	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T		T

HELIX II

aa		aa	mou	gfsh	can	bud	bov	xen	chk	hum	can	gfsh	bud	chk	can	duc	chk	bud	can	duc	chk	bud	hum	can	chk
posn		no.	360	365	366	371	blu	viol	418	420	440	441	444	455	500	502	507	508	505	505	506	509	560	567	571
3		72	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	V		L
4		73	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		N
5		74	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	W		W
6	F	* 75	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I		I
7	I L	* 76	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L		L
8		77	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	L	L	L	L	V		V
9	NS	78	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N		N
10	L	* 79	V	I	I	I	V	I	I	V	L	L	L	L	L	L	L	L	L	L	L	L	L		L
11	AS	80	S	S	S	S	S	T	S	S	A	S	A	A	A	A	A	A	A	A	V	A	A		A
12		81	L	L	V	F	L	V	A	F	V	I	V	L	V	V	V	V	V	V	V	V	V		V
13	AS	* 82	G	G	S	C	G	G	S	G	A	A	A	A	A	A	A	A	A	A	A	A	A		A
14	D	* 83	G	G	G	G	G	G	G	G	N	N	N	N	D	D	D	D	D	D	D	D	D		D
15	LF	84	F	F	L	F	F	F	F	F	L	L	L	L	L	L	L	L	L	L	L	L	L		L
16		85	L	I	M	L	I	L	V	L	L	F	L	L	C	F	F	F	F	F	F	F	A		G
17		* 86	F	F	C	A	Y	M	S	L	V	V	V	V	M	M	M	M	M	M	M	M	E		E
18		87	C	D	C	C	C	C	C	C	V	A	L	I	A	A	A	A	V	V	V	V	T		T
19		88	I	T	V	I	I	I	V	I	C	I	L	L	C	C	C	C	F	F	F	F	V		V
20		89	F	F	F	I	F	F	L	F	V	F	V	V	F	F	F	F	G	G	G	G	I		I
21		* 90	S	S	C	C	S	S	S	S	G	G	G	G	G	G	G	G	G	G	G	G	A		A
22		91	V	V	I	I	V	I	V	V	S	S	S	S	F	F	F	F	F	F	F	F	S		S
23		92	F	S	F	F	F	F	F	F	T	P	T	T	T	T	T	T	T	T	T	T	T		T
24		93	T	Q	T	T	I	P	V	P	T	L	T	T	V	V	V	V	T	T	T	T	I		I
25		94	V	V	V	V	V	V	V	V	A	S	A	A	T	T	T	T	T	T	T	T	S		S

HELIX III

aa posn		aa no.	mou 360	gfsh 365	can 366	bud 371	bov blu	xen viol	chk 418	hum 420	can 440	gfsh 441	bud 444	chk 455	can 500	duc 502	chk 507	bud 508	can 505	duc 505	chk 506	bud 509	hum 560	can 567	chk 571
3	*	113	E	E	E	E	E	D	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E
4	**	114	A	A	G	G	A	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
5		115	F	A	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	Y	Y	Y
6		116	L	M	A	M	L	V	V	L	T	L	T	A	F	L	F	F	F	F	F	F	T	T	T
7	*	117	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	V	V	V
8	*	118	S	S	A	A	C	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	S	S	S
9		119	V	I	T	T	T	L	H	V	L	L	L	L	L	L	L	L	L	L	L	L	L	A	A
10		120	A	A	G	A	A	T	G	A	G	G	G	G	G	G	G	G	G	G	G	G	C	C	C
11	**	121	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
12	*	122	L	L	M	L	L	L	L	L	M	M	M	M	Q	Q	Q	Q	E	E	E	E	I	I	I
13		123	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	I	I	I	I	T	T	T
14	S *	124	T	T	T	T	T	T	T	T	S	G	S	S	A	A	A	A	A	A	A	A	G	A	A
15	I **	125	G	G	G	G	G	G	G	G	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
16		126	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
17	*	127	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
18	L**	128	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
19	*	129	A	A	A	A	A	A	A	A	A	A	A	A	V	V	V	V	V	V	V	V	A	A	A
20		130	F	V	F	F	F	F	F	F	V	V	V	V	V	V	V	V	V	V	V	V	I	I	I
21	I L*	131	L	L	L	L	L	L	L	L	V	V	V	V	L	L	L	L	L	L	L	L	I	I	I
22	SA**	132	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	S	S	S
23		133	F	F	F	F	F	F	F	F	F	F	F	F	I	I	I	I	I	I	V	I	W	W	W
24	DE	134	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E
25	R	135	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
26	Y	136	Y	Y	Y	Y	Y	Y	Y	Y	F	W	F	F	Y	Y	Y	Y	Y	Y	Y	Y	W	W	W

HELIX IV

aa posn		aa no	mou 360	gfsh 365	can 366	bud 360	bov blu	xen viol	chk 418	hum 420	can 440	gfsh 441	bud 444	chk 455	can 500	duc 502	chk 507	bud 508	can 505	duc 505	chk 506	bud 509	hum 560	can 567	chk 571
3		153	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
4		154	L	L	L	L	L	L	L	L	V	I	L	V	M	M	M	M	I	I	I	I	I	V	V
5		155	M	G	L	V	M	A	L	T	L	A	L	L	M	M	M	M	M	M	M	M	V	A	A
6		156	V	A	V	V	V	V	V	V	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
7	I **	157	V	V	V	V	V	V	V	V	C	C	C	C	I	I	I	I	V	V	V	V	I	V	I
8		158	L	A	A	V	V	I	V	L	A	I	A	V	A	A	A	A	A	V	A	V	A	L	L
9		159	A	L	A	A	A	C	A	A	I	L	V	A	F	F	F	F	F	F	F	F	F	F	F
10		160	T	T	T	T	T	T	T	T	T	P	T	T	T	T	T	T	S	T	S	T	S	S	S
11	W	161	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
12		162	I	I	I	V	T	I	L	T	I	I	V	V	V	I	V	I	I	I	I	I	I	I	L
13		163	I	I	I	I	I	I	I	I	F	S	C	L	M	M	M	M	M	M	M	M	W	W	W
14	SA**	164	G	G	G	G	G	G	G	G	G	A	G	G	A	A	A	A	A	A	A	A	S	S	S
15		165	I	I	V	I	I	I	V	I	L	L	L	F	I	F	F	F	L	L	M	L	A	C	C
16		166	G	G	G	G	G	V	G	G	I	A	A	V	S	S	S	S	A	A	A	A	V	A	A
17		167	V	C	V	V	V	V	V	V	A	A	A	A	C	C	C	C	C	C	C	C	W	W	W
18	*	168	S	A	A	A	S	S	G	S	S	S	A	S	A	A	A	A	A	A	A	A	T	T	T
19		169	I	T	I	I	I	V	L	I	V	L	T	A	A	A	A	A	A	A	A	A	A	A	A
20	P	170	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
21	P *	171	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
22		172	F	F	F	F	F	F	F	F	L	L	L	L	L	L	L	L	L	L	L	L	I	I	I
23		173	F	W	F	F	F	L	F	F	F	F	L	F	F	F	F	F	F	F	F	F	F	F	F
24		174	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
25		175	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
26		176	S	S	S	S	S	S	S	S	S	S	N	S	S	S	S	S	S	S	S	S	S	S	S

HELIX V

aa		aa	mou	gfsh	can	bud	bov	xen	chk	hum	can	gfsh	bud	chk	can	duc	chk	bud	can	duc	chk	bud	hum	can	chk
posn		no	360	365	366	360	blu	viol	418	420	440	441	444	455	500	502	507	508	505	505	506	509	560	567	571
3		204	T	T	T	T	T	T	T	T	L	V	F	V	V	V	V	V	V	V	V	V	M	M	M
4		205	W	W	W	W	W	W	W	W	I	M	L	L	L	L	L	L	I	I	I	I	I	V	V
5		206	F	F	F	F	F	F	F	F	F	F	F	F	Y	Y	Y	Y	Y	Y	Y	Y	V	V	V
6		207	L	L	L	L	L	I	L	L	S	L	L	L	M	M	M	M	M	M	M	M	L	L	L
7	**	208	F	F	F	F	F	F	F	F	S	F	F	F	F	F	F	F	F	F	F	F	M	M	M
8		209	I	I	I	I	I	I	I	I	C	C	L	T	V	I	V	I	V	V	V	V	V	V	V
9		210	F	F	F	F	F	F	F	F	F	F	F	F	I	I	I	T	V	V	V	V	T	T	T
10	*	211	C	C	C	C	C	C	C	C	C	C	C	C	H	H	H	H	H	H	H	H	C	C	C
11	FY*	212	F	F	F	F	Y	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	C	C	C
12		213	I	I	I	I	I	V	I	I	G	A	G	G	I	I	I	I	M	T	M	M	I	F	F
13		214	I	I	I	V	V	I	V	V	F	V	V	V	I	I	I	I	I	I	I	I	I	F	F
14	P**	215	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	R	P	P	P
15	L *	216	L	L	L	L	L	L	L	L	L	F	L	L	V	V	V	V	L	L	L	L	L	L	L
16		217	S	S	S	S	S	S	S	S	S	G	S	A	V	V	V	V	L	M	A	M	A	A	A
17		218	L	L	L	L	L	L	L	L	V	T	V	I	I	V	V	V	I	V	V	I	I	V	I
18	MI**	219	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
19		220	C	I	I	I	C	C	I	C	V	V	V	V	F	F	F	F	F	F	F	F	M	I	I
20		221	F	F	F	F	F	F	F	F	F	F	V	F	F	F	F	F	F	F	F	F	L	F	L
21	*	222	S	S	S	S	S	S	S	S	S	C	C	S	S	S	S	S	C	C	C	C	C	C	C
22	Y *	223	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
23		224	S	S	S	S	S	G	S	T	G	G	G	G	G	G	G	G	G	G	G	G	L	L	L
24		225	Q	Q	Q	Q	Q	R	Q	Q	R	Q	R	R	R	R	R	R	N	N	N	N	Q	Q	Q
25	I V	226	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	V	V	V
26		227	L	L	L	L	L	L	L	L	L	L	L	L	V	I	I	F	V	V	V	V	W	W	S

HELIX VI

aa posn		*aa no.*	mou 360	gfsh 365	can 366	bud 371	bov blu	xen viol	chk 418	hum 420	can 440	gfsh 441	bud 444	chk 455	can 500	duc 502	chk 507	bud 508	can 505	duc 505	chk 506	bud 509	hum 560	can 567	chk 571
3	KR	*252*	H	R	R	R	H	R	R	R	K	K	R	K	R	R	R	R	R	R	R	R	R	R	R
4		*253*	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
5	L**	*254*	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
6		*255*	V	V	V	V	V	I	V	V	V	V	V	V	I	I	I	I	I	I	I	I	V	V	V
7		*256*	V	V	V	V	V	F	V	V	V	V	V	V	L	L	L	L	I	I	I	I	V	V	V
8	I *	*257*	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
9	V *	*258*	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	I	I	I
10		*259*	G	G	G	G	G	G	G	G	L	L	L	L	L	L	L	L	I	I	I	I	F	L	V
11		*260*	S	S	S	S	S	S	S	S	G	G	G	G	G	G	G	G	S	A	A	A	A	A	A
12	F**	*261*	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	Y	Y	Y
13		*262*	C	V	C	C	C	C	C	C	L	L	L	L	M	M	M	M	L	L	L	L	C	C	C
14		*263*	L	V	M	V	L	L	L	V	V	V	T	V	L	L	L	L	I	I	I	I	V	F	F
15	CS*	*264*	C	C	C	C	C	C	C	C	C	C	C	C	A	A	A	A	C	C	C	C	C	C	C
16	W**	*265*	Y	Y	Y	Y	Y	Y	Y	Y	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
17		*266*	V	G	V	V	T	V	V	V	L	A	L	A	T	T	T	T	V	V	V	G	G	G	G
18	P	*267*	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
19	YF*	*268*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
20	*	*269*	A	A	A	A	A	A	A	A	C	A	E	T	A	A	A	A	A	A	A	A	T	T	T
21		*270*	A	I	A	A	A	A	A	A	S	S	T	A	V	V	V	V	S	S	S	S	F	I	F
22	*	*271*	L	T	L	L	L	M	L	F	F	F	F	F	V	V	V	V	V	V	V	V	F	F	F
23		*272*	A	A	A	A	A	A	A	A	A	S	A	A	A	A	A	G	A	A	A	A	A	A	A
24		*273*	M	L	M	M	M	M	M	M	L	L	F	L	F	F	F	F	F	F	F	F	C	S	C
25		*274*	Y	Y	Y	Y	Y	Y	Y	Y	W	W	W	W	W	W	W	W	Y	Y	Y	Y	F	F	F
26		*275*	M	F	M	M	I	M	M	M	V	I	A	V	I	I	I	I	I	I	I	I	A	A	A

HELIX VII

aa posn		*aa no.*	mou 360	gfsh 365	can 366	bud 371	bov blu	xen viol	chk 418	hum 420	can 440	gfsh 441	bud 444	chk 455	can 500	duc 502	chk 507	bud 508	can 505	duc 505	chk 506	bud 509	hum 560	can 567	chk 571
3		*288*	V	V	V	V	V	V	V	V	A	A	A	A	M	M	M	M	M	M	M	M	A	A	A
4		*289*	T	A	T	T	T	T	T	T	S	T	S	S	A	S	A	S	T	T	T	T	A	A	A
5		*290*	I	I	I	I	I	I	I	I	I	I	V	I	V	V	V	V	I	I	I	I	L	L	L
6	*	*291*	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
7	*	*292*	A	S	A	A	A	A	A	S	S	S	S	S	A	A	A	A	A	A	A	A	R	A	A
8		*293*	F	L	F	F	F	F	F	F	V	C	V	V	F	F	F	F	F	F	F	F	Y	F	Y
9	LF	*294*	F	F	F	F	F	F	F	F	F	L	F	F	F	F	F	F	F	F	F	F	F	F	F
10		*295*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	A	A	A	A	A	A	A
11	*	*296*	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
12		*297*	S	S	S	S	S	S	S	S	A	A	A	S	S	S	S	S	S	S	S	S	S	S	S
13	NS	*298*	S	S	S	S	A	S	A	A	S	S	S	S	S	S	S	S	S	S	S	S	A	A	A
14	S *	*299*	C	C	C	C	C	C	C	C	T	T	T	T	S	S	S	S	A	A	A	S	T	T	T
15		*300*	V	V	V	V	V	V	V	I	V	V	V	V	L	L	L	L	I	I	I	I	I	I	I
16		*301*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
17	*	*302*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
18	P *	*303*	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
19		*304*	I	L	I	I	I	I	I	I	I	V	I	V	I	I	I	V	V	V	V	V	V	I	I
20	I	*305*	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
21	Y *	*306*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
22	*	*307*	C	A	C	C	C	S	C	C	V	V	V	V	V	L	V	V	I	I	I	I	V	V	V
23		*308*	F	F	F	F	F	F	F	F	F	L	L	L	L	L	L	L	V	V	V	V	F	F	F
24		*309*	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
25		*310*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
26		*311*	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	R	R	R

Figure 7.16b »

A three dimensional model of an opsin molecule showing the position of residues which are predicted to face the retinal binding pocket (red rings) (Baldwin, 1993; Schertler, 1993). The thicker rings denote residue in closest proximity to the chromophore.

The relative positions of the seven α-helical transmembrane regions (large circles) when viewed from the cytosolic side of the molecule are shown. The lines represent the orientation of the of amino acids around the helix, the length of each line giving an indication of the depth at which the amino acid is located. The numbers denote the relative positions of amino acids within the helix. The approximate position of the retinal chromophore and its attachment to Lys-296 via a Schiff's base linkage are shown schematically.

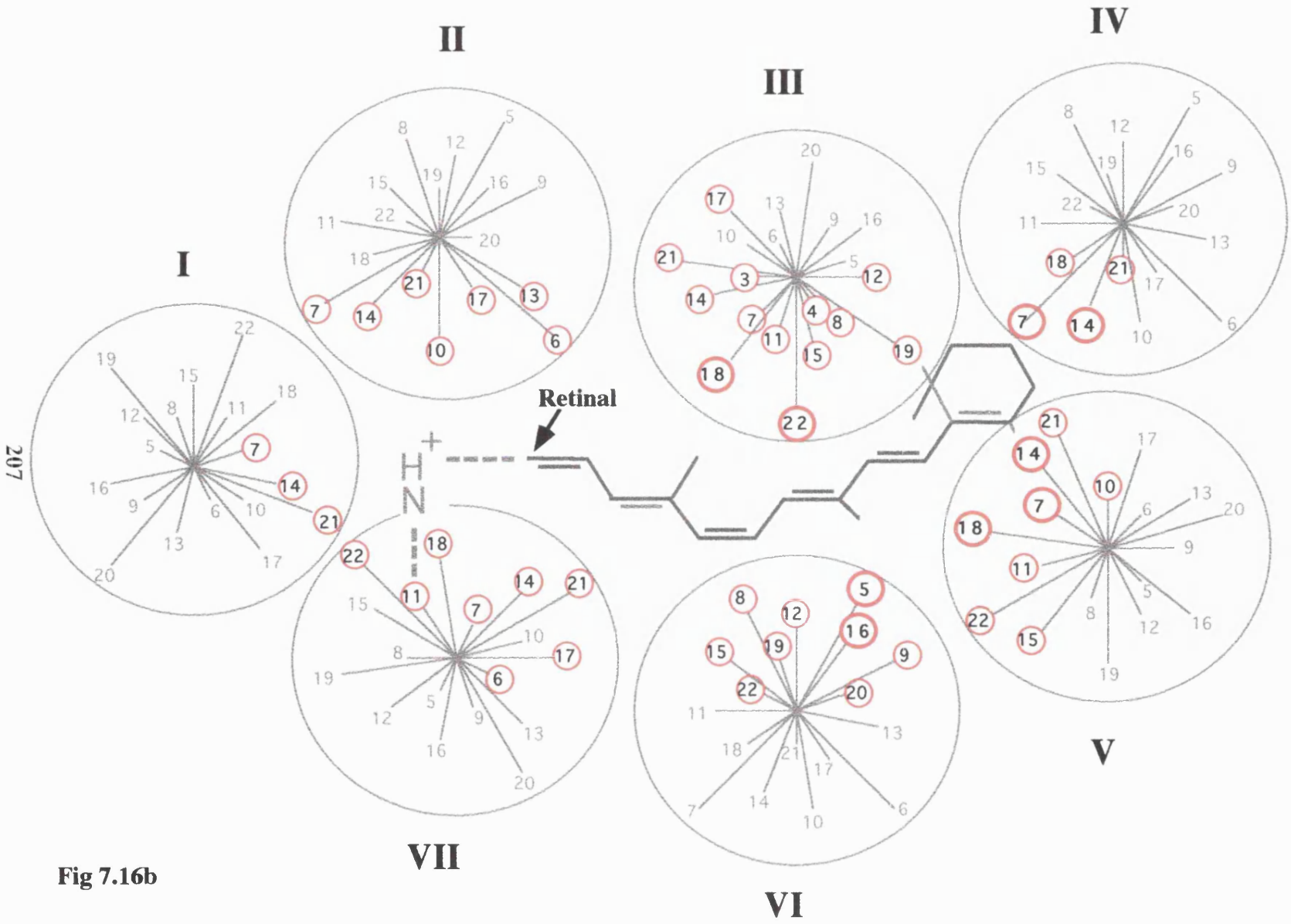


Fig 7.16b

7.17. Ultraviolet spectral tuning sites

Table 7.17a shows the results of a more comprehensive alignment of 12 vertebrate opsin sequences from the violet sensitive lineage, (some of which were included in table 7.16a). Residues which are found exclusively within VS opsins are underlined (in grey text) and have been listed in table 7.17b. These residues (table 7.17b) may play a role in tuning VS visual pigments down to wavelengths of 420 nm and below - particularly those which are polar and face the retinal binding pocket. For example, Tyr-265 is a chromophore-facing, hydroxyl bearing residue found only within the VS group of opsins. A Trp to Tyr substitution at site 265 in bovine rod opsin has been shown experimentally to cause a 15 nm blue shift (Nakayama and Khorana, 1991). Thr-124 is another *OH-* bearing polar residue which faces the retinal binding pocket. Polar residues which do not point directly into the retinal binding site in VS opsins include Gln-38, Thr-204, and Ser-260. These residues should not be ignored and may play a minor role in spectral tuning since they make up the unique helical environment that distinguishes a VS opsin. For instance substituting an amino acid on the outer surface of a helix with a different amino acid with a slightly larger residue, may slightly alter the distance to the next residue, and so on. In this way, substitutions in the outward facing residues of a helix could have subtle effects on the spatial positioning of the residues facing inwards.

Table 7.17a »
Alignment of 12 opsins from the violet sensitive lineage.

Only residues located in helical portions of the opsin are shown. The position of residues within a helix are indicated on the left hand side of each table, together with highly conserved residues (Baldwin, 1993). An asterisk indicates residues which are predicted to point into the retinal binding groove (Baldwin, 1993). Amino acid numbers in bold italics refer to the numbering according to bovine rod opsin (Wang *et al.,* 1980). Letters in light and dark pink represent ultraviolet- and violet- opsin sequences respectively. Numbers in the top margin are the pigment λ_{max} if known. Residues which are highly conserved across all VS opsins are indicated in grey, underlined lettering.

HELIX I

aa posn		*aa no.*	mou 360-370	gfsh 365	can 366	bud 371	rat blu	bov blu	xen viol	cmln viol	chk 418	hum 420	mar 423	tal 429
1		*38*	R	Y	Y	Y	R	H	T	Y	Y	Y	Y	Y
2		*39*	L	L	L	L	L	Q	L	F	L	L	L	L
3		*40*	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
4		*41*	A	A	T	T	A	A	A	T	T	A	A	A
5		*42*	A	A	I	A	A	V	I	A	A	A	A	A
6		*43*	F	F	F	F	F	F	F	F	F	F	F	F
7	*	*44*	M	M	M	M	M	M	M	M	M	M	M	M
8		*45*	G	G	G	G	G	G	G	G	G	G	G	G
9		*46*	F	F	L	F	F	F	M	F	I	T	I	T
10	I	*47*	V	V	V	V	V	V	V	V	V	V	V	V
11		*48*	F	F	F	F	F	F	F	F	F	F	F	F
12		*49*	F	F	V	M	F	F	L	F	A	L	L	L
13		*50*	V	V	A	V	V	V	I	A	V	I	I	A
14	G *	*51*	G	G	G	G	G	G	G	G	G	G	G	G
15		*52*	T	T	T	T	T	T	T	T	T	F	L	F
16		*53*	P	P	P	P	P	P	P	P	P	P	P	P
17	G	*54*	L	L	L	L	L	L	L	L	L	L	L	L
18	N	*55*	N	N	N	N	N	N	N	N	N	N	N	N
19		*56*	A	A	A	A	A	A	F	A	A	A	T	A
20	L	*57*	I	I	I	I	I	T	I	I	V	M	M	M
21	V *	*58*	V	V	V	V	V	V	V	I	V	V	V	V
22	I	*59*	L	L	L	L	L	L	L	L	L	L	L	L
23		*60*	V	F	I	V	V	V	L	I	W	V	V	V

HELIX II

aa posn		*aa no.*	mou 360-370	gfsh 365	can 366	bud 371	rat blu	bov blu	xen viol	cmln viol	chk 418	hum 420	mar 423	tal 429
4		*73*	N	N	N	N	N	N	N	N	N	N	N	N
5		*74*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
6	F *	*75*	I	I	I	I	I	I	I	I	I	I	V	I
7	I L*	*76*	L	L	L	L	L	L	L	L	L	L	L	L
8		*77*	V	V	V	V	V	V	V	V	V	V	V	V
9	NS	*78*	N	N	N	N	N	N	N	N	N	N	N	N
10	L *	*79*	V	I	I	I	V	V	I	I	I	V	V	V
11	AS	*80*	S	S	S	S	S	S	T	S	S	S	S	S
12		*81*	L	L	V	F	L	L	V	F	A	F	V	F
13	AS*	*82*	G	G	S	C	G	G	G	A	S	G	G	G
14	D *	*83*	G	G	G	G	G	G	G	G	G	G	G	G
15	LF	*84*	F	F	L	F	F	F	F	F	F	F	F	F
16		*85*	L	I	M	L	L	I	L	L	V	L	L	L
17	*	*86*	F	F	C	A	F	Y	M	F	S	L	L	L
18		*87*	C	D	C	C	C	C	C	C	C	C	C	C
19		*88*	I	T	V	I	I	I	I	T	V	I	I	I
20		*89*	F	F	F	I	F	F	F	F	L	F	F	F
21	*	*90*	S	S	C	C	S	S	S	S	S	S	S	S
22		*91*	V	V	I	I	V	V	I	V	V	V	V	V
23		*92*	F	S	F	F	F	F	F	F	F	F	F	F
24		*93*	T	Q	T	T	T	I	P	T	V	P	P	P
25		*94*	V	V	V	V	V	V	V	V	V	V	V	V
26		*95*	F	F	F	F	F	F	F	F	F	F	F	F

HELIX III

aa posn		*aa no.*	mou 360-370	gfsh 365	can 366	bud 371	rat blu	bov blu	xen viol	cmln viol	chk 418	hum 420	mar 423	tal 429
1		*111*	A	A	R	A	A	A	S	A	E	A	A	G
2		*112*	L	M	F	F	L	L	I	M	L	L	L	F
3	*	*113*	E	E	E	E	E	E	D	E	E	E	E	E
4	**	*114*	A	A	G	G	A	A	A	A	A	G	G	A
5		*115*	F	A	F	F	G	F	F	F	F	F	F	F
6		*116*	L	M	A	M	L	L	V	L	V	L	L	L
7	*	*117*	G	G	G	G	G	G	G	G	G	G	G	G
8	*	*118*	S	S	A	A	S	C	T	S	T	T	T	T
9		*119*	V	I	T	T	V	T	L	V	H	V	V	V
10		*120*	A	A	G	A	A	A	T	A	G	A	A	A
11	**	*121*	G	G	G	G	G	G	G	G	G	G	G	G
12	*	*122*	L	L	M	L	L	L	L	L	L	L	L	L
13		*123*	V	V	V	V	V	V	V	V	V	V	V	V
14	S *	*124*	T	T	T	T	T	T	T	T	T	T	T	T
15	I **	*125*	G	G	G	G	G	G	G	G	G	G	G	G
16		*126*	W	W	W	W	W	W	W	W	W	W	W	W
17	*	*127*	S	S	S	S	S	S	S	S	S	S	S	S
18	L **	*128*	L	L	L	L	L	L	L	L	L	L	L	L
19	*	*129*	A	A	A	A	A	A	A	A	A	A	A	A
20		*130*	F	V	F	F	F	F	F	F	F	F	F	F
21	I L*	*131*	L	L	L	L	L	L	L	L	L	L	L	L
22	SA**	*132*	A	A	A	A	A	A	A	A	A	A	A	A
23		*133*	F	F	F	F	F	F	F	F	F	F	F	F

HELIX IV

aa posn		*aa no.*	mou 360-370	gfsh 365	can 366	bud 371	rat bla	bov blu	xen viol	cmln viol	chk 418	hum 420	mar 423	tal 429
3		*153*	A	A	A	A	A	A	A	A	A	A	A	A
4		*154*	L	L	L	L	L	L	L	L	L	L	L	L
5		*155*	M	G	L	V	M	M	A	L	L	T	M	T
6		*156*	V	A	V	V	V	V	V	V	V	V	V	V
7	I **	*157*	V	V	V	V	V	V	V	V	V	V	V	V
8		*158*	L	A	A	V	L	V	I	A	V	L	L	L
9		*159*	A	L	A	A	A	A	C	A	A	A	T	A
10		*160*	T	T	T	T	T	T	T	T	T	T	T	T
11	W	*161*	W	W	W	W	W	W	W	W	W	W	W	W
12		*162*	I	I	I	V	I	T	I	F	L	T	T	I
13		*163*	I	I	I	I	I	I	I	I	I	I	I	I
14	SA**	*164*	G	G	G	G	G	G	G	G	G	G	G	G
15		*165*	I	I	V	I	I	I	I	I	V	I	I	I
16		*166*	G	G	G	G	G	G	V	G	G	G	G	G
17		*167*	V	C	V	V	V	V	V	V	V	V	V	V
18	*	*168*	S	A	A	A	S	S	S	S	G	S	S	S
19		*169*	I	T	I	I	I	I	V	I	L	I	I	I
20	P	*170*	P	P	P	P	P	P	P	P	P	P	P	P
21	P *	*171*	P	P	P	P	P	P	P	P	P	P	P	P
22		*172*	F	F	F	F	F	F	F	F	F	F	F	F
23		*173*	F	W	F	F	F	F	L	F	F	F	F	F
24		*174*	G	G	G	G	G	G	G	G	G	G	G	G
25		*175*	W	W	W	W	W	W	W	W	W	W	W	W

HELIX V

aa posn		*aa no.*	mou 360-370	gfsh 365	can 366	bud 371	rat blu	bov blu	xen viol	cmln viol	chk 418	hum 420	mar 423	tal 429
2		*203*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3		*204*	T	T	T	T	T	T	T	T	T	T	T	T
4		*205*	W	W	W	W	W	W	W	W	W	W	W	W
5		*206*	F	F	F	F	F	F	F	F	F	F	F	F
6		*207*	L	L	L	L	L	L	I	L	L	L	L	L
7	**	*208*	F	F	F	F	F	F	F	F	F	F	F	F
8		*209*	I	I	I	I	I	I	I	I	I	I	I	I
9		*210*	F	F	F	F	F	F	F	F	F	F	F	F
10	*	*211*	C	C	C	C	C	C	C	C	C	C	C	C
11	FY*	*212*	F	F	F	F	F	Y	F	F	F	F	F	F
12		*213*	I	I	I	I	I	I	V	I	I	I	I	I
13		*214*	I	I	I	V	I	V	I	V	V	V	V	V
14	P**	*215*	P	P	P	P	P	P	P	P	P	P	P	P
15	L *	*216*	L	L	L	L	L	L	L	L	L	L	L	L
16		*217*	S	S	S	S	S	S	S	T	S	S	A	S
17		*218*	L	L	L	L	L	L	L	L	L	L	L	L
18	MI**	*219*	I	I	I	I	I	I	I	I	I	I	I	I
19		*220*	C	I	I	I	C	C	C	I	I	C	C	C
20		*221*	F	F	F	F	F	F	F	F	F	F	F	F
21	*	*222*	S	S	S	S	S	S	S	S	S	S	S	S
22	Y *	*223*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
23		*224*	S	S	S	S	F	S	G	S	S	T	A	T
24		*225*	Q	Q	Q	Q	Q	Q	R	Q	Q	Q	Q	Q

HELIX VI

aa posn		*aa no.*	mou 360-370	gfsh 365	can 366	bud 371	rat blu	bov blu	xen viol	cmln viol	chk 418	hum 420	mar 423	tal 429
4		*253*	M	M	M	M	M	M	M	M	M	M	M	M
5	L**	*254*	V	V	V	V	V	V	V	V	V	V	V	V
6		*255*	V	V	V	V	V	V	I	V	V	V	V	V
7		*256*	V	V	V	V	V	V	F	V	V	V	V	V
8	I *	*257*	M	M	M	M	M	M	M	M	M	M	M	M
9	V *	*258*	V	V	V	V	V	V	V	V	V	V	V	V
10		*259*	G	G	G	G	G	G	G	G	G	G	G	G
11		*260*	S	S	S	S	S	S	S	S	S	S	S	S
12	F**	*261*	F	F	F	F	F	F	F	F	F	F	F	F
13		*262*	C	V	C	C	C	C	C	C	C	C	C	C
14		*263*	L	V	M	V	L	L	L	L	L	V	V	V
15	CS*	*264*	C	C	C	C	C	C	C	C	C	C	C	C
16	W**	*265*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
17		*266*	V	G	V	V	V	T	V	V	V	V	V	V
18	P	*267*	P	P	P	P	P	P	P	P	P	P	P	P
19	YF*	*268*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
20	*	*269*	A	A	A	A	A	A	A	A	A	A	A	A
21		*270*	A	I	A	A	A	A	A	S	A	A	A	A
22	*	*271*	L	T	L	L	L	L	M	L	L	F	L	F
23		*272*	A	A	A	A	A	A	A	A	A	A	A	A
24		*273*	M	L	M	M	M	M	M	M	M	M	M	M
25		*274*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
26		*275*	M	F	M	M	M	I	M	M	M	M	M	M

HELIX VII

aa posn		*aa no.*	mou 360-370	gfsh 365	can 366	bud 371	rat blu	bov blu	xen viol	cmln viol	chk 418	hum 420	mar 423	tal 429
1		*286*	R	R	R	R	R	R	R	R	R	R	R	R
2		*287*	L	L	L	L	L	L	L	L	L	L	L	L
3		*288*	V	V	V	V	V	V	V	V	V	V	V	V
4		*289*	T	A	T	T	T	T	T	T	T	T	T	T
5		*290*	I	I	I	I	I	I	I	I	I	I	I	I
6	*	*291*	P	P	P	P	P	P	P	P	P	P	P	P
7	*	*292*	A	S	A	A	A	A	A	A	A	S	A	A
8		*293*	F	L	F	F	F	F	F	F	F	F	F	F
9	LF	*294*	F	F	F	F	F	F	F	F	F	F	F	F
10		*295*	S	S	S	S	S	S	S	S	S	S	S	S
11		*296*	K	K	K	K	K	K	K	K	K	K	K	K
12		*297*	S	S	S	S	S	S	S	S	S	S	S	S
13	NS	*298*	S	S	S	S	S	A	S	S	A	A	S	A
14	SA*	*299*	C	C	C	C	C	C	C	C	C	C	C	C
15		*300*	V	V	V	V	V	V	V	V	V	I	I	I
16		*301*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
17	*	*302*	N	N	N	N	N	N	N	N	N	N	N	N
18	P *	*303*	P	P	P	P	P	P	P	P	P	P	P	P
19		*304*	I	L	I	I	I	I	I	I	I	I	I	I
20	I	*305*	I	I	I	I	I	I	I	I	I	I	I	I
21	Y *	*306*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
22	*	*307*	C	A	C	C	C	C	S	C	C	C	C	C
23		*308*	F	F	F	F	F	F	F	F	F	F	F	F

Table 7.17b

Residues which are found exclusively within VS opsins (See table 7.17a).

Helix	aa posn in helix	*aa no. bov. rod*	residue found in VS opsins	predicted before ? *and by whom*			
				a	*b*	*c*	*d*
I	3	*38*	**Gln^{N}**				
I	8	*45*	Gly			√	√
II	14*	*83*	Gly				
II	15	*84*	Phe			√	√
III	7*	*117*	Gly				
III	14*	*124*	**Thr^{OH}**	√			
III	15**	*125*	Gly		√	√	√
III	21	*131*	Leu				
IV	4	*154*	Leu				
IV	13	*163*	Ile				
V	3	*204*	**Thr^{OH}**		√		√
V	4	*205*	Trp				√
V	17	*218*	Leu				
VI	10	*259*	Gly			√	
VI	11	*260*	**Ser^{OH}**			√	
VI	16**	*265*	**Tyr^{OH}**		√	√	√
VII	3	*288*	Val				
VII	14*	*299*	Cys			√	√
VII	22*	*307*	Cys				√

An asterisk beside the residue number indicates the degree of proximity to the retinal binding pocket, (two asterisks indicate very close proximity). Polar residues are indicated in bold lettering. ***a***, Chang, *et al.*, 1995; ***b***, Yokoyama, 1994, 1995; ***c***, Hisatomi *et al.*, 1996; ***d***, Kawamura and Yokoyama, 1996

7.18. Avian- or canary-specific VS tuning sites

Inspection of the alignment of 12 VS opsins in table 7.17a shows several sites which display a certain degree of variability and may indicate species-specific ultraviolet tuning sites, which possibly fine-tune the λ_{max} of the VS visual pigment. Possible avian- or canary ultraviolet opsin-specific tuning sites have been extracted from table 7.17a and are highlighted in table 7.18a.

When VS opsins are arranged according to their respective vertebrate groups as in table 7.18a, it appears that individual violet-specific tuning sites have evolved independently in each group. A Gly/Ala residue is found at site 82 (helix II, position 13) of the VS opsins in fish, amphibia, reptiles and mammals. In contrast, the presence of a polar Ser residue in the canary and chicken VS opsins, suggests that this site may be a putative avian VS spectral tuning site. However the budgerigar (VP λ_{max} = 371 nm) ultraviolet opsin possesses a non-polar Cys residue at this site. If this were a major avian VS tuning site, one might expect the budgerigar ultraviolet opsin also to posses a Ser, or at least a polar residue. Nevertheless, this site may play a minor role in tuning.

An *OH-* bearing polar Ser is found in the chicken violet opsin at Site 86 (helix II, position 17), whereas non-polar residues are found in both canary and budgerigar ultraviolet opsins. This suggests that a polar to non-polar substitution at site 86 may be involved in the shift in pigment λ_{maxs} from the violet region (400-420 nm) down into the ultraviolet region (< 370 nm) in birds. At site 90 (helix II, position 21) a polar Ser in the chicken violet opsin is again replaced by non-polar Cys residues in both canary and budgerigar ultraviolet opsins. This is a strong candidate ultraviolet tuning site in avians at least, possibly affecting the shift in λ_{max} from the violet region (400-420 nm) down into the ultraviolet region (<370nm) of the spectrum. Site 118 (helix III, position 8) is another strong candidate ultraviolet tuning site in avians, where a polar, *OH-* bearing, Thr residue in the chicken violet opsin is replaced by a non-polar alanine in both the canary and budgerigar ultraviolet opsins (See fig 7.18a).

At site 114 (helix III, position 4), the homologous substitution, Ala to Gly is seen in avian and mammalian VS opsins. In birds, an Ala residue occurs in the chicken violet opsin, whereas a Gly residue is seen in both canary and budgerigar ultraviolet opsins. In the mammals the converse is seen; a Gly residue occurs in the mouse ultraviolet opsin whereas the human and marmoset violet opsins possess an Ala (as do rat and bovine VS opsins although the λ_{maxs} of these pigments have yet to be measured). Although not a convincing candidate site, position 114 is predicted to be in very close proximity to the retinal chromophore (see fig. 7.16b) and it may be that the extra methyl group in alanine has a very slight, perhaps steric effect on the chromophore. Site 168 (helix IV,

position 18) is another Gly to Ala homologous substitution (violet to ultraviolet, respectively) specific to avians, which probably has very little, if any, effect on the chromophore. The positions of these sites in a three dimensional opsin model are shown in fig. 7.18b.

Table 7.18a
Putative canary ultraviolet opsin-specific tuning sites

Helix	aa posn in helix	*aa no. (bov rod)*	**fish**	**amphibian**	**avian / reptilian**				**mammalian**				
			gfsh 365	xen viol	cmln viol	**chk 418**	**can 366**	**bud 371**	mou 365	rat blu	bov blu	mar 423	hum 420
II	**13***	***82***	G	G	A	**S**	**S**	**C**	G	G	G	G	G
II	**17***	***86***	F	M	F	**S**	**C**	**A**	F	F	Y	L	L
II	**21***	***90***	S	S	S	**S**	**C**	**C**	S	S	S	S	S
III	**4****	***114***	A	A	A	**A**	**G**	**G**	A	A	A	G	G
III	**8***	***118***	S	T	S	**T**	**A**	**A**	S	S	C	T	T
IV	**18***	***168***	A	S	S	**G**	**A**	**A**	S	S	S	S	S

All residues face into the retinal binding pocket. An asterisk beside the residue number indicates the degree of proximity to the retinal binding pocket (two asterisks indicate very close proximity). (see page 166 for list of abbreviations).

Figure 7.18b »

A three dimensional model of an opsin molecule (Baldwin, 1993; Schertler, 1993) showing the relative positions of the seven α-helical transmembrane regions (circles) when viewed from the cytosolic side of the molecule. The lines represent the orientation of the of amino acids around the helix, the length of each line giving an indication of the depth at which the amino acid is located. The numbers denote the relative positions of amino acids within the helix. The approximate position of the retinal chromophore and its attachment to Lys-296 via a Schiff's base linkage are shown schematically.

Putative UV tuning sites in the canary ultraviolet sensitive opsin are shown, using the single letter amino acid code. Mauve and pink coloured circles represent equivalent residues in the canary ultraviolet and chick violet opsins, respectively.

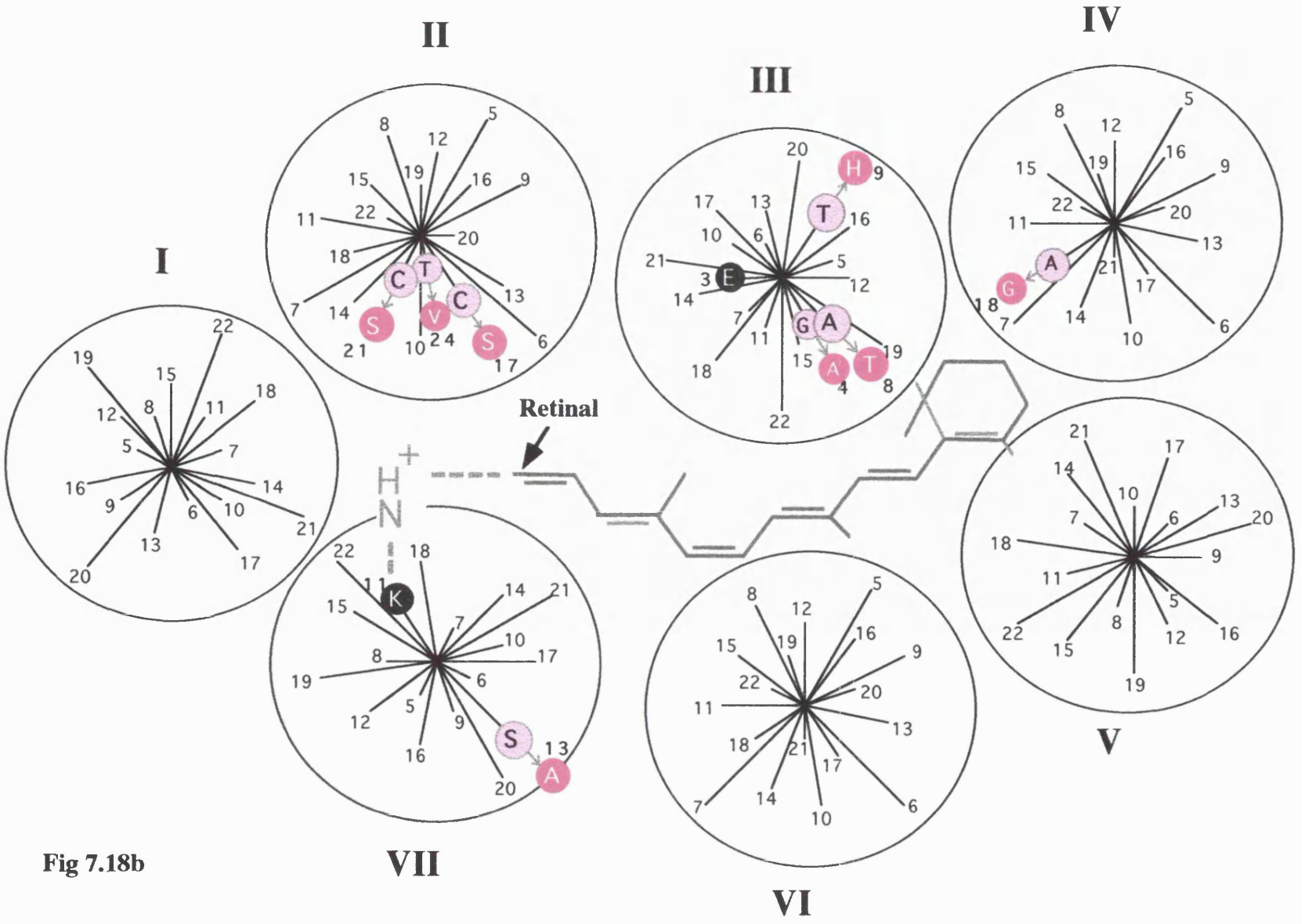


Fig 7.18b

7.19. Conclusions

The alignment of 12 vertebrate VS opsins used in the present study represents the most comprehensive line up of violet- and ultraviolet sensitive opsins to date. With the exception of residues which are common to all members of the VS group of opsin, the putative tuning sites drawn schematically in fig. 7.18b (Helical wheel model) are deemed to be most important in tuning the canary ultraviolet sensitive opsin from violet wavelengths (400-420) down to ultraviolet wavelengths (<370 nm). Particularly salient are the non-homologous substitutions at chromophore facing sites, 90 (Ser to Cys) and 118 (Thr to Ala), both of which involve the loss of an OH group in the canary ultraviolet sensitive opsin. Several of the putative ultraviolet/violet specific tuning sites identified in the present study have been suggested previously. So far, only one residue, Tyr-265 has been shown, by site directed mutagenesis studies, to cause a blue shift in bovine rod opsin (Nakayama and Khorana, 1991). Clearly, more site-directed mutagenesis studies need to be carried out in order to verify the growing number of predicted spectral tuning sites.

7.20. Possible Spectral tuning sites in the canary SWS opsin

Highly conserved polar/charged residues in SWS opsins which face the retinal binding pocket have been extracted from table 7.16a and are presented in table 7.20a. The chameleon and cavefish blue opsin sequences have also been included. The polar residues, Asn-83 (helix II, position 14), Ser-124 (helix III, position 14) and Ser-292 (helix VII, position 7) are found exclusively in SWS opsins and may play a significant role in the spectral tuning of SWS opsins down to wavelengths of 430-460 nm. The other listed residues are highly conserved although not SWS opsin-specific.

Site 222 is a putative avian/reptilian SWS tuning site. At this position, a polar *OH*-bearing Ser is seen in the canary, chicken and chameleon SWS opsins, whereas the cavefish and goldfish SWS opsins possess a non-polar Cys. The budgerigar SWS opsin, however, does not fit this trend and shares the Cys residue found in fish. Interestingly, in the phylogenetic trees, (Figures 7.11f and 7.11g), the older, goldfish SWS opsin branched off before the avian SWS subclade, and the budgerigar SWS opsin formed a separate branch within the avian subclade, indicating an earlier divergence from the ancestral opsin than the canary and chicken SWS opsins. In support of its position in the evolutionary trees, the budgerigar SWS opsin appears to have retained the 'older' Cys residue, also present in fish. Ser-208 (helix V, position 7) which is predicted to lie in very close proximity to the chromophore may be a putative canary-specific SWS tuning site. A non-polar Phe residue occurs in all other SWS opsin sequences at this site.

7.21. Conclusions

Several highly conserved polar residues which face the chromophore binding site have been identified in SWS opsins and are illustrated in fig. 7.21a. Of these, the residues at sites 83, 124 and 292 (Asn, Ser and Ser, respectively) are exclusive to the SWS class of opsins, and are therefore strong candidate SWS spectral tuning sites. In support of this, substitution of Asp-83 in bovine rod opsin with Asn has previously been found to cause an 8.5 nm shift to shorter wavelengths (Nathans, 1990b).

Table 7.20a
Highly conserved polar/charged residues in SWS opsins.

Helix	aa posn in helix	*aa no. (bov rod)*	fish		avian / reptilian			
			cfsh blu	gfsh 441	cmln 447	can 440	bud 444	chk 455
I	21*	*58*	T	T	T	T	T	T
II	14*	*83*	**N**	**N**	**N**	**N**	**N**	**N**
III	8*	*118*	T	T	T	T	T	T
III	14*	*124*	**S**	**G**	**S**	**S**	**S**	**S**
IV	18*	*168*	S	S	S	S	A	S
V	7**	*208*	F	F	F	**S**	F	F
V	21*	*222*	C	C	**S**	**S**	C	**S**
VII	7*	*292*	**S**	**S**	**S**	**S**	**S**	**S**
VII	14*	*299*	T	T	T	T	T	T

All residues face into the retinal binding pocket. An asterisk beside the residue number indicates the degree of proximity to the retinal binding pocket (two asterisks indicate very close proximity). Residues which are conserved only in the SWS group of opsins are indicated in blue lettering. See page 166 for a list of abbreviations used.

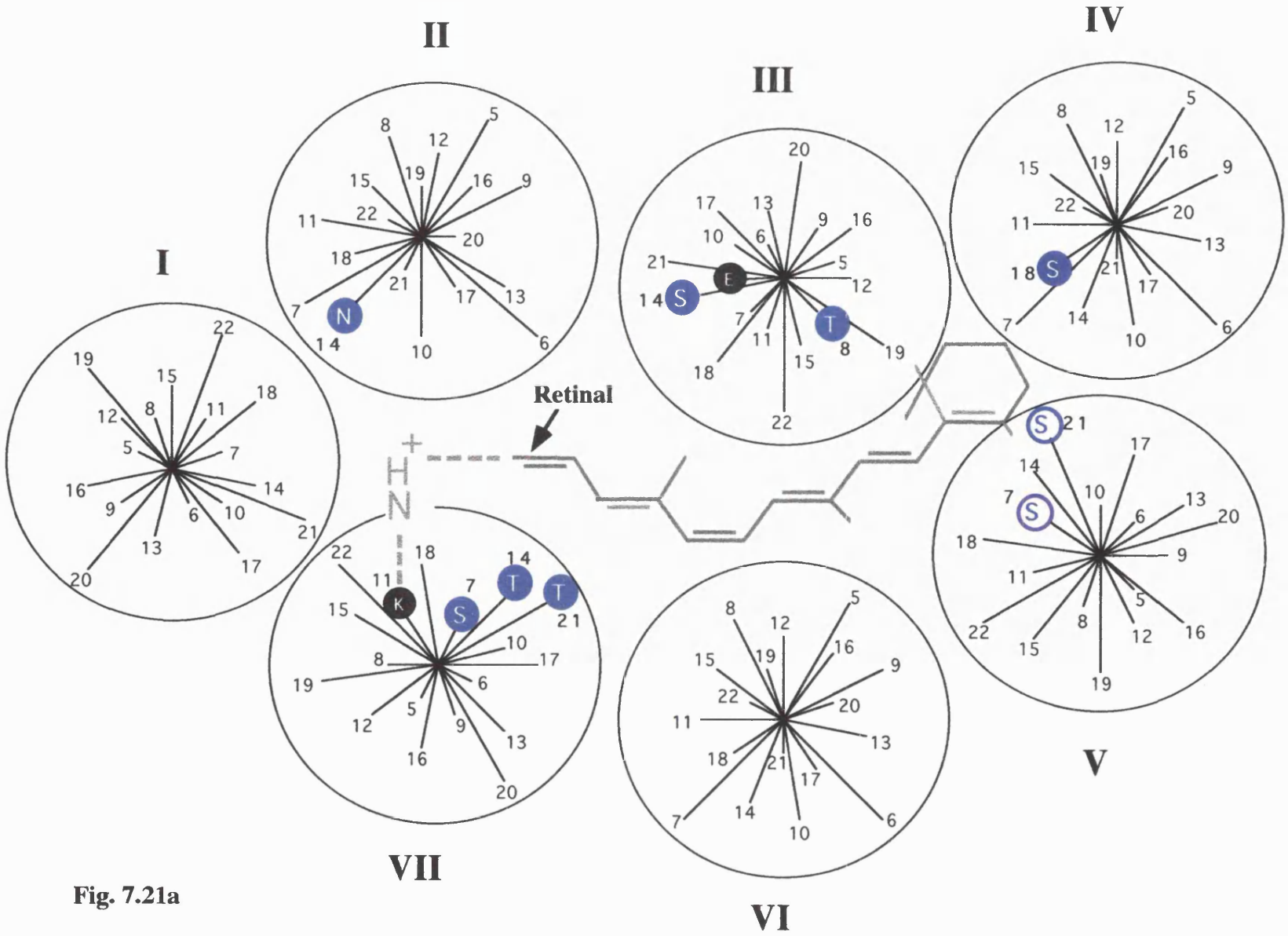


Fig. 7.21a

7.22. Possible Spectral tuning sites in the canary MWS and rod opsins

The almost identical λ_{maxes} of avian MWS cone and rod visual pigments raises the question of whether similar spectral tuning mechanisms are used at the molecular level in these two closely related opsin classes. The very high degree of homology between avian MWS and rod opsins is apparent, upon inspection of table 7.16a on page 205. Table 7.22a lists only polar/charged residues found in both MWS- and rod opsins in avians, at sites facing the retinal binding pocket. Residues which are highly conserved in all opsins, such as Lys-296 and Glu-113, have been omitted. The negatively charged Asp-83 and positively charged His-211 are likely to have direct interactions with the chromophore and be involved in spectral tuning in avian rod and MWS opsins. The presence of a His residue exclusively in members of the M2 branch of opsins (Okano *et al.,* 1992) suggests that it serves an important functional role. Infact, site directed mutagenesis experiments, in bovine rod opsin suggest that protonation of His-211 stabilizes the biologically active Meta II conformation of bovine rod opsin, (Weitz and Nathans, 1992). The other polar *OH-* bearing Thr residues at sites 58 and 118 may also exert a direct polarizing effect on the retinal chromophore.

The few chromophore facing sites at which amino acid residues differ between avian MWS and rod opsins, are listed in table 7.22b. The Gln (MWS opsins) to Glu (rod opsins) substitution at site 122 constitutes a non-conservative change (polar, *N*-bearing to charged residue) which is predicted to be in very close proximity to the chromophore. A Glu to Gln substitution, when replicated in site-directed mutants of bovine rod opsin (expressed, reconstituted opsin λ_{max} = 500 nm) leads to a 20 nm shift to shorter wavelengths (480 nm) (Zhukovsky and Oprian, 1989; Sakmar and Khorana, 1989). It has been suggested that the effect of Glu-122 is steric rather than charge based since substitution of a polar, uncharged Asn caused an even greater 25 nm shift to shorter wavelengths (475 nm), (Zhukovsky and Oprian, 1989; Sakmar and Khorana, 1989). Given that avian MWS and rod opsins are very similar (both in visual pigment λ_{max} and in opsin sequence (≈70%, see amino acid identity tables on pages 185 and 193) the fact that a Gln change causes a 20 nm shift to shorter wavelengths in bovine rod opsin, raises the question of which substitutions will affect the shift in λ_{max} of the avian MWS pigment back up to 500 nm. With this in mind, it is interesting that the remaining two chromophore facing, amino acid substitutions of a non-conservative nature in table 7.22b are both polar to non-polar changes. These are at sites 222 (Ser to Cys), and 299 (Ser to Ala), and in both cases the polar residue occurs in MWS opsins. Perhaps the polar Ser-222 and Ser-299 in avian MWS opsins partially 'correct' the putative shift in λ_{max} to 480 nm (as a result of the Gln-122 substitution in MWS opsins) and cause a 20 nm shift back up to 500 nm. Site directed mutagenesis studies on these sites may be able to verify this. The remaining amino acid differences between

avian MWS and rod opsins are all homologous, non-polar to non-polar changes and will probably have very little effect on the chromophore.

The putative MWS and rod spectral tuning sites discussed above are shown in fig. 7.22c

Table 7.22a
Polar/charged residues found in both MWS- and rod opsins in avians, at sites facing the retinal binding pocket.

Helix	aa posn in helix	*aa no. (bov rod)*	MWS opsins	rod opsins	
I	21*	*58*	Thr^{OH}	Thr^{OH}	
II	14*	*83*	Asp^{-}	Asp^{-}	D83G, +1.5 D83N, - 8.5 (Nathans, 1990b)
III	8*	*118*	Thr^{OH}	Thr^{OH}	
V	10**	*211*	His^{+}	His^{+}	•exclusive to avian MWS and rod opsins. Protonation of His-211 thought to stabilise the biologically active Meta II conformation in bovine rod opsin. Weitz and Nathans, 1992)

•and other members of M2 branch (Okano, 1992). An asterisk beside the residue position indicates the degree of proximity to the chromophore (Baldwin, 1993). Two Asterisks indicate very close proximity. +/- shift to longer or shorter wavelengths, respectively.

Table 7.22b
Amino acid residues which differ between avian MWS and rod opsins.

Helix	aa posn in helix	*aa no. (bov rod)*	MWS opsins	rod opsins	
I	7*	*44*	Ile	Met	
III	12**	*122*	Gln^{N}	Glu^{-}	E122N, - 20 nm E122D, - 25 nm (Zhukovsky and Oprian, 1989; Sakmar and Khorana, 1989)
IV	7**	*157*	Ile	Val	
V	15*	*216*	Val	Leu	
V	21*	*222*	Ser^{OH}	Cys	
VI	15*	*264*	Ala	Cys	
VII	14*	*299*	Ser^{OH}	Ala/S	Ser-299 also found in budgerigar rod opsin
VII	22*	*307*	Val/Leu	Ile	

An asterisk beside the residue position indicates the degree of proximity to the chromophore (Baldwin, 1993). Two Asterisks indicate very close proximity.

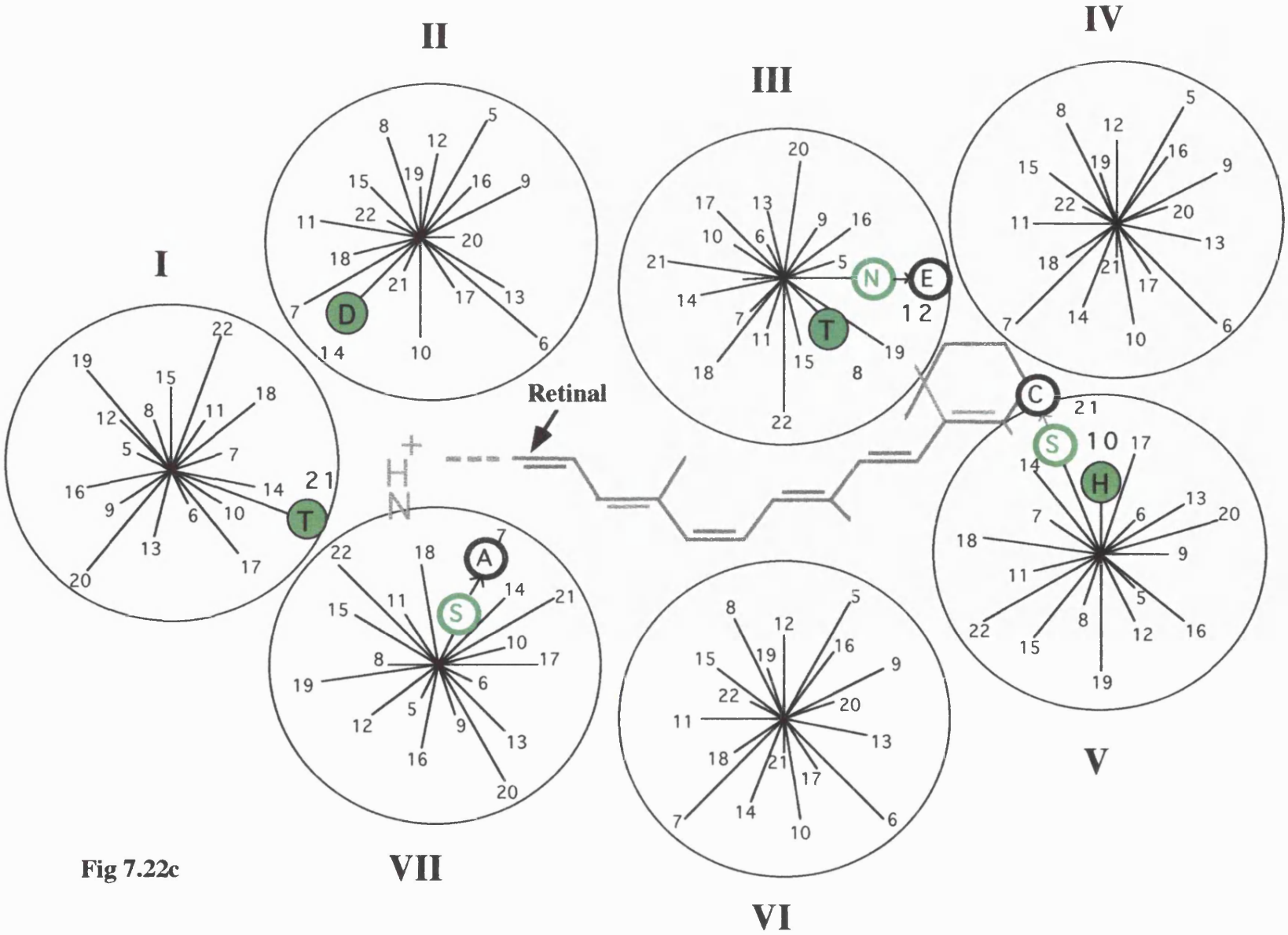


Fig 7.22c

7.23. Possible spectral tuning sites in the LWS opsin from *Serinus canaria*

Table 7.23a lists the seven key amino acid residues previously identified by Asenjo, *et al.* (1994) as being important in the spectral tuning of the human L_G pigment from a λ_{max} of 532 nm up to 563 nm (λ_{max} of the human L_R pigment) (see Visual Pigments chapter). The amino acid residues occupying the equivalent sites in the canary LWS opsin are given along with those found in the chicken LWS opsin, for purposes of comparison. The canary LWS opsin shares the same residues that are found in the human L_R opsin at five of the positions listed. However, the Ile at position 214 (using bovine rod opsin numbering) in the human L_R opsin is replaced by a Phe residue in both canary and chicken LWS opsins. This is an homologous change since phenylalanine possesses a non-polar, uncharged side chain, despite its aromatic ring structure. As can be seen from fig. 7.16b, this site, although located within helix V (position 13) actually faces away from the retinal binding pocket. Conservation of amino acid residue at this site may not be crucial in all LWS opsins and Phe-214 probably exerts an indirect effect, if at all, on the chromophore by influencing the conformation of surrounding helices, for example.

Interestingly, the canary LWS opsin is similar to the human L_G opsin in substituting a non-polar Phe residue at site 309 instead of the polar, hydroxyl bearing, Tyr-309 which is seen in both the human L_R- and chicken LWS opsins. Site 309 is located close to the top of helix VII, but is not one of the 26 helical residues, suggesting that it has an indirect effect, if any, on the chromophore. The fact that some of the residues identified by Asenjo *et al.* (1994) are not conserved amongst avian LWS opsins suggests that these sites may not be crucial to all LWS opsins, but specifically fine tune the human L_R opsin.

Most vertebrate LWS opsins possess the hydroxyl bearing amino acid residues Ser-164, Tyr-261, Thr-269 (using bovine rod opsin numbering) which all point into the retinal binding groove (Okano *et al.,* 1992; Johnson *et al.,* 1993; Hisatomi *et al.,* 1994; Yokoyama, 1994; Chang *et al.,* 1995). This may be evidence for a common ancestral gene. However, since the MW gene duplication event which gave rise to the LW pigment only appears to have occurred in fish and humans, and is not seen in other vertebrates, it may be that these sites have evolved as longwave spectral tuning sites independently in each class of vertebrates, and as such are a good example of convergent evolution.

Table 7.23a
The seven key amino acid residues previously identified by Asenjo *et al.* (1994) as being important in the spectral tuning of the human $L_{G/R}$ pigments.

no. in bov. rod	no. in hum LWS/ MWS	Residue in human L_G opsin λ_{max} = 532 nm	Residue in human L_R opsin λ_{max} = 563 nm	**Residue in canary LWS opsin λ_{max} = 569 nm**	Residue in chicken LWS opsin λ_{max} = 571 nm
100	116	Tyr	Ser	**Ser**	Ser
164	180	Ala	Ser	**Ser**	Ser
214	230	Thr	Ile	**Phe**	Phe
217	233	Ser	Ala	**Ala**	Ala
261	277	Phe	Tyr	**Tyr**	Tyr
269	285	Ala	Thr	**Thr**	Thr
293	309	Phe	Tyr	**Phe**	Tyr

At around 570 nm, the LWS pigment λ_{max} values obtained from MSP studies on the canary and chicken (Bowmaker and Knowles, 1977; Bowmaker *et al.,* 1997) are relatively long in comparison to values obtained from birds such as the tawny owl (λ_{max} = 555 nm), *Strix aluco,* (Bowmaker and Martin, 1978) and the penguin (λ_{max} = 543 nm), *Spheniscus humboldti,* (Bowmaker and Martin, 1985) which have been found to possess slightly shorter longwave pigments. It is interesting that these three different λ_{max} values resemble those obtained from the three allelic variants of the single polymorphic middle- to longwave pigment in New world primates (i.e. 563, 556 and 543 nm) (Williams *et al.,* 1992; Ibbotson *et al.,* 1992; Bowmaker *et al.,* 1984, 1985; Jacobs, 1984; Travis *et al.,* 1988; Neitz *et al.,* 1991). That avian LWS pigments should also cluster around exactly the same three λ_{max} values that are seen in primates seems to indicate a certain rigidity in the location of λ_{max}s since the avian (or reptilian) line diverged from the mammalian line approximately 350-450 million years ago (Benton, 1993). While adaptive evolution may be a convenient explanation, the underlying reason may be to do with specific structural constraints on opsins which will only allow certain amino acid residues to occupy a particular position. If a mutation changes the residue, perhaps a non-viable pigment is produced which cannot bind retinal as efficiently and has a reduced ability to activate transducin, similar to the situation in some site directed mutants (see Zhukovsky and Oprian, 1989, for example). Due to this, visual pigment λ_{max}s tend to cluster around certain values (Dartnall and Lythgoe, 1965). It would be interesting to see if the same amino acid substitutions that occur in the three forms of the primate middle- to longwave opsin also occur in avian LWS opsins. On the basis of the above evidence, it seems likely that identical spectral tuning sites will exist, although the reason why certain avian species have evolved LWS pigment with slighter shorter λ_{max} is still unclear.

8. CONCLUSIONS

In the present study, the technique of microspectrophotometry has been used to identify the different classes of photoreceptor that are present in the retina of the canary, *Serinus canaria.* The results of the study show that the canary has a duplex retina, containing both rods, and single and double cones. The canary possesses a complement of photoreceptors which is typical of avian species. As in other passerine birds, four spectrally distinct cone pigments are found which include an ultraviolet sensitive pigment.

Typical pigment-oil droplet combinations are found in the canary retina. In the LWS single cones the P568 is combined with an R-type droplet. The P568 is also found in both members of the double cones and is associated with a P-type droplet in the Principal member. In the MWS cones the P506 is combined with a Y-type droplet, in the SWS cones the P442 is associated with a C-type droplet and in the UV cones the P366 is paired with a T-type droplet. With at least four spectrally distinct cone visual pigments the canary has the potential for a tetrachromatic colour vision system which extends into the ultraviolet.

The uses of UV light may vary depending on the ecology of a certain species. It has previously been suggested that UV wavelengths may be used in birds for regulating circadian rhythms, orientatation/navigation, foraging and inter- and intraspecies communication such as sexual displays.

The strong, hard, conical bills of canaries suggest that they are predominantly seed eaters. It is possible that UV sensitivity in the canary could be used in locating and perhaps distinguishing seeds on the basis of UV reflectance. Some seeds have a UV reflecting-waxy coating which, upon removal can affect the rate at which the seeds are taken by birds (Bennet and Cuthill, 1994). Fruits, such as berries, have been found to reflect in the UV (Burkhardt, 1982). The wild canary is a fruit-eater too, and may well use its UV sensitivity in distinguishing different berries on the basis of their UV reflectance. The plumage of the female wild canary is usually duller than that of the male and it is possible that differences in the plumage UV reflectance of male canaries exists. The sexual selection hypothesis suggests that UV vision may also have a role to play in signalling between birds, since much evidence has been obtained for the presence of UV-reflecting plumage in different birds (Burkhardt, 1982; 1989; Burkhardt and Finger, 1991). Reports of ultraviolet light being used in mate choice and/or species recognition have come from behavioural experiments on two other passerine species, the Pekin robin, *Leiothrix lutea,* (Maier, 1994b) and the zebra finch,

Taeniopygia guttata, (Bennett *et al.,* 1996).

The rods in the retina of *Serinus canaria* contain a P506, spectrally similar to the MWS cone pigment. There were relatively few rods in the canary retina which may reflect the highly diurnal lifestyle of the wild canary.

The carotenoid concentration in the retinal oil droplets of the three strains of canary (Clear, variegated and heavily variegated) did not show a corresponding increase with the degree of carotenoid concentration in the feathers. However, the R-type droplets did vary considerably between birds - regardless of strain or length of time in captivity. Some birds had typically highly pigmented 'red' R-type droplets, whereas others appeared to have 'reduced' R-type oil droplets which were spectrally indistinguishable from P-type oil droplets. Since the canaries used in the present study were all inbred it is likely that the apparent absence of typical 'red' oil droplets in some birds was a consequence of domestication. For example, a mutation may have occurred in one of the genes coding for a critical enzyme in the astaxanthin synthesis pathway rendereding the R-type droplets astaxanthin-free. A mutation such as this is more likely to be retained through inbreeding of 'type' canaries. If the mutation theory is correct, identification of the exact step in the astaxanthin synthesis pathway would be an interesting subject for future research.

The complete nucleotide sequence of the ultraviolet sensitive opsin gene from *Serinus canaria* including the 5' and 3' untranslated regions was cloned and sequenced from retinal cDNA. Translation of the canary UVS cDNA shows that it encodes an opsin of 350 amino acid residues. The Kyte-Doolittle hydrophobicity plot of the deduced amino acid sequence of the UVS opsin indicates the presence of seven potential transmembrane domains, separated by hydrophillic regions, typical of the family of G-protein coupled receptors. Comparison of the deduced amino acid sequence of the canary UVS opsin with that of 28 other vertebrate opsins revealed that it shares the highest percentage identity with the budgerigar UVS opsin (87%) followed by the putative chameleon violet- (85%) and chicken violet- (84%) opsins.

Results from the phylogenetic analyses suggest that the ultraviolet sensitive opsins in birds, mammals and teleosts probably evolved as a subgroup of the violet sensitive group of opsins. Furthermore, when VS opsins are arranged according to their respective vertebrate groups, each group appears to have evolved independent ultraviolet-specific spectral tuning mechanisms.

The most important putative spectral tuning sites thought to be responsible for tuning

the canary ultraviolet sensitive opsin from violet wavelengths (400-420) down to ultraviolet wavelengths (<370 nm) are both non-homologous substitutions at chromophore facing sites. These sites at residues 90 (Ser to Cys) and 118 (Thr to Ala) both involve the loss of an OH group in the canary ultraviolet sensitive opsin and may provide a useful starting point for future site-directed mutagenesis studies on spectral tuning of opsins.

Approximately 1 Kb of partial cDNA sequence, encompassing exons 1- 5 of the canary SWS-, MWS- and rod opsins was also cloned and sequenced, together with 620 bp of the LWS opsin. The deduced amino acid translations for each of these partial sequences have been presented and comparisons of percentage nucleotide and amino acid sequence identity with other opsins made. These comparisons show that the sequences for the rod and four cone opsins in *Serinus canaria* belong to the five ancestral vertebrate opsin groups.

The similar λ_{maxes} of the canary MWS cone and rod visual pigments raises the question of whether similar spectral tuning mechanisms are used at the molecular level in these two closely related opsin classes. The very high degree of homology between other avian MWS and rod opsins is apparent, from the alignments presented. The negatively charged Asp-83 and positively charged His-211 are likely to have direct interactions with the chromophore and be involved in spectral tuning in both avian rod and MWS opsins (see Weitz and Nathans, 1992).

Of the few chromophore facing sites at which amino acid residues differ between avian MWS and rod opsins, the Gln (MWS opsins) to Glu (rod opsins) substitution at site 122 constitutes a non-conservative change (polar, *N*- bearing to charged residue) which is predicted to be in very close proximity to the chromophore. A Glu to Gln substitution, when replicated in site-directed mutants of bovine rod opsin (expressed, reconstituted opsin λ_{max} = 500 nm) leads to a 20 nm shift to shorter wavelengths (480 nm) (Zhukovsky and Oprian, 1989; Sakmar and Khorana, 1989). If the Gln-122 residue in MWS pigments also leads to a 480 nm absorbing pigment then it is possible that the polar, chromophore facing Serine substitutions at sites 222 (Cys-222 in the rod opsin), and 299 (Ala-299 in the rod opsin), may affect the shift back up to 500 nm in the avian MWS pigment. Future site directed mutagenesis studies may be able to verify this.

APPENDIX A

Abbreviations for amino acids

Amino acid	Three-letter abbreviation	Single letter symbol	Nature of side chain (polar / charged)
Alanine	Ala	A	nonpolar
Cysteine	Cys	C	nonpolar
Aspartic acid	**Asp**	**D**	**charged: acidic**
Glutamic acid	**Glu**	**E**	**charged: acidic**
Phenylalanine	Phe	F	nonpolar
Glycine	Gly	G	nonpolar
Histidine	**His**	**H**	**charged: basic**
Isoleucine	Ile	I	nonpolar
Lysine	**Lys**	**K**	**charged: basic**
Leucine	Leu	L	nonpolar
Methionine	Met	M	nonpolar
Asparagine	**Asn**	**N**	**uncharged: polar**
Proline	Pro	P	nonpolar
Glutamine	**Gln**	**Q**	**uncharged: polar**
Arginine	**Arg**	**R**	**charged: basic**
Serine	**Ser**	**S**	**uncharged: polar**
Threonine	**Thr**	**T**	**uncharged: polar**
Valine	Val	V	nonpolar
Tryptophan	Trp	W	nonpolar
Tyrosine	**Tyr**	**Y**	**uncharged: polar**

APPENDIX B

Blue-White screening for recombinant colonies

The enzyme, β-galactosidase, is coded by the *lac Z* gene on the *E.coli* chromosome and is involved in the breakdown of lactose to glucose plus galactose. Certain strains of *E.coli* contain a modified *lac Z* gene, known as *lac Z'* which lacks the segment which codes for the α-peptide portion of the β-galactosidase enzyme. These mutant bacteria can only synthesize β-galactosidase when they are carrying a plasmid such as pUC8 which carries the missing portion of the *lac Z* gene.

In cloning experiments, transformants (i.e. competent cells which have successfully taken up the plasmid) are first selected on ampicillin agar, since the plasmid confers ampicillin resistance on transformant cells. The transformants are then screened for β-galactosidase activity to distinguish recombinants from non-recombinants; recombinant colonies will be unable to synthesize β-galactosidase due to the inserted DNA interrupting the *Lac Z* α peptide sequence. The lactose analogue, X-Gal, can be used for this purpose and is broken down by β-galactosidase into a deep blue coloured product. The X-Gal and a β-galactosidase inducer such as IPTG can simply be added to the agar as well as ampicillin. Non-recombinant colonies which are able to synthesize β-galactosidase, are blue and can be distinguished from recombinant colonies which are white (since they cannot synthesize β-galactosidase due to the inserted DNA interrupting the *Lac Z'* gene sequence).

APPENDIX C

Buffers:

Loading buffer for sequencing reactions:

For 39 ml 10X:	12 ml	glycerol
	0.06 g	bromophenol blue
	0.06 g	xylene cyanol FF
	6 ml	50X TAE

made up to 30 ml in ddH2O.

Loading buffer for agarose gels:

For 30 ml 10X:	7.6 g	ficoll (Mr 400kD)
	0.75 g	Orange G, 0.25M

NE buffer

150 mM	sodium chloride
25 mM	EDTA

TAE buffer

For 500 ml of 50X:	121 g	Tris (0.04M)
	28.6 ml	glacial acetic acid
	9.3 g	EDTA pH 8.0 (0.001M)

made up to 500 ml in ddH2O.

TBE buffer

For 1 litre of 10X:	108 g	Tris (0.045M)
	55 g	boric acid
	9.5 g	EDTA (0.001M)

made up to 1 litre in dd H2O.

TE buffer

For 1 litre of 10X:	12.11 g	Tris (0.01M)
	3.7 g	EDTA (0.001M)

made up to 1 litre in ddH2O.

Denaturing Solution: 43.83 g sodium chloride (1.5M)
10 g sodium hydroxide (0.5M)
made up to 500 ml in ddH2O.

Neutralising solution: 43.83 g sodium chloride (1.5M)
30.28 g Tris pH 7.2 (0.5M)
186 g EDTA (0.001M)
made up to 500 ml in ddH2O.

ANTIBIOTICS

Ampicillin

A 50 mg/ml stock solution of ampicillin was made in ddH2O, filter sterilised through a 0.22-micron filter and stored at -20 °C.

Kanamycin

A 10 mg/ml stock solution of kanamycin was made in ddH2O, filter sterilised through a 0.22-micron filter and stored at -20 °C.

Tetracyclin

A 5 mg/ml stock solution of tetracyclin was made in 95 % ethanol and stored at -20 °C.

Laurita Bautaria (LB) medium

For 1 litre of broth:

10 g	tryptone
5 g	yeast extract
10 g	sodium chloride
1 ml	1M sodium hydroxide

made up to 1 litre with ddH2O. Sterilised by autoclaving at 15 psi for 20 minutes.

For LB + ampicillin (or kanamycin)

A final antibiotic concentration of 100 ug/ml was added to cooled (approximately 50 °C), autoclaved LB.

IPTG (100 mM) stock solution.

A 24 mg/ml stock solution of IPTG was made in ddH2O, filter sterilised through a 0.22-micron filter and stored at -20 °C.

5% X-Gal stock solution.

A 50 mg/ml stock solution of X-Gal was made in N,N' dimethyl-formamide in a sterile glass bottle/tube covered with silver foil and stored at -20 °C.

LB Agar plates with antibiotic and IPTG / X-Gal

15 g Agar were added to 1 litre of LB medium and sterilized by autoclaving for 20 mins and allowed to cool to 40-50 °C.
The following were then added:

-IPTG to 0.5 μM (5ml IPTG 100 mM stock solution).
-X-Gal to 80 μg/ml (1.6 ml 5% X-Gal stock solution).
-Ampicillin to 50 μg/ml (1 ml of 50 mg/ml solution) or kanamycin to 50 μg/ml (5 ml of 10 mg/ml solution).
-Tetracycline to 15 50 μg/ml (3 ml of 5 mg/ml solution)

20-25 ml of the medium were used per plate. Once the agar had hardened, the plates were stored for up to 30 days, inverted at 4 °C in the dark.

9. REFERENCES

Amemiya, T. (1975). Electron microscopic and cytochemical study on paraboloid glycogen of the accessory cone of the chick retina. *Histochemistry,* **43** (185-192).

Anderson, D. H., Fisher, S.K. (1975). Disc shedding in rodlike and conelike photoreceptors of tree squirrels. *Science,* **187**, 953-955.

Applebury, M. L. and Hargrave, P.A. (1986). Molecular biology of visual pigments. *Vision Research,* **26**, 1881-1885.

Asenjo, A. B., Rim, J. and Oprian, D.D. (1994). Molecular determinants of human red/green color discrimination. *Neuron,* **12**, 1131-1138.

Baldwin, J. M. (1993). The probable arrangement of the helices in G protein-coupled receptors. *European Molecular Biology Organization Journal,* **12**, 1693-1703.

Bennett, A. T. D. and Cuthill, I. C. (1994). Ultraviolet Vision in Birds: What is its Function. *Vision Research,* **31**, 1471-1478.

Bennett, A. T. D., Cuthill, I.C., Partridge, J.C. and Maier, E.J. (1996). Ultraviolet vision and mate choice in zebra finches. *Nature,* **380**, 433-435.

Benton, M. (1993). Life and time. In Gould, S.J. (Ed) *The book of life.* (pp. 22-36) London: Hutchinson

Bliss, A. F. (1946). The chemistry of daylight vision. *Journal of General Physiology,* **29**, 277-297.

Bloch, S. and Martinoya, C. (1971). Are colour oil droplets the basis of the pigeon's chromatic space? *Vision Research Supplement,* **3**, 411-418.

Blough, D. S. (1957). Spectral sensitivity in the pigeon. *Journal of the Optical Society of America,* **47**, 827-833.

Blough, P. M. (1972). Wavelength generalization and discrimination in the pigeon.

Perception and Psychophysics, **12**, 342-348.

Bok, D. and Hall, M.O. (1971). The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. *Journal of Cell Biology,* **49**, 664.

Bowmaker, J. K. and Knowles, A. (1977). The visual pigments and oil droplets of the chicken retina. *Vision Research,* **17**, 755-764.

Bowmaker, J. K. (1977). The visual pigments, oil droplets and spectral sensitivity of the pigeon, *Columba livia. Vision Research,* **17**, 1129-1138.

Bowmaker, J. K. and Martin, G.R. (1978). Visual pigments and colour vision in a nocturnal bird, *Strix aluco* (Tawny Owl). *Vision Research,* **18**, 1125-1130.

Bowmaker, J. K. (1979). Visual pigments and Oil Droplets in the Pigeon Retina, as Measured by Microspectrophotometry, and Their Relationship to Spectral Sensitivity. In Granda, A. M. and Maxwell J.H. (Eds), *Neural Mechanisms of Behavior in the Pigeon.* New York and London: Plenum Press.

Bowmaker, J.K. and Dartnall, H.J. (1980). Visual pigments of rods and cones in a human retina. *Journal of Physiology,* **298**, 501-511.

Bowmaker, J. K. (1984). Microspectrophotometry of vertebrate photoreceptors: a brief review. *Vision Research,* **24**, 1641-1650.

Bowmaker, J. K. and Martin, G.R. (1985). Visual pigments and oil droplets in the penguin, *Spheniscus humboldti. Journal of Comparative Physiology A,* **156**, 71-77.

Bowmaker, J. K., Jacobs, G.H., Spiegelhalter, D.J. and Mollon, J.D. (1985). Two types of trichromatic squirrel monkey share a pigment in the red-green spectral region. *Vision Research,* **25**, 1937-1946.

Bowmaker, J. K. (1991a). Photoreceptors, photopigments and oil droplets. In Gouras, P.(Ed.), *Vision and Visual Dysfunction, 6, The perception of Colour* (pp. 108-127). London: Macmillan.

Bowmaker, J. K. (1991b). Evolution of visual pigments and photoreceptors. In Gregory, R.L. and Cronley-Dillon, J. R. (Eds), *Evolution of the Eye and Visual System: Part II, Vision and Visual Dysfunction* (pp. 63-81). London: Macmillan.

Bowmaker, J. K., Thorpe, A. and Douglas, R.H. (1991c). Ultraviolet-sensitive cones in the goldfish. *Vision Research,* **31**, 349-352.

Bowmaker, J. K., Kovach, J.K., Whitmore, A.V., and Loew, E.R. (1993). Visual pigments and oil droplets in genetically manipulated and carotenoid deprived quail: a microspectrophotometric study. *Vision Research,* **33**, 571-578.

Bowmaker, J. K. (1995). The visual pigments of fish. In Osborne, N. N. and Chader, G.J. (Ed.), *Progress in Retinal and Eye Research* (pp. 1-31). Oxford: Pergamon.

Bowmaker, J. K., Heath, L.A., Wilkie, S.E., Hunt, D.M. (1997). Visual pigments and oil droplets from six classes of photoreceptor in the retinas of birds. *Vision Research,* **37**, 2183-2194.

Bowmaker, J. K. and Hunt, D.M. (1997). Molecular genetics of spectral sensitivity variation in photoreceptors. In Archer, S.N., Djamgoz, M.B.A., Loew, E.R., Partridge, J.C. and Valerga, S. (Eds) *Adaptive Mechanisms in the Ecology of Vision.* London: Chapman and Hall.

Bownds, D. (1967). Site of attachment of retinal in rhodopsin. *Nature,* **216**, 1178-1181.

Boynton, R. M. (1963). Contributions of threshold measurements to color discrimination theory. *Journal of the Optical Society of America,* **53**, 165-178.

Braekevelt, C. R. (1993a). Fine structure of the retinal photoreceptors of the Great Horned Owl *(Bubo virginianus). Histology and Histopathology,* **8**, 25-34.

Braekevelt, C. R. (1993b). Retinal photoreceptor fine structure in the red-tailed hawk *(Buteo jamaicensis). Anatomia Histologia Embryologia,* **22**, 222-232.

Braekevelt, C. R. (1994). Retinal photoreceptor fine structure in the great blue heron *(Ardea herodias). Anatomia Histologia Embryologia,* **23**, 281-292.

Braekevelt, C. R. (1994). Retinal photoreceptor fine structure in the American crow *(Corvus brachyhynchos). Anatomia Histologia Embryologia,* **23**, 376-387.

Braekevelt, C. R., Smith, S.A. and Smith, B.J. (1996). Fine structure of the retinal photoreceptors of the barred owl *(Strix Varia). Histology and Histopathology,* **11**, 79-88.

Bridges, C. D. B. (1962). Visual pigments of the pigeon *(Columba livia). Vision Research,* **2**, 125-137.

Burkhardt, D. (1982). Birds, berries and UV. *Die Naturwissenschaften,* **69**, 153-157.

Burkhardt, D. M., E. (1989). The spectral sensitivity of a passerine bird is highest in the UV. *Die Naturwissenschaften,* **76**, 82-83.

Burkhardt, D. (1989). UV vision: A bird's eye view of feathers. *Journal of Comparative Physiology A,* **164**, 787-796.

Burkhardt, D. and Finger, E. (1991). Black, white and UV: How birds see birds. *Die Naturwissenschaften,* **78**, 279-280.

Carlson, S. D. (1972). Microspectrophotometry of visual pigments. *Quarterly Review in Biophysics,* **5**, 349-393.

Chan, T., Lee, M. and Sakmar, T.P. (1992). Introduction of hydroxyl-bearing amino acids causes bathochromic spectral shifts in rhodopsin. Amino acid substitutions responsible for red-green color pigment spectral tuning. *Journal of Biological Chemistry,* **267**, 9478-9480.

Chance, B., Perry, R., Åkerman, L. & Thorell, B. (1959). Highly sensitive recording microspectrophotometer. *Review of Scientific Instruments,* **830**, 735.

Chang, B. S. W., Crandall, K.A., Carulli, J.P. and Hartl, D.L. (1995). Opsin phylogeny and evolution: a model for blue shifts in wavelength regulation. *Molecular Phylogenetics and Evolution,* **4**, 33-43.

Chen, D. M. and Goldsmith, T.H. (1986). Four spectral classes of cones in the retinas of birds. *Journal of Comparative Physiology A,* **159**, 473-479.

Chiu, M. I., Zack, D.J., Wang, Y. and Nathans, J. (1994). Murine and Bovine Blue Cone Pigment Genes: Cloning and Characterization of Two New Members of the S Family of Visual Pigments. *Genomics,* **21**, 440-443.

Coemans, M. A. J. M., Vos Hzn, J.J. and Nuboer, J.F.W. (1994). The Relation Between Celestial Colour Gradients and the Position of the Sun, with Regard to the Sun Compass. *Vision Research,* **34**, 1461-1470.

Cohen, A. I. (1963). The fine structure of the visual receptors of the pigeon. *Experimental Eye Research,* **2**, 88-97.

Cohen, A. I. (1972). Rods and cones. In Fuortes, M. G. F. (Ed.), *Handbook of Sensory Physiology* (pp. 63-110). Berlin: Springer-Verlag.

Cook, N. J. and Kaupp, U.B. (1988). Solubilization, purification and reconstitution of the sodium-calcium exchanger from bovine retinal rod outer segments. *Journal of Biological Chemistry,* **263**, 11382-11388.

Cooper, T. G. and Meyer, D.B. (1968). Ontogeny of retinal oil droplets in the chick embryo. *Experimental Eye Research,* **7**, 434-442.

Crescitelli, F. (1958). The natural history of visual pigments. *Annals New York Academy of Sciences,* **74**, 230-255.

Crescitelli, F., Wilson, B.W. and Lilyblade, A.L. (1964). The visual pigments of birds. I. The turkey. *Vision Research,* **4**, 275-280.

Crescitelli, F. (1972). The visual cells and visual pigments of the vertebrate eye. In Dartnall, H. J. A.(Ed.), *Handbook of Sensory Physiology* (pp. 245-363). Berlin: Springer.

Crescitelli, F. (1977). The visual pigments of geckos and other vertebrates: an essay in comparative biology. In Crescitelli, F. (Ed.), *Handbook of Sensory Physiology* (pp. 391-449). Berlin: Springer.

Crescitelli, F., and Karvaly, B. (1991). The gecko visual pigment : the anion hypsochromic effect. *Vision Research,* **31**, 945-950.

Dartnall, H.J. and Lythgoe, J.N. (1965). The spectral tuning of visual pigments. *Vision Research,* **5**, 81-100.

Deeb, S. S., Lindsey, D.T., Hibiya, Y., Sanocki, E., Winderickx, J., Teller, D.Y. and Motulsky, A.G. (1992). Genotype-phenotype relationships in human red/green color-vision defects: molecular and psychophysical studies. *American Journal of Human Genetics,* **51**, 687-700.

Delius, J. and Emmerton, J. (1979). Visual performance of pigeons. In Granda, A. M. and Maxwell, J.H. (Ed.), *Neural Mechanisms of Behavior in the Pigeon.* New York and London: Plenum Press.

Donnor, K. O. (1953). The spectral sensitivity of the pigeon's retinal elements. *Journal of Physiology,* **122**, 524-537.

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

Dratz, E. and Hargrave, P. (1983). The structure of rhodopsin in the rod outer segment disk membrane. *Trends in Biochemical Sciences B,* **8**, 128-133.

Duke-Elder, S. (1958). *"System of Ophthalmology".* London: Henry Kimpton.

Dunn, R. F. (1966). Studies on the retina of the gecko Coleonyx variegatus. II. The rectilinear visual cell mosaic. *Journal of Ultrastructure Research,* **16**, 672-684.

Eisner, T., Aneshansley, D.J. and Eisner, M. (1978). UV viewing with a color television camera. *Bioscience,* **38**, 496-498.

Emmerton, J. and Delius, J.D. (1980). Wavelength discrimination in the 'visible' and ultraviolet spectrum by pigeons. *Journal of Comparative Physiology A,* **141**, 47-52.

Emmerton, J. S. J., Muth, I., Schlecht, P. (1980). Spectral transmission of the ocular media of the pigeon. *Investigative Ophthalmology & Visual Science,* **19**, 1382-1387.

Emmerton, J. and Remy, M. (1983). The pigeon's sensitivity to ultraviolet and 'visible' light. *Experientia,* **39**, 1161-1163.

Emmerton, J. (1983). Pattern discrimination in the near ultraviolet by pigeons. *Perception and Psychophysics,* **34**, 555-559.

Engström, K. (1958). On the cone mosaic in the retina of Parus major. *Acta Zoologica,* **39**, 65-69.

Engström, K. (1968). Cone types and cone arrangements in teleost retinae. *Acta Zoologica,* **42**, 179-243.

Fager, L. Y. and Fager, R.S. (1979). Halide control of colour of the chicken cone pigment iodopsin. *Experimental Eye Research,* **29**, 401-408.

Fager, L. Y. and Fager, R.S. (1981). Chicken blue and chicken violet, short wavelength sensitive visual pigments. *Vision Research,* **21**, 581-586.

Farber, D.B. (1995). From mice to men - the cyclic GMP phosphodiesterase gene in vision and disease. *Investigative Ophthalmology & Visual Science,* **36**, 261.

Findlay, J. B. C. and Pappin, D.J.C. (1986). The opsin family of proteins. *Biochemical Journal,* **238**, 625-642.

Finger, E., Burkhardt, D. and Dyck, J. (1992). Avian plumage colors: Origin of UV reflection in a black parrot. *Die Naturwissenschaften,* **79**, 187-188.

Fox, H. M. and Vevers, G. (1960). *The nature of animal colours.* New York: Macmillan.

Franke, R. R., Konig, B., Sakmar, T.P., Khorana, H.G. and Hofman, K.P. (1990). Rhodopsin mutants that bind but fail to activate transducin. *Science,* **250**, 123-125.

Franke, R. R., Sakmar, T.P., Graham, R.M. and Khorana, H.G. (1992). Structure and function in rhodopsin: studies of the interaction between the rhodopsin cytoplasmic domain and transducin. *Journal of Biological Chemistry,* **267**, 14767-14774.

Fujimoto, K., Yanase, T. and Hanaoka T. (1957). Spectral transmittance of retinal colored oil globules re-examined with microspectrophotometer. *Japanese Journal of Physiology,* **7**, 339-346.

Goldsmith, T. H. (1980). Hummingbirds see near UV light. *Science,* **207**, 786-788.

Goldsmith, T. H., Collins, J.S. and Licht, S. (1984). The cone oil droplets of avian retinas. *Vision Research,* **24**, 1661-1671.

Goldsmith, T. H. (1994). Ultraviolet Receptors and Color Vision: Evolutionary Implications and a Dissonance of Paradigms. *Vision Research,* **34**, 1479-1487.

Govardovskii, V. I. and Zueva, L.V. (1977). Visual pigments of chicken and pigeon. *Vision Research,* **17**, 537-543.

Graf, V. and Norren, D.V. (1974). A blue sensitive mechanism in the pigeon retina: λmax 400 nm. *Vision Research,* **14**, 1203-1209.

Granda, A. M. and Yazulla, S. (1971). The spectral sensitivity of single units in the nucleus rotundus of a pigeon, (Columba livia). *Journal of General Physiology,* **57**, 363-384.

Hamilton, W. F. and Coleman, T.B. (1933). Trichromatic vision in the pigeon as illustrated by the spectral hue discrimination curve. *Journal of Comparative. Psychology,* **15**, 183-191.

Hanaoka, T. and Fujimoto, K. (1957). Absorption spectra of a single cone in carp retina. *Japanese Journal of Physiology,* **7**, 276-285.

Hargrave, P. A. (1982). Rhodopsin chemistry, structure and topology. *Progress in Retinal Research,* **1**, 1-51.

Harosi, F. I. and MacNicol, E.F. (1974). Visual pigments of goldfish cones: spectral properties and dichroism. *Journal of General Physiology,* **63**, 279-304.

Hazlett, L. D., Meyer, D.B. and Susan, S.R. (1974). Visual cell ultrastructure in the Japanese Quail *(Coturnix coturnix japonica). Anatomical Record,* **178**, 371-372.

Heath, L. A., Wilkie, S.E., Bowmaker, J.K. and Hunt, D.M. (1997). The rod and green cone opsins of two avian species, the budgerigar, *(Melopsittacus undulatus)* and the mallard duck, *(Anas platyrhynchos).* Submitted.

Higgins, D. G., Bleasby, A.J. and Fuchs, R. (1992). CLUSTAL V: Improved software for multiple sequence alignment. *Computer Applications in the Biosciences,* **8**, 189-191.

Hisatomi, O., Kayada, S., Aoki, Y., Iwasa, T. and Tokunaga, F. (1994). Phylogenetic relationships among vertebrate visual pigments. *Vision Research,* **34**, 3097-3102.

Hisatomi, O., Satoh, T., Barthel, L.K., Stenkamp, D.L., Raymond, P.A. and Tokunaga, F. (1996). Molecular Cloning and Characterization of the Putative Ultraviolet-sensitive Visual Pigment of Goldfish. *Vision Research,* **36**, 933-939.

Honig, B., Dinur, U., Nakanishi, K., Balogh-Nair, V., Gawinowicz, M.A., Arnaboldi, M. and Motto, M.G. (1979). An external point-charge model for wavelength regulation in visual pigments. *Journal of the American Chemical Society,* **101**, 7084-7086.

Hubbard, R., and Sperling, L (1973). The colors of the visual pigment chromophores, *Experimental Eye Research,* **17**, 581-589.

Hunt, D. M., Cowing, J.A. Patel, R., Appukuttan, B., Bowmaker, J.K. and Mollon, J.D. (1995a). Sequence and evolution of the blue cone pigment gene in Old and New World primates. *Genomics,* **27**, 535-538.

Hunt, D. M., Cowing, J.A. Patel, R., Appukuttan, B., Bowmaker, J.K. and Mollon, J.D. (1995b). Sequence and spectral tuning of blue cone photopigments in Old and New World primates. *Investigative Ophthalmology & Visual Science,* **36**, 889.

Hunt, D. M., Fitzgibbon, J., Slobodyanyuk, S. and Bowmaker, J.K. (1996). Spectral tuning and molecular evolution of rod visual pigments in the species flock of cottoid fish in Lake Baikal. *Vision Research,* **36**, 1217-1224.

Ibbotson, R. E., Hunt, D.M., Bowmaker, J.K. and Mollon, J.D. (1992). Sequence divergence and copy number of the middle-wave and long-wave photopigment genes in Old World monkeys. *Proceedings of the Royal Society of London B*, **247**, 145-154.

Ikeda, H. (1965). The spectral sensitivity of the pigeon (Columba livia). *Vision Research,* **5**, 19-36.

Imai, H., Imamoto, Y., Yoshizawa, T. and Shichida, Y. (1995). Difference in molecular properties between chicken green and rhodopsin as related to the functional difference between cone and rod photoreceptor cells. *Biochemistry,* **34**, 10525-10531.

Jacobs, G. H. (1981). *Comparative Color Vision.* New York: Academic press.

Jacobs, G. H. (1984). Within-species variations in visual capacity among squirrel monkeys (Saimiri sciureus) : Color vision. *Vision Research,* **24**, 1267-1277.

Jacobs, G. H., Crognale, M. and Fenwick, J. (1987). Cone pigment of the Great Horned Owl. *The Condor,* **89**, 434-436.

Jacobs, G. H., Neitz, J. and Deegan, J.F. (1991). Retinal receptors in rodents maximally sensitive to ultraviolet light. *Nature,* **353**, 655-656.

Jacobs, G. H. (1992). UV vision in vertebrates. *American Zoologist,* **32**, 544-554.

Jacobs, G. H. (1993). The distribution and nature of colour vision among the mammals. *Biological Reviews,* **68**, 413-471.

Jane, S. D., and Bowmaker, J.K. (1988). Tetrachromatic colour vision in the duck (Anas platyrhynchos) : microspectrophotometry of visual pigments and oil droplets. *Journal of Comparative Physiology A,* **165**, 225-235.

Jitsumori, M. (1976). Anomaloscope experiment for a study of color mixture in the pigeon. *Japanese Psychological Research,* **18**, 126-135.

Johnson, R. L., Grant, K.B., Zankel, T.C., Boehm, M.F., Merbs, S.L., Nathans, J. and Nakanishi, K. (1993). Cloning and expression of goldfish opsin sequences. *Biochemistry,* **32**, 208-214.

Johnston, D. and Hudson, R.A. (1976). Isolation and composition of the carotenoid-containing oil droplets from cone photoreceptors. *Biochim. Biophys. Acta,* **424**, 235-245.

Karnik, S. S., and Khorana, H.G. (1990). Assembly of functional rhodopsin requires a disulfide bond between cysteine residues 110 and 187. *Journal of Biological Chemistry,* **265**, 17520-17524.

Kawamura, S. and Yokoyama, S. (1993). Molecular characterization of the red visual pigment gene of the American chameleon *(Anolis carolinensis). Febs Letters,* **323**, 247-251.

Kawamura, S. and Yokoyama, S. (1996). Phylogenetic Relationships among Short Wavelength-sensitive Opsins of American Chameleon (Anolis carolinensis) and Other Vertebrates. *Vision Research,* **36**, 2797-2804.

Kevan, P. G. (1978). Floral colouration, its colourimetric analysis and significance in anthecology. In A. F. Richards (Ed.), *The pollination of flowers by insects. Linnean society symposium series 6* (pp. 51-78). London: Linnean Society.

King-Smith, P. E. (1969). Absorption spectra and function of the coloured oil drops in the pigeon retina. *Vision Research,* **9**, 1391-1401.

King-Smith, P. E. (1971). Special senses. In Bell, D. J. and Freeman, B.M. (Ed.), *Physiology and biochemistry of the domestic fowl.* New York: Academic Press.

Kirschfeld, K. (1982). Carotenoid pigments: their possible role in protecting against photooxidation in eyes and photoreceptor cells. *Proceedings of the Royal Society of London B,* **216**, 71-85.

Kito, Y., Partridge, J.C., Seidou, M., Narita, K., Hamanaka, T., Michinomae, M., Sekiya, N. and Yoshihara, K. (1992). The absorbance spectrum and photosensitivity of a new synthetic 'visual pigment' based on 4-hydroxyretinal. *Vision Research* **32**: 3-10.

Kleinschmidt, J. and Harósi, F.I. (1992). Anion sensitivity and spectral tuning of cone visual pigments *in situ. Proceedings of the National Academy of Sciences,*

89, 9181-9185.

Knowles, A. (1976). The effects of chloride ions upon chicken visual pigments. *Biochemical and Biophysical Research Communications,* **73**, 56-62.

Knowles, A. and Dartnall, H.J.A. (1977). The photobiology of vision. In H. Davson (Ed.), *The eye, 2B,* London, Academic Press.

Knowles, A. (1980). The chloride effect in chicken red cone receptors. *Vision Research,* **20**, 475-483.

Knox, B.E. (1995). *Xenopus laevis* violet cone opsin mRNA, complete coding sequence. Genbank, Accession U23463.

Kojima, D., Okano, T., Fukada, Y., Shichida, Y., Yoshizawa, T. and Ebrey, T.G. (1992). Cone visual pigments are present in gecko rod cells. *Proceedings of the National Academy of Sciences,* **89**, 6841-6845.

König, B., Arendt, A., McDowell, J.H., Kahlert, M., Hargrave, P.A. and Hofmann, K.P. (1989). 3 cytoplasmic loops of rhodopsin interact with transducin. *Proceedings of the National Academy of Sciences,* **86**, 6878-6882.

Kozak, M. (1981). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Research,* **9**, 5233-5252.

Kreithen, M. L. and Eisner, T. (1978). Ultraviolet light detection by the homing pigeon. *Nature,* **272**, 347-348.

Kropf, A. and Hubbard, R. (1958). The Mechanism of Bleaching Rhodopsin. *Annals New York Academy of Sciences,* **74**, 266-280.

Kühne, W. and Ayres, W.C. (1878). On the stable colours of the retina. Journal of Physiology, **1**, 109-130.

Kumar, S., Tamura, K. and Nei, M. (1993). *MEGA: Molecular evolutionary genetics analysis, version 1.01.* Pennsylvania: Pennsylvania State University.

Kuwata, O., Imamoto, Y., Okano, T., Kokame, K., Kojima, D., Matsumoto, H., Morodome, A., Fukada, Y., Schichida, Y., Yasuda, K., Shimura, Y. and Yoshizawa, T. (1990). The primary structure of iodopsin, a chicken red-sensitive cone pigment. *Febs Letters,* **272**, 128-132.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology,* **157**, 105-132.

La Vail, M. M. (1976). Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. *Science,* **194**, 1071.

Lamb, T. D. and Pugh, E.N. (1990). Physiology of transduction and adaptation in rod and cone photoreceptors. *Trends in Neurosciences,* **2**, 3-13.

Land, M. F. (1991). Polarizing the world of fish. *Nature,* **353,** 118-119.

Liebman, P. A. and Entine, G. (1964). Sensitive low-light level microspectrophotometer detection of photosensitive pigments of retinal cones. *Journal of the Optical Society of America,* **54**, 1451-1459.

Liebman, P. A. and Granda, A.M. (1971). Microspectrophotometric measurements of visual pigments in two species of turtle, *Pseudemys scripta* and *Chelonia mydas. Vision Research,* **11**, 105-114.

Liebman, P. A. (1972). Microspectrophotometry of photoreceptors. In H. J. A. Dartnall (Ed.), *Handbook of Sensory Physiology.* Berlin: Springer.

Liebman, P. A. and Granda, A.M. (1975). Superdense carotenoid spectra resolved in single cone oil droplets. *Nature,* **253**, 170-172.

Lin, S. W., Sakmar, T.P. Franke, R.R., Khorana, H.G. and Mathies, R.A. (1992). Resonance Raman microprobe spectroscopy of rhodopsin mutants: Effect of substitutions in the third transmembrane helix. *Biochemistry,* **31**, 5105-5111.

Lipetz, L. E. and MacNicol, E.F. (1982). Photoreceptors of freshwater turtles: cell types and visual pigments. *Biological Bulletin,* **163**, 396.

Lipetz, L. E. (1984a). A new method for determining peak absorbance of dense pigment samples and its application to the cone oil droplets of Emydoidea blandingii. *Vision Research,* **24**, 597-604.

Lipetz, L. E. (1984b). Pigment types, Densities and Concentrations in Cone Oil Droplets of *Emydoidea Blandingii. Vision Research,* **24**, 605-612.

Loew, E. R. (1994). A third, ultraviolet-sensitive, visual pigment in the Tokay gecko (Gekko gekko). *Vision Research,* **34**, 1427-1431.

Lythgoe, J. N. (1979). *The Ecology of Vision.* Oxford: Clarendon Press.

Lythgoe, J. N. and Partridge, J.C. (1989). Visual Pigments and the Acquisition of Visual Information. *Journal of Experimental Biology,* **146**, 1-20.

Lythgoe, J.N. and Partridge, J.C. (1991). The modelling of optimal visual pigments of dichromatic teleosts in green coastal waters. *Vision Research* **31**: 361-371.

MacNichol, E.F., Jones, G.I., Cornwall, M.C., Mansfield, R.J.W. (1987) On the relation between the spectral absorbance functions of visual pigments. *Biophysical journal,* (meeting abstract). **51**, 267.

Maier, E. J. (1992). Spectral sensitivities including the ultraviolet of the passeriform bird Leiothrix lutea. *Journal of Comparative Physiology A,* **170**, 709-714.

Maier, E. J. and Bowmaker, J.K. (1993). Colour vision in a passeriform bird, Leiothrix lutea: correlation of visual pigment absorbance and oil droplet transmission with spectral sensitivity. *Journal of Comparative Physiology A,* **172**, 295-301.

Maier, E. J. (1994). Ultraviolet Vision in a Passeriform Bird: from Receptor Spectral Sensitivity to Overall Spectral Sensitivity in Leiothrix lutea. *Vision Research,* **34** (11), 1415-1418.

Maier, E. J. (1994b). To deal with the "Invisible" - on the biological significance of UV sensitivity in birds. *Die Naturwissenschaften,* **80**, 476-478.

Mariani, A. P. and Leure-DuPree, A.E. (1978). Photoreceptors and oil droplet colors in the red area of the pigeon retina. *Journal of Comparative Neurology,* **182**, 821-837.

Martin, G. R. (1985). *Eye.* In *Form and Function in Birds.* (pp. 311 - 373). London: Academic Press Inc. Ltd.

Merbs, S. L. and Nathans, J. (1992). Absorption spectra of the hybrid pigments responsible for anomalous color vision. *Science,* **258**, 464-466.

Merbs, S. L. and Nathans, J. (1993). Role of hydroxyl-bearing amino acids in differentially tuning the absorption spectra of the human red and green cone pigments. *Photochemistry and Photobiology,* **58**, 706-710.

Meyer, D. B., Cooper, T.G. and Gernez, C. (1965). Retinal Oil Droplets. In J. W. Rohen (Ed.), *The structure of the Eye.* (pp. 521-533). Stuttgart.: Schattauer-Verlag.

Meyer, D. B. and Cooper, T.G. (1966). The visual cells of the chicken as revealed by phase contrast microscopy. *American Journal of Anatomy,* **118**, 723-734.

Meyer, D. B., Stuckey, S.R., Hudson, R.A. (1971a). Oil droplet carotenoids of avian cones I. Dietary exclusion: models for biochemical and physiological studies. *Comparative Biochemistry and Physiology.* **40B**, 61-70.

Meyer, D. B. (1971b). The effect of dietary carotenoid deprivation on avian retinal oil droplets. *Ophthalmic Research,* **2**, 104-109.

Meyer, D. B. and May, H.C. (1973). The topographical distribution of rods and cones in the adult chicken retina. *Experimental Eye Research,* **17**, 347-355.

Meyer, D. B. (1977). The avian eye and its adaptations. In *Handbook of Sensory Physiology,* Berlin and New York: Springer-Verlag.

Miller, W. H. (1979). Ocular optical filtering. In Autrum, H. (Ed.), *Handbook of Sensory Physiology* Berlin and New York: Springer-Verlag.

Mollon, J. D. (1993). Mixing genes and mixing colours. *Current Biology,* **3** (2), 82-85.

Morris, V. B. and Shorey, C.D. (1967). An electronmicroscope study of types of receptor in the chick retina. *Journal of Comparative Neurology,* **129**, 313-340.

Morris, V. B. (1970). Symmetry in a receptor mosaic demonstrated in the chick from frequencies, spacing and arrangement of the types of retinal receptor. *Journal of Comparative Neurology,* **140**, 359-398.

Muntz, W. R. A. (1972). Inert absorbing and reflecting pigments. In H. J. A. Dartnall (Ed.), *Handbook of Sensory Physiology: Photochemistry of Vision.* (pp. 530-565). Berlin: Springer-Verlag.

Nakayama, T. A. and Khorana, H.G. (1991). Mapping of the amino acids in membrane-embedded helices that interact with the retinal chromophore in bovine rhodopsin. *Journal of Biological Chemistry,* **266**, 4269-4275.

Nathans, J., Thomas, D. and Hogness, D.S. (1986). Molecular genetics of human color vision: the genes encoding blue, green and red pigments. *Science,* **232**, 193-203.

Nathans, J. (1990a). Determinations of visual pigment absorbance: identification of the retinylidene Schiff's base counterion in bovine rhodopsin. *Biochemistry,* **29**, 9746-9752.

Nathans, J. (1990b). Determinations of visual pigment absorbance: role of charged amino acids in the putative transmembrane segments. *Biochemistry,* **29**, 937-942.

Neitz, M., Neitz, J. and Jacobs, G.H. (1991). Spectral tuning of pigments underlying red-green color vision. *Science,* **252**, 971-974.

Norren, D. V. (1975). Two short wavelength sensitive cone systems in pigeon, chicken and daw. *Vision Research,* **15**, 1164-1166.

O'Day, W. T. and Young, R. W. (1978). Rhythmic daily shedding of outer segment membranes by visual cells in the goldfish. *Journal of Cell Biology* , **76**, 593.

O'Tousa, J. E., Baehr, W., Martin, R.L., Hirsh, J., Pak, W.L. and Applebury, M.L. (1985). The Drosophila ninaE gene encodes an opsin. *Cell,* **40**, 839-850.

Ohtsuka, T. (1984). Fluorescence from colourless oil droplets: a new criterion for identification of cone photoreceptors. *Neuroscience Letters,* **52**, 241-245.

Oishi, T., Kawata, A., Hayashi, T., Kukada, Y., Shichida, Y. and Yoshizawa, T. (1990). Immunohistochemical localization of iodopsin in the retina of the chicken and Japanese quail. *Cell and Tissue Research,* **261**, 397-401.

Okano, T., Fukada, T., Artamonov, I.D. and Yoshizawa, T. (1989). Purification of cone visual pigments from chicken retina. *Biochemistry,* **28**, 8848-8856.

Okano, T., Kojima, D., Fukada, Y., Shichida, Y. and Yoshizawa, T. (1992). Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. *Proceedings of the National Academy of Sciences,* **89**, 5932-5936.

Ovchinnikov, Y. A., Abdulaev, N.G. and Bogachuk, A.S. (1988). Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitylated. *Febs Letters,* **230**, 1-5.

Palacios, A. G., and Varela, F.J. (1992). Color mixing in the pigeon (Columba livia) II. A psychophysical determination in the middle, short and near-UV wavelength range. *Vision Research,* **32**, 1947-1953.

Partridge, J. C. (1989). The visual ecology of avian cone oil droplets. *Journal of Comparative Physiology A,* **165**, 415-426.

Pedler, C. and Boyle, M. (1969). Multiple oil droplets in the photoreceptors of the pigeon. *Vision Research,* **9**, 525-528.

Pohl, H. (1992). UV radiation: A zeitgeber for the circadian clock in birds. *Die Naturwissenschaften,* **79**, 227-229.

Remy, M., and Emmerton, J. (1989). Behavioral spectral sensitivities of different retinal areas in pigeons. *Behavioral Neuroscience,* **103**, 170-177.

Roaf, H. E. (1929). The absorption of light by the coloured globules in the retina of the domestic hen. *Proceedings of the Royal Society of London B,* **105**, 371-374.

Rodieck, R. W. (1973). *The vertebrate retina: principles of structure and function.* San Francisco: Freeman.

Röhlich, P and Szél, Å. (1993). Binding sites of photoreceptor-specific antibodies COS-1, OS-2 and AO. *Current Eye Research,* **12**, 935-944.

Romeskie, M. and Yager, D. (1976). Psychophysical studies of pigeon color vision I. Photopic spectral sensitivity. *Vision Research,* **16**, 501-505.

Saibil, H. (1990). Cell and molecular biology of photoreceptors. *Seminars in the Neurosciences,* **2**, 15-23.

Saitou, N. and Nei, M. (1987). The neighbour-joining method: A new method for reconstructing trees. *Molecular biology and Evolution,* **4**, 406-425.

Sakmar, T. P., Franke, R.R. and Khorana, G.H. (1989). Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. *Proceedings of the National Academy of Sciences,* **86**, 8309-8313.

Sakmar, T. P., Frank, R.R. and Khorana, H.G. (1991). The Role of the Retinylidene Schiff Base Counterion in Rhodopsin in Determining Wavelength Absorbance and Schiff Base pKa. *Proceedings of the National Academy of Sciences, USA,* **88**, 3079-3083.

Schertler, G. X., Villa, C. and Henderson, R. (1993). Projection structure or rhodopsin. *Nature,* **362**, 770-772.

Schneider, B. (1972). Multidimensional scaling of color differences in the pigeon. *Perception and Psychophysics,* **12**, 373-378.

Serle, W., Morel, G.J., Hartwig, W. (1972). *A Field Guide to the Birds of West Africa.* Collins.

Shichida, Y., Kato, T., Sasayama, S., Fukada, Y., and Yoshizawa, T. (1990). Effects of chloride on chicken iodopsin and the chromophore transfer reactions from iodopsin to scotopsin and B-photopsin. *Biochemistry,* **29**, 5843-5848.

Silberglied, R. E. (1979). Communication in the UV. *Annual Review of Ecology and Systematics,* **10**, 373-398.

Sillman, A. J. (1969). The visual pigments of several species of birds. *Vision Research,* **9**, 1063-1077.

Sillman, A. J. (1973). Avian Vision. In Farner, D. S., King, J.R. and Parkes, K.C. (Eds), *Avian Biology.* New York and London: Academic Press.

Sillman, A. J., Bolnick, D.A., Hayes, L.W., Walter, A.E., Loew, E.R. (1981). Microspectrophotometry of the photoreceptors of palaeognathus birds - the Emu and Tinamou. *Journal of Comparative Physiology A,* **144**, 271-276.

Spekreijse, H., Wagner, H.G. and Wolbarsht, M.L. (1972). Spectral and spatial coding of ganglion cell responses in goldfish retina. *Journal of Neurophysiology,* **35**, 73-86.

Strother, G. K. and Wolken, J.J. (1960). Microspectrophotometry. I. Absorption spectra of colored oil globules in the chicken retina. *Experimental Cell Research,* **21**, 504-512.

Szél, Å., Takacs, L., Monostori E., Diamantstein, T., Vigh-Teichmann I., Röhlich, P. (1986). Monoclonal antibody recognizing cone visual pigment. *Experimental Eye Research,* **43**, 871-883.

Thompson, P. and Findlay, J.B.C. (1984). Phosphorylation of ovine rhodopsin: Identification of the phosphorylated sites. *Biochemical Journal,* **22**, 773-780.

Travis, D. S., Bowmaker, J.K. and Mollon, J.D. (1988). Polymorphism of visual pigments in a callitrichid monkey. *Vision Research,* **28**, 481-490.

Varela, F. J., Palacios, A.G. and Goldsmith, T.H. (1993). The Avian Eye View. In Zeigler, P.H. and Bischof, H (Eds), *Vision, Brain and Behavior in Birds :*(pp77-98).

Viitala, J., Korpimaki, E., Palokangas, P. and Koivula, M. (1995). Attraction of kestrels to vole scent marks visible in ultraviolet light. *Nature,* **373**, 425-427.

Vos Hzn, J. J., Coemans, M.A.J.M. and Nuboer, J.F.W. (1994). The photopic sensitivity of the yellow field of the pigeon's retina to ultraviolet light. *Vision Research,* **34**, 1419-1425.

Wald, G. (1937). Photo-labile pigments of the chicken retina. *Nature,* **140**, 545-546.

Wald, G. and Zussman, H. (1938). Carotenoids of the chicken retina. *Journal of Biological Chemistry,* **122**, 449-460.

Wald, G. (1945). Human vision and the spectrum. *Science , New York,* **101**, 653-658.

Wald, G. (1948). Galloxanthin, a carotenoid from the chicken retina. *Journal of General Physiology,* **31**, 377-383.

Wald, G., Brown, P.K. and Smith, P.H. (1955). Iodopsin. *Journal of General Physiology,* **38**, 623-681.

Walker, G. B. R. A., D. (1993). *Coloured, Type & Song Canaries - A Complete Guide to Keeping, Breeding and Showing* (2nd ed.). London: Blandford Press.

Wallman, J. (1979). Role of the Retinal Oil Droplets in the Color Vision of Japanese Quail. In Granda, A. M. and Maxwell, J.H. (Eds), *Neural Mechanisms of Behavior in the Pigeon* (pp. 327-351). New York and London: Plenum Press.

Walls, G. L. and Judd, H. D. (1933). The intra-ocular colour-filters of vertebrates. *British Journal of Ophthalmology,* **17**, 641-675; 705-725.

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

Walls, G. L. (1963). *The Vertebrate Eye And Its Adaptive Radiation.* New York: Hafner.

Wang, J. K., McDowell, J.H. and Hargrave, P.A. (1980). Site of attachment of 11-cis retinal in bovine rhodopsin. *Biochemistry,* **19**, 5111-5117.

Wang, S. Z., Adler, R. and Nathans, J. (1992). A visual pigment from chicken that resembles rhodopsin: amino acid sequence, gene structure, and functional expression. *Biochemistry,* **31**, 3309-3315.

Wang, Z., Asenjo, A.B. and Oprian, D.D. (1993). Identification of the Cl^- -binding site in the human red and green color vision pigments. *Biochemistry,* **32**, 2125-2130.

Weitz, C. J., Miyake, Y., Shinzato, K., Montag, E., Zrenner, E., Went, L.N. and Nathans, J. (1992a). Human tritanopia associated with two amino acid substitutions in the blue-sensitive opsin. *American Journal of Human Genetics,* **50**, 498-507.

Weitz, C. J., Went, L.N. and Nathans, J. (1992b). Human tritanopia associated with a third amino acid substitution in the blue-sensitive visual pigment. *American Journal of Human Genetics,* **51**, 444-446.

Weitz, C. J. and Nathans, J. (1992c). Histidine residues regulate the transition of photoexcited rhodopsin to its active conformation, metarhodopsin II., *Neuron,* **8**, 465-472.

Wessels, R. H. A. (1974). *Tetrachromatic Vision In The Daw.* Doctoral Thesis, Utrecht.

Wilkie, S. E., Vissers, P.M.A.M., Das, D., DeGrip, W.J. Bowmaker, J.K. and Hunt, D.M. (1997). UV vision in birds: spectral characteristics, cDNA sequence and retinal localization of the UV-sensitive visual pigment of the budgerigar. (Submitted).

Williams, A. J., Hunt, D.M., Bowmaker, J.K. and Mollon, J.D. (1992). The polymorphic photopigments of the marmoset: spectral tuning and genetic basis. *European Molecular Biology Organization Journal,* **11**, 2039-2045.

Wiltschko, R., Munro, U., Ford, H. and Wiltschko, R. (1993). Red light disrupts magnetic orientation of migratory birds. *Nature,* **364**, 525-527.

Winderickx, J., Lindsey, D.T., Sanocki, E., Teller, D.Y., Motulsky, A.G. and Deeb, S.S. (1992). Polymorphism in red photopigment underlies variation in colour matching. *Nature,* **356**, 431-433.

Wolbarsht, M. L. (1976). The function of intraocular colour filters. *Federation Proceedings,* **35**, 44-50.

Wood, C. A. (1917). *The Fundus Oculi Of Birds Especially As Viewed By The Ophthalmoscope.* Chicago: Lakeside Press.

Wortel, J. F. W., R.J., and Nuboer, J.F. (1984). Photopic spectral sensitivity of the red and the yellow field of the pigeon retina. *Vision Research,* **24**, 1107-1113.

Wright, A. A., and Cumming, W.W. (1971). Color-naming functions for the pigeon. *Journal of the Experimental Analysis of Behavior,* **15**, 7-17.

Wright, A. A. (1972). Psychometric and psychophysical hue discrimination functions for the pigeon. *Vision Research,* **12**, 1447-1464.

Wright, A. A. (1979). Color-vision psychophysics: a comparison of pigeon and human. In Granda, A. M. and Maxwell, J.H. (Ed.), *Neural mechanisms of Behavior in the Pigeon.* (pp. 89-127). New York: Plenum Press.

Yau, K. (1994). Phototransduction mechanisms in retinal rods and cones. *Investigative Ophthalmology & Visual Science,* **35**, 9-32.

Yazulla, S., and Granda, A.M. (1973). Opponent-color units in the thalamus of the pigeon. *Vision Research,* **13**, 1555-1563.

Yen, L. and Fager, R.S. (1984). Chromatographic resolution of the rod pigment from the four cone pigments of the chicken retina. *Vision Research,* **24**, 1555-1562.

Yokoyama, R. and Yokoyama, S. (1990). Convergent evolution of the red- and green-like visual pigment genes in fish, *Astyanax fasciatus,* and human. *Proceedings of the National Academy of Sciences,* **87**, 9315-9318.

Yokoyama, S. (1994). Gene duplications and evolution of the short wavelength-sensitive visual pigments in vertebrates. *Molecular Biology and Evolution,* **11**, 32-39.

Yokoyama, S. (1995). Amino acid replacements and wavelength absorption of visual pigments in vertebrates. *Molecular Biology and Evolution,* **12**, 53-61.

Young, R. W. (1974). Biogenesis and renewal of visual cell outer segment membranes. *Experimental Eye Research,* **18**, 215-223.

Young, R. W. (1977). The daily rhythm of shedding and degradation of cone outer segment membranes in the lizard retina. *Journal of Ultrastructure,* **61**, 172.

Young, R. W. (1978). The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina. *Investigative Ophthalmology & Visual Science,* **17**, 105.

Young , S. R. and Martin, G.R. (1984). Optics of retinal oil droplets: A model of light collection and polarization detection in the avian retina. *Vision Research,* **24**, 129-137.

Zhukovsky, E. A. and Oprian, D.D. (1989). The effect of carboxylic acid side chains on the absorption maximum of visual pigments. *Science,* **246**, 928-930.