RETINAL PHOTORECEPTOR COMPLEMENT OF PALEOGNATHOUS BIRDS

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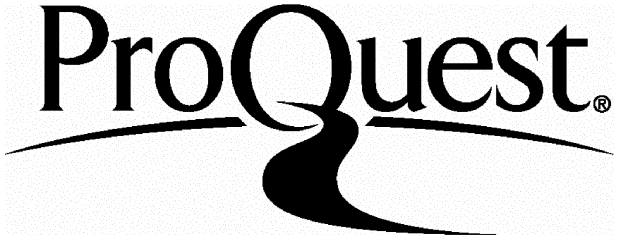


ABSTRACT

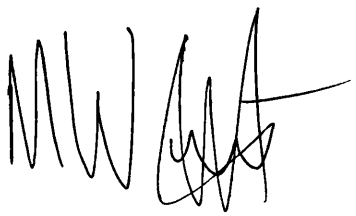
Studies of the cone visual pigment complement from a wide range of representatives of the vertebrate classes have provided data on the origins and evolution of vertebrate colour vision. Tetrachromatic visual systems have been identified in many diurnal birds, as well as in some reptiles and fish. However, little data are available for the paleognathous birds, a group that represents the earliest offshoot of the main avian stem and may provide an important link between the visual system of reptiles and those of the neognathous birds.

Microspectrophotometry (MSP) was used to determine the absorbance spectra of both rod and cone visual pigments and oil droplets from the retinae of the ostrich (*Struthio camelus*) and rhea (*Rhea americana*). Light and fluorescence microscopy of whole fresh tissue mounts was used to determine the relative numbers of oil droplets in the retinae. Both species possess rods, double cones and four classes of single cone identified by their oil droplets. The rods have λ_{max} at about 505-nm, whereas three cone pigments were recorded with λ_{max} at about 570, 505 and 445 nm. The 570-nm pigment is located in both members of the double cones and in a class of single cone containing a red (R-type) oil droplet with λ_{cut} at about 560-nm. The 510-nm and 445-nm cone pigments were found in populations of single cones containing yellow (Y-type) and clear (C-type) oil droplets with λ_{cut} at about 500 and 420-nm respectively. Double cones possess a pale (P-type) droplet in the principal member and a small droplet containing low concentrations of carotenoid (A-type) in the accessory member. The fourth class of single cone contains a transparent (T-type) droplet and in the ostrich a 405-nm pigment has been characterised. The complement of visual pigments and oil droplets, and the relative ratio of cone types in the ostrich and rhea are remarkably similar to that found in neognathous birds.

Nucleotide sequences from opsin genes of both the ostrich and rhea have been identified. Comparison of these sequences with those from other avian species indicates that paleognathous opsins are highly homologous to the opsins found for neognathous avian species.

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.



Mathew William Wright, B.Sc.

This thesis is dedicated to my brother,

Paul James Wright,

who has always been there when I needed support.

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ABBREVIATIONS

A-type	accessory droplet
bp	base pair
°C	degrees Celsius
C-type	clear oil droplet
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
dATP	deoxy-adenosine-5'triphosphate
dCTP	deoxy-cytosine-5'triphosphate
DEPC	diethylpyrocarbonate
dITP	deoxy-inosine-5'triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide-5'triphosphate
dUTP	deoxy-uracil-5'triphosphate
EDTA	ethylene-diamine-tetra acetic acid
ELM	external limiting membrane
e.r.g.	electroretinography
g	gram (s)
GDP	guanosine diphosphate
GTP	guanosine triphosphate
IHC	immunohistochemistry
IPTG	isopryl-B-D-thiogalactoside
Kb	kilobase(s)
L	Long wavelength-absorbing cone opsin class
LWS	Long wavelength sensitive
µg	microgram
µl	microlitre
M	molar
M_C	Middle wavelength-absorbing cone opsin class
ml	millilitre
M_{Rd}	Middle wavelength-absorbing rod opsin class
MSP	Microspectrophotometry
MWS	Middle wavelength sensitive

ng	nanogram
nm	nanometre
OSS	outer segment sensitivity
PDE	phosphodiesterase
PE	pigment epithelium
P-type	principal oil droplet
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
R-type	red oil droplet
RNA	ribonucleic acid
rpm	revolutions per minute
S_B	Short wavelength-absorbing cone opsin class
SDW	sterile distilled water
S_V	Ultraviolet/violet wavelength-absorbing cone opsin class
Taq	*Thermus aquaticus*
Tm	annealing temperature
T-type	transparent oil droplet
TAE	Tris / acetate / EDTA buffer
TBE	Tris / borate / EDTA buffer
TM	transmembrane
UV	ultraviolet
UVS	ultraviolet sensitive
VS	violet sensitive
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
Y-type	yellow oil droplet

GENERAL INTRODUCTION

1.0 PALEOGNATHOUS BIRDS

Paleognathous birds have traditionally been defined by their distinctive morphological features, chief among these is the paleognathous palate, first pointed out by Huxley (1867), from which this family of birds gained its name. The structural organisation of the paleognathous palate resembles a condition well-developed in reptiles, one that enables the upper jaw to enlarge gape of mouth opening (Olson, 1985). Characters similar to the paleognathous palate are only found in neognathous birds during early developmental stages (Jollie, 1958).

There are nine main groups of paleognathous birds, but only six of them have living representatives. These are the cassowaries (*Casuariinae*) of New Guinea, emus (*Dromiceinae*) of Australia, kiwis (*Apterygidae*) of New Zealand, ostriches (*Struthionidae*) of Africa, rheas (*Rheidae*) of South America and tinamous (*Tinamidae*) of the Neotropics, ranging from north-eastern Mexico to Tierra del Fuego (Blake, 1979). The extinct groups include moas (*Dinornithidae*) of New Zealand, elephant birds (*Aepyornithidae*) of Africa and Madagascar and mihirung birds (*Dromornithidae*) of Australia (Rich, 1979). Except for the tinamou, these birds are entirely flightless and are known collectively as ratites. The *Tinamidae* are only capable of semi-sustained flight. How these groups are related, their origins and whether or not paleognathous birds represent a monophyletic group remain debatable questions. Numerous different approaches have been utilised to study these questions and on at least some of these issues a consensus now exists.

Charles Darwin in his "On the origin of species by means of natural selection". (published in 1859) believed that paleognathous birds were all descended from ancestors which originally flew, but through lack of use of their wings and an increased use of their hind limbs had evolved into flightless birds. This early theory by Darwin probably has some elements of truth, though it is more likely that the ancestral 'flier' of paleognathous birds could not perform sustained flight and was in fact a semi-sustained flier like the tinamou (Prager *et al.*, 1976; McGowan, 1982, 1986).

Although some controversy still exists for outlying members of the paleognathous group (Cooper, 1993), there is now a consensus belief that paleognathous birds are likely to have a monophylous origin, evolving from a common ancestor (e.g. Sibley & Ahlquist, 1972; Cracraft, 1974; Bledsoe, 1988; Sibley & Ahlquist, 1990). It has been suggested that this common ancestor lived at a time when only one large landmass (Gondwanaland) existed, and that this ancestor walked across land bridges which connected the modern day continents. The divergence from the ancestral form occurred during or after this time, since these land bridges ceased to exist between fifty and ninety million years ago (Prager *et al.*, 1976; Hedges *et al.*, 1996; Van Tuinen *et al.*, 1998).

Although various approximate dates of paleognathous divergence have been fielded over the years, there was always a consensus that paleognathous birds were the first offshoot of the main avian stem. For instance, morphological comparison of paleognathous skeletal structure (Cracraft, 1974), analysis of the avian karyotypes (Boer, 1980), comparison of the amino acids of the eye lens protein α-crystallin A (Stapel *et al.*, 1984; Caspers *et al.*, 1999), ultra-structural comparison of avian spermatozoa (Soley, 1993; Ausió *et al.*, 1999), and DNA-DNA hybridisation (Sibley & Ahlquist 1981, 1983, 1990; Sibley *et al.*, 1988) all support the paleognathous birds as being evolved from the oldest surviving offshoot of the Class *Aves* with the Galliformes and Anseriformes as their closest relatives. However, some evidence now exists that the paleognathous birds are not the first offshoot of the main avian stem. This difference of opinion is restricted to research utilizing comparison of mitochondrial DNA sequences, which indicate that the Passeriformes branched off from the main avian stem prior to the paleognathous offshoot (Härlid *et al.*, 1997; Härlid *et al.*, 1998; Härlid & Arnason, 1999). These findings are questioned in recent research which suggests that the evolution of mitochondrial sequences is too rapid for accurate evaluations of the ancient evolution of birds (Groth & Barrowclough, 1999), and that due to the reduction of alignment ambiguities, studies using nuclear exons should provide a more objective analysis. Many further comparisons will be required to decide which of these studies has defined the correct phylogeny, and it may be a great many years before there is a definitive answer. If the *Paleognathae* do represent a primary divergence from the main avian stem it would suggest that

the visual systems of paleognathous birds may be more closely related to an ancestral avian form than those identified in the neognathous birds.

1.1 THE OSTRICH (Family – *Struthionidae*; Species - *Struthio camelus*)

The adult ostrich stands at an average height of 2.5m (8ft 2in), which makes it the world's tallest bird species. Both sexes possess soft and fluffy feathers. However its plumage is sexually dimorphic: in the male there are distinctively jet black feathers **(Fig. 1.1)** which in females are a ruddy grey-brown colour. In addition, the males possess loose-textured drooping white feathers on their wings and tail.

The birds have large strong legs (ending uniquely in only two toes), which enable an easy striding gait allowing a top speed when running of 40mph (60 km/h). It is their great speed in combination with a keen sight that provides them with a means of outpacing any predating animal that might seek to attack them. The ostriches inhabit landscapes such as desert, semi-desert, and grasslands where the terrain is open enough for speed to be an effective deterrent. Ostriches are herbivorous foragers and feed via ballistic pecking of leaves, flowers and seeds of a wide variety of plants.

Today, wild ostriches can be found across Africa from Senegal to Ethiopia, and south as far as Tanzania with separate populations in Southern Africa. The former wild population in the Arabian Peninsula is now extinct. Ostriches are also now found extensively across the world on farms, where they are bred mainly for their feathers and meat.

1.2 THE RHEA (Family – *Rheidae*; Species - *Rhea americana*)

The rhea stands at an average height of 1.3m (4ft 3in). Unlike the ostrich, the plumage is not sexually dimorphic with both sexes possessing grey/ruddy feathers that are longer and shaggier to protect the rhea from its colder environment. This large flightless bird lives on open plains, particularly where tall grasses and bushes are in abundance. The rhea conceals itself amongst tall plants to avoid detection by predators **(Fig. 1.2)** and, like the ostrich, escapes most attacks with its keen sight and rapid sprinting ability. Rheas feed on tender leaves, roots and seeds, also eating many insects, especially grasshoppers, and small vertebrates.

FIGURE 1.1
Male ostrich (*Struthio camelus*) with distinctive black and white plumage.

FIGURE 1.2
Rhea (*Rhea americana*) with chicks, concealed amongst the grasses and shrubs.

Wild rheas are found in open country from Brazil south of the river Amazon to the Argentinean pampas. Rheas are also now farmed for their meat, however, this industry is not as widespread as ostrich farming.

1.3 AVIAN EYES

Avian eyes "represent the culmination of a process of development which is traceable back to the earliest land-living animals, and which has been continuously in one direction, towards the perfection of daylight vision."

R.J.Pumphrey (1948)

The eye structure of diurnal birds is specifically adapted to a daylight environment and is designed to utilize as much of the available incident light as possible. Avian eyes tend to be large, occupying most of the skull (Meyer, 1977). Large eyes improve visual acuity by allowing the formation of a large retinal image. Paleognathous birds all have large eyes, in particular the ostrich which has the largest eyes of any land vertebrate.

The shape of avian eyes varies between species and three main shapes have been proposed: flat, globose and tubular (Walls, 1942). Ostriches and rheas possess globose eyes (**Fig. 1.3**), which have almost equal equatorial and axial diameters (50 mm bulbar axial length in the ostrich; Martin, 1985). The larger axial diameter projects a larger image on the retina thus giving greater visual acuity. This eye type is also typically seen in diurnal birds that require good resolution over great distances, such as the Corvidae and Falconiformes (Duke-Elder, 1958).

1.4 OCULAR MEDIA

The media within avian eyes that is interposed between the incident light and the retinal photoreceptor cells (such as the cornea, lens and humours) is mostly transparent. This is in stark contrast to some other vertebrates, in which the lens is sometimes pigmented and acts as a pre-retinal filter e.g. human lenses absorb strongly at wavelengths below about 420 nm (Wyszecki & Stiles, 1982). However, UV light provides useful visual information for some avian species and so the ocular media of most birds is relatively transparent down to near-UV wavelengths

(Govardovskii & Zueva, 1977; Emmerton *et al.*, 1980; Goldsmith, 1990; Das *et al.*, 1997). The ocular media of the canary (*Serinus canaria*) for example, is virtually transparent at wavelengths down to about 380nm, and only has an absorbance of 0.2 at about 350 nm (Das *et al.*, 1999). However, some avian lenses do appear to filter some of the light at shorter wavelengths, this is particularly apparent in those birds which lack ultraviolet sensitivity, such as the Mallard duck, *Anas platyrhynchos* (Jane & Bowmaker, 1988). Unlike the UV-transparent canary lens, the duck lens shows significant absorbance below 400 nm with absorbance reaching as high as 2.0 at about 350 nm.

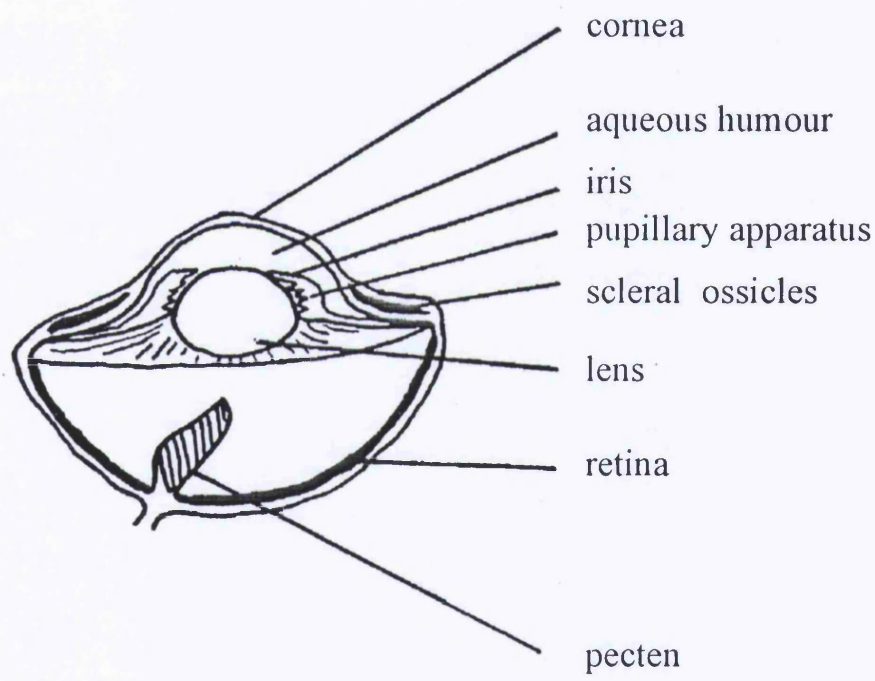


FIGURE 1.3

Schematic diagram of a cross-section through a typical globose shape eye (left eye)

1.5 VISUAL FIELDS

Of the paleognathous birds, only the visual fields of the ostrich have been examined (Martin & Katzir, 1995). Birds have their eyes on opposite sides of their head and each eye has its own monocular visual field. In the ostrich, these fields are 155° and probably function to alert the ostrich to dangers on the horizon. Birds also have a large 'apparent binocular field' in the region of overlap between the two monocular fields, however only a small central area of this field is served by functional retina in the avian eyes and this restricted region represents a true binocular visual field (Martin & Katzir, 1999). Ostriches have been shown to have a binocular area in front of their bill that extends vertically through 80° and up to a width of about 20°, this small binocular field is utilized by ostriches for foraging food on the ground. It has been suggested by Martin & Katzir (1995) that foraging behaviour is the principal determinant of the characteristics of a bird's visual field. Indeed, other species using visually guided pecking to feed possess a small binocular field like that of the ostrich (e.g. the pigeon, *Columba livia*, Martin & Young, 1983). More recent studies on the Short-Toed Eagle, *Circaetus gallicus*, (Martin & Katzir, 1999) which feed by taking live reptilian prey in their feet, suggests that a small frontal binocular field guides the acquisition of singular food items whether they are taken directly in the bill or in the feet. Since the rhea and other paleognathous birds have a similar diet to the ostrich, they are likely to have similar visual fields.

1.6 HORIZONTAL STREAK

In the retina of most vertebrate taxa a central region known as the *area centralis* can be observed. Such regions have a high resolving power since they are richly cone-dominated (Pumphrey, 1948). In some birds, including the ostrich, the *area* is not a circular, but a ribbon-like structure (**Fig. 1.6**) visible as a horizontal ridge when the retina is dissected. The fovea is seen as a depression within this ridge.

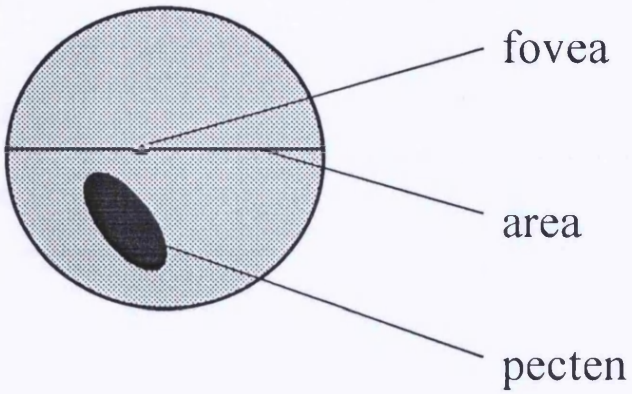


FIGURE 1.6
Schematic diagram of the ophthalmoscopic appearance of the inner aspect of the retina of the ostrich (*Struthio camelus*) as seen through the pupil (adapted from Wood, 1917; Pumphrey, 1948).

The horizontal streak (*area*) of ostriches, as with their small frontal binocular field (Martin & Katzir, 1995), probably represents an adaptation to their visual requirements in their native habitat. For instance, horizontal streaks are never found in birds of prey or inhabitants of forests, instead they are mainly seen in birds that live in open spaces (Duijm, 1958). Also, they are characterised typically in birds that procure their food from the ground (such as the ostrich) or in aquatic birds (e.g. Anseriforms) (Duke-Elder, 1958).

1.7 PECTEN

Attached to the retina at the blind spot, i.e. the point of entry of the optic nerve, birds possess a structure known as the pecten (*Pecten oculi)*. It is a convolute of blood vessels, which appears in the ventral part of the fundus and projects freely into the vitreous. Duke-Elder (1958) identified three types of pecten classified by their morphology: vaned, conical and pleated (reviewed by Martin, 1985). Paleognathous birds (except the cassowary and kiwi) have been shown to possess a vaned pecten, which consists of a central vertical lamina with laterally disposed vanes. Conical pecten are only found in kiwis, *Apterygidae*, and with no vanes or pleats have a form resembling the conus papillaris of reptiles (Wood, 1917; Duke-Elder, 1958). All

other avian species possess the pleated pecten, in which the whole organ is pleated upon itself like an accordion (Duke-Elder, 1958; Meyer, 1977).

Many functions have been proposed for the pecten, including regulation of intraocular pH and as a device to alleviate increased intraocular pressure during accommodation of the eye. However, the two prevailing theories suggest that either the shadow pecten casts on the retina performs various functions (reviewed by Martin, 1985) or that the pecten acts as a means to deliver nutrients to the avascular retina by diffusion. Functions of pecten utilizing its shadow have been criticised because, under normal viewing conditions, the pecten shadow falls mainly on its base and slightly to each side (Walls, 1942), although this has not been proved for all bird species.

In support of the latter theory, each fold (vane or pleat) contains a single arteriole which branches into a capillary network which would be ideal for diffusion into the vitreous (Duke-Elder, 1958). Also, the number of these folds depends on the activity of the retina. For instance, the most active diurnal species like the ostrich and rhea have 25 to 30 folds, whereas crepuscular cassowaries have six in total, bush-dwelling emus have 3 to 4 folds (Braekevelt, 1998a) and the nocturnal kiwi has none. The role of the pecten as a provider of substrates and oxygen to the inner retina (Wingstrand & Munk, 1965), is now largely criticised since there is little oxidative metabolism in avian retinal tissue (Hughes *et al*, 1972; Buono & Sheffield, 1991; Ruggerio & Sheffield, 1998). Instead, it has been shown that avian retinae have a very high glycolytic rate (Hughes *et al.*, 1972). The glucose levels of avian blood plasma and vitreous humour are very high (Krebs, 1972), suggesting that the pecten acts to pump glucose into the vitreous. This glucose is then utilized by the retinal tissue for glycolytic energy production, thus circumventing the need for oxidative processes and their high oxygen demands (Buono & Sheffield, 1991; Ruggerio & Sheffield, 1998). Moreover, this theory offers an acceptable explanation as to how the avian retina survives without vascularisation.

1.8 AVIAN RETINAE

"Histologically the retina of birds is the most beautiful and elaborate in its architecture in the animal kingdom; layers and sub-layers are clearly defined with each cell accurately in place."

S. Duke-Elder (1958)

"La rétine des vertébratés" was first published by S. Ramon y Cajal in 1893 (Rodieck, 1973) and represented the first extensive study of avian retinal structure. The observations made by Cajal were reviewed and further studied by Rodieck (1973) and Martin (1985), and are summarised below. Morphologically the avian retina is divided into two major layers, the outer pigmented layer and the inner neural layers (all retinal layers illustrated in **Fig. 1.8**).

1.9 PIGMENT EPITHELIUM

The outer pigmented layer comprises a thin layer of cuboidal cells known as the pigment epithelium (PE). PE cells have long ciliary processes containing light-absorbing melanin pigment granules, which extend out into the photoreceptor layer to surround the outer segments of the photoreceptors. The pigment granules migrate along the processes towards the tips of the cells (away from the photoreceptors) in the dark and towards the photoreceptors in response to light. The employment of pigment granules in this way is thought to provide a barrier to stray light reaching the outer segments and aids the transition between scotopic and photopic vision (Miller, 1979). PE cells also provide oxygen and substrates to the retina from the choroidal circulation. However, it is assumed that the primary role of the avian PE is in the regeneration of visual pigments and the breakdown of membrane discs shed from the outer segments of photoreceptors.

1.10 NEURAL RETINA

There are four acknowledged layers in the neural retina: the **neuroepithelial (photoreceptor) layer, bipolar layer, ganglion cell layer** and **optic fibre layer**. The **photoreceptor layer** is sandwiched between the pigment epithelium and the external limiting membrane (ELM). It comprises the photoreceptor outer segments and the outer nuclear layer (photoreceptor nuclei). The **bipolar layer** consists of the

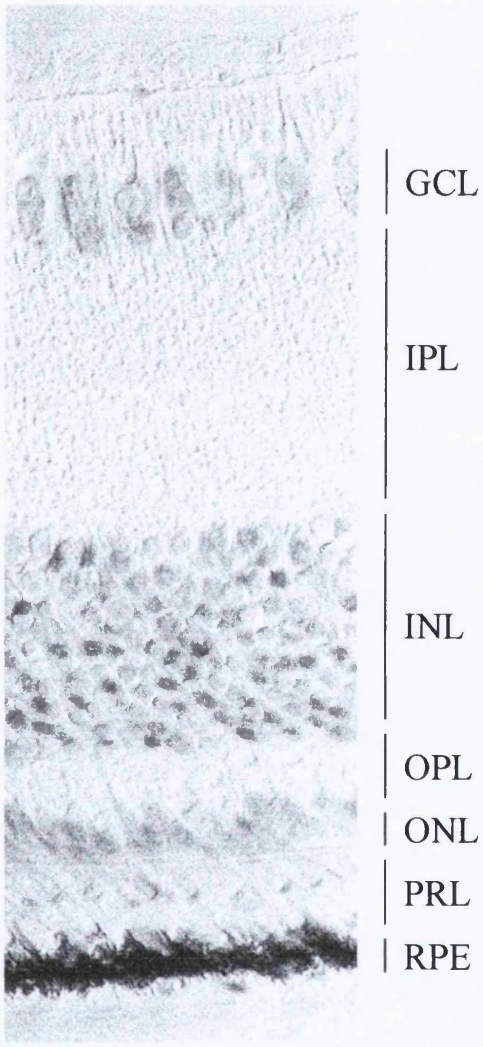


FIGURE 1.8
Layers of an avian retina. Photomicrograph of cross-section through a budgerigar retina (relabelled from Jeffery & Williams, 1994). Key: **GCL** – Ganglion Cell Layer; **IPL** – Inner Plexiform Layer; **INL** – Inner Nuclear Layer; **OPL** - Outer Plexiform Layer; **ONL** – Outer Nuclear Layer; **PRL** – Photoreceptor Layer; **RPE** – Retinal Pigment Epithelium.

outer plexiform layer (in which the dendrites of each bipolar cell synapse with either rods or cones), the inner nuclear layer (containing the nuclei of bipolar, amacrine, horizontal and Müller cells) and the inner plexiform layer (where the bipolar cells synapse onto ganglion cell dendrites). The outermost layers are the **ganglion cell layer** (which contains the nuclei of the ganglion cells) and the **optic fibre layer** (through which the axons of the ganglion cells form nerve fibres and leave the eye as the optic nerve).

The avian bipolar layer is the site of primary neural processing of the visual input. The neural input from the rod photoreceptors undergoes summation, with several rods synapsing onto a single bipolar cell. Rods therefore lose spatial acuity but gain overall sensitivity. In contrast, an individual cone will usually synapse with a single bipolar cell, thus gaining spatial acuity but losing overall sensitivity. Consequently, the inner nuclear layer is thicker in the cone-dominated retinae of diurnal birds than the rod-dominated retinae of nocturnal birds (Sillman, 1973). Amacrine and horizontal cells integrate visual input from the photoreceptors, conducted by bipolar cells, by acting as lateral interneurones across the bipolar layer. Sillman (1973) suggested that the relative abundance of amacrine and horizontal cells in avian retinae indicates that these cells may perform complex visual functions, such as movement detection, that in mammals are normally accomplished at higher centres in the brain. Müller cells are glial cells which provide structural support to the retina. The fibres that Müller cells send out surround most neurones in the retina and the terminal bars of these fibres form the external and internal limiting membranes.

1.11 AVIAN PHOTORECEPTORS

Both rods and cones comprise a **soma** that synapses with the bipolar cells, a central **inner segment** and an **outer segment** that contains the visual pigment. The inner and outer segments are connected together by a ciliary stalk, through which visual pigment molecules synthesised in the inner segment travel into the outer segment (Fein & Szuts, 1982). Calycal processes originate from the tips of the inner segments and run parallel with the outer segments (Cohen, 1963). Their function is unknown, however it has been suggested that they offer structural support and may even prevent the outer segment from rotating about the ciliary stalk (Rodieck, 1973).

The **soma** of both rods and cones synapse onto the bipolar cells via chemical synapses. However, cones do so via cone pedicles with flat bases that have numerous synapses, including synaptic invaginations which often contain synaptic ribbons, whereas rods synapse via spherules that are smaller, rounded and possess fewer synapses (Fein & Szuts, 1982).

The **inner segment** consists of the ellipsoid and myoid regions (Fein & Szuts, 1982). The ellipsoid lies closest to the base of the outer segment and is the 'powerhouse' of the cell containing numerous mitochondria, which provide metabolic energy. Also located in the cone ellipsoids of some vertebrates, including many birds, are large oil droplets that are often heavily pigmented (see next chapter). Immediately proximal to the ellipsoid is a conglomeration of smooth endoplasmic reticulum and glycogen granules, known as the paraboloid in rods and hyperboloid in cones, which is believed to act as a glycogen store for the metabolic activity of the myoid region (Rodieck, 1973). The myoid region, which lies adjacent to the soma, is the primary site of protein synthesis in the cell and thus contains free ribosomes, rough endoplasmic reticulum and Golgi apparatus. It also contains contractile proteins which in some species are thought to act like muscles, contracting to enable light-dependent photomechanical movements in the retina (hence the name myoid which means muscle-like). For instance, in some species elongation of the myoid is thought to move the outer segments so that they are shielded by the PE (Rodieck, 1973).

The **outer segment** contains a stack of lamellar membranes oriented perpendicular to the photoreceptor long axis, in which the visual pigment molecules are embedded. In cones, these lamellae are all infoldings of the plasma membrane and their internal space is continuous with the extracellular environment. However in rods, the lamellae are closed off from the plasma membrane as they mature to form stacks of isolated intracellular disks, with only those disks located near the base of the outer segment remaining continuous with the plasma membrane (Fein & Szuts, 1982). This stacked lamellar structure with its large membrane surface area in a compact space enables a photoreceptor to accommodate the large number of visual pigment molecules it requires to attain a sensible level of photosensitivity (Rodieck, 1973).

Morris & Shorey (1967) described and illustrated (**Fig. 1.11**) rods, double cones and two types of single cone in the chicken retina. Morris (1970) later identified a third type of single cone distinguished by its oil droplet electron density. Analyses of the pigeon retina using Golgi impregnation distinguished five morphological types of photoreceptor cell (Mariani & Leure-Dupree, 1978) namely rods, the two members of double cones and two classes of single cone (straight and oblique). The straight single cones were upright and contained either a red or orange oil droplet, which today would be identified as long-wave sensitive (LWS) and middle-wave sensitive (MWS) cone types (Bowmaker, 1977; Bowmaker *et al.*, 1997). The oblique cones, so called because the inner segment is laterally displaced, were probably a short-wave sensitive (SWS) cone class since they were said to contain a pale yellow-green droplet.

In the chicken and pigeon four spectrally distinct cone visual pigments, including a fourth violet- or ultraviolet sensitive (UV/VS) pigment, have been identified using microspectrophotometric measurements of retinal photoreceptors *in situ* (Bowmaker, 1977; Bowmaker & Knowles, 1977; Bowmaker *et al.*, 1997). Microspectrophotometry (MSP) of the retinae of a variety of avian species (detailed in **Table 4.3**) indicates that most birds have four different cone visual pigments. It is suggested that each cone pigment is found in the outer segments of a specific class of single cones (either LWS, MWS, SWS or UV/VS) as identified by the associated presence of a specific oil droplet type. The LWS pigment is also found in the outer segments of both members of double cones. Evidence from MSP studies is corroborated by immunocytochemistry of the avian retina (e.g. pigeon; Cserháti, 1989), chromatography of visual pigments (e.g. pigeon and chicken; Fager & Fager, 1981; Fager & Fager, 1982; Yen & Fager, 1984; Yoshizawa & Fukada, 1993) and the extraction and expression of the four cone opsins (e.g. chicken; Takao *et al.*, 1988; Okano *et al.*, 1989; Wang *et al.*, 1992). In contrast, some studies indicate that paleognathous birds may not possess the typical photoreceptor complement of visual pigment and oil droplet observed in most neognathous birds (Sillman *et al.*, 1981; Braekevelt, 1998b), these are discussed later in section 4.11.

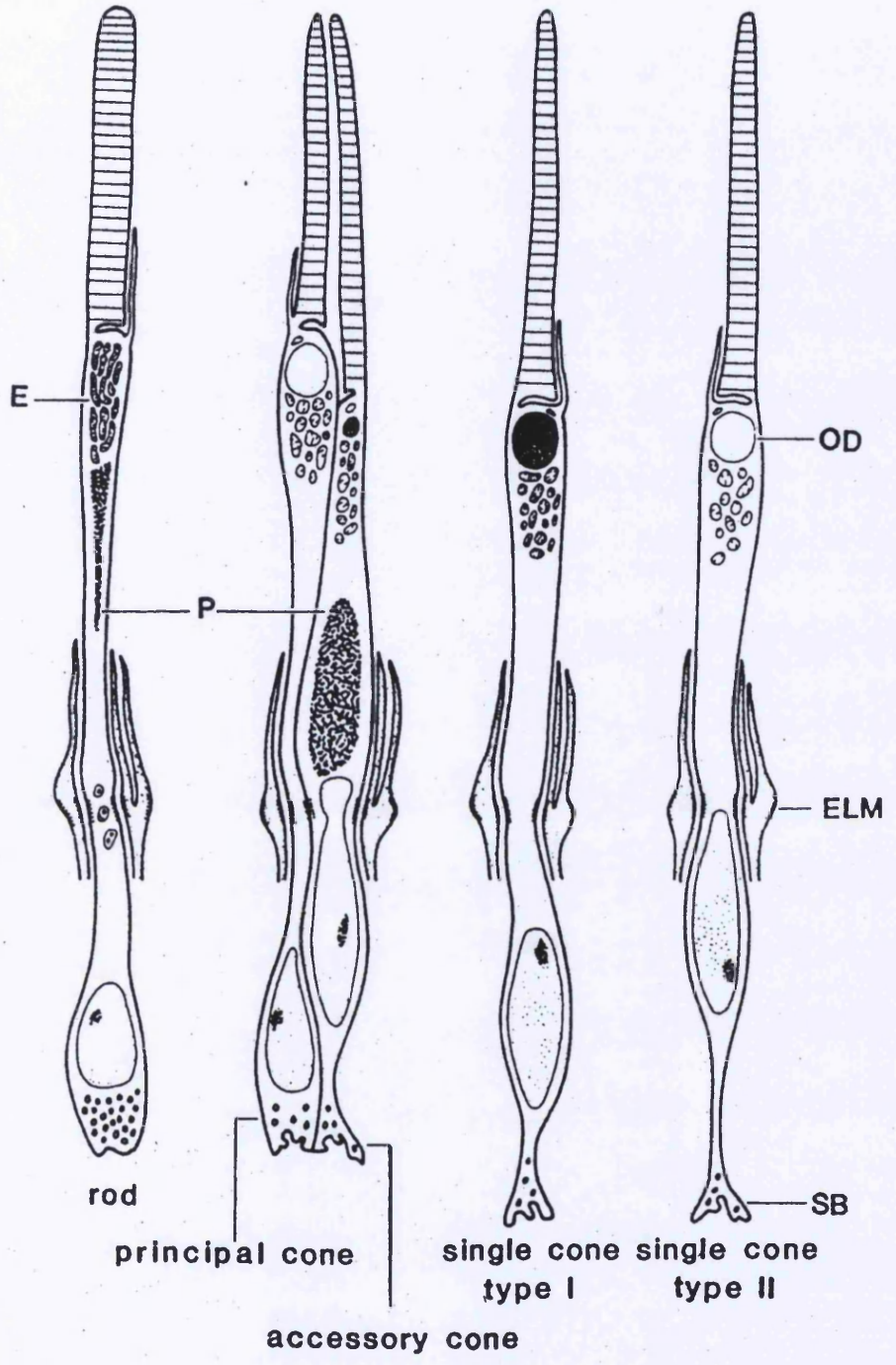


FIGURE 1.11
Schematic drawing illustrating differences between the five types of visual receptor in the chicken retina as identified by Morris & Shorey (1967): **E**, ellipsoid; **OD**, oil droplet; **ELM**; external limiting membrane; **P**, paraboloid; **SB**, synaptic body. (Morris & Shorey, 1967).

1.12 DOUBLE CONES

Double cones typically comprise a principal member which is long and thin and contains a large pale (P-type) oil droplet, and a smaller accessory cone which is broader and shorter and may contain a small oil droplet (A-type) with very low absorbance (Bowmaker & Knowles, 1977; Jane & Bowmaker, 1988; Hart *et al.*, 1998). This cone type has been known about since the earliest retinal studies (Rodieck, 1973) and makes up the largest relative proportion of cone types in most avian retinae (e.g. chicken, *Gallus gallus*, Bowmaker & Knowles, 1977; duck, *Anas platyrhynchos*, Jane & Bowmaker, 1988; budgerigar, *Melopsittacus undulatus*, Wilkie *et al.*, 1998). However, whereas a vast literature is available on the role of single cones in colour discrimination, the specific function of double cones is still unclear. Electroretinographic (e.r.g.) studies have determined the photopic spectral sensitivity of avian retinae, and indicate a significant broad peak around 570 nm which could be attributed to double cones e.g. pigeon, *Colomba livia* (Blough *et al.*, 1972). However, behavioural increment threshold measures of photopic spectral sensitivity, show only the peaks which correspond to the relative spectral sensitivities of the four single cone types e.g. Pekin robin, *Leiothrix lutea* (Maier & Bowmaker, 1993). This implies that colour vision is primarily subserved by the four classes of single cone without any major input from double cones. Other proposed functions for double cones include the detection of polarised light (Young & Martin, 1984), a role in enhanced movement detection (Hart *et al.*, 1998) and a function in brightness discrimination.

The absence of double cones observed in some avian retinae, may be due merely to the two members (principal and accessory) separating during tissue preparation for MSP. No double cones have as yet been identified in the tawny owl, *Strix aluco*, (Bowmaker & Martin, 1978) or the Humboldt penguin, *Spheniscus humboldti*, (Bowmaker & Martin, 1985). Also, a microspectrophotometric study of tinamous and emus found no double cones and suggested that paleognathous birds might not possess this cone-type (Sillman *et al.*, 1981). However, fine structural analysis of the retinal photoreceptors of other owls (great horned owl, *Bubo virginianus* and barred owl, *Strix varia*) and the emu has revealed the presence of double cones (Braekevelt *et al.*, 1996; Braekevelt, 1993, 1998b).

Hart *et al.* (1998) studied the spectral absorption characteristics of the P-type oil droplets in the double cones of the European starling, *Sturnus vulgaris,* and discovered a dorso-ventral gradient in the absorbance of these droplets. It was suggested that such a gradient may serve to allow good vision of the ground whilst reducing excessive irradiance from the sky, as this would reduce luminance differences between the two visual hemifields. Alternatively, the ventral retina may need more protection from harmful ultraviolet radiation (Kirschfeld, 1982).

OIL DROPLETS

The ellipsoid of the cones of many birds, some fish, amphibians, reptiles and non-placental mammals contain a large spherical oil droplet (Walls, 1942; Meyer *et al.*, 1965). These highly refractile droplets occupy the entire diameter of the distal end of the inner segment, thus indicating that some of the light reaching the visual pigment in the outer segments is via the oil droplets. Any such 'filtering' of light, whether from oil droplets or pre-retinal filters (e.g. cornea and lens), must be included in calculations of the effective spectral sensitivity of a photoreceptor cell (Bowmaker, 1977). Oil droplets are usually only found in cones, however, they have been observed in the rod photoreceptors of some geckos, the African lungfish (*Protopterus aethiopicus*) and the 'primitive' reptile, *Sphenodon punctatum* (Walls, 1942).

2.0 EVOLUTION OF OIL DROPLETS

"The evolution of avian retinal oil droplets is more influenced by the visual ecology of different species than by their phylogeny."

J.C. Partridge (1989)

'Colourless' oil droplets have been identified in a variety of species **(Table 2.0)**, from cartilaginous fish to metatherian mammals. The only Classes from which they are notably absent are the teleost fishes and eutherian mammals. As these droplets seemed to be so ubiquitous and were the only droplet type located in the most 'primitive' vertebrates (e.g. lungfishes), it would be tempting to believe that colourless oil droplets were the first to evolve. However, Walls (1942) in his extensive review of vertebrate retinal oil droplets suggested that colourless oil droplets could equally feasibly have evolved from coloured oil droplets that had become adapted to a nocturnal habitat.

In many birds and some reptiles and amphibians, carotenoid pigments are found dissolved in some of the oil droplets (Liebman & Granda, 1971;Goldsmith *et al.*, 1984). This pigmentation embues these droplets with a distinct colouration (usually

Table 2.0
A brief summary of the phylogenetic distribution of oil droplet types.
Adapted from Walls (1942).

	ENVIRONMENT	OIL DROPLET TYPES
FISHES:		
Chondrosteans	Benthic	Colourless
Holosteans	Diurnal	Absent
Lungfish	Nocturnal	Colourless
Teleosts	Diurnal	Absent
AMPHIBIANS:		
Frogs	Nocturnal / Diurnal	Yellow & Colourless
Toads	Nocturnal	Absent
Urodeles	Fossorial	Absent
REPTILES:		
Sphenodon	Nocturnal / Fossorial	Pale Yellow
Lizards	Diurnal / Fossorial	Colourless or Yellow & Colourless
Geckos	Nocturnal / Diurnal	Colourless or Yellow & Colourless
Snakes	Crepuscular / Fossorial	Absent
Turtles	Diurnal	Red, Orange, Yellow & Colourless.
Crocodilians	Nocturnal	Absent
BIRDS:		
Most birds	Diurnal	Red, Orange, Yellow & Colourless.
Some birds	Crepuscular or Nocturnal	Yellow, but mostly Colourless.
Nocturnal birds	Nocturnal	Colourless
MAMMALS:		
Eutherian	Nocturnal	Absent
Metatherian	Nocturnal / Diurnal	Colourless

red, orange or yellow) and allows them to be classified into different types. Yellow is the most commonly observed colour of oil droplets across species. The biggest variety of droplet types is seen in the retinae of turtles and most diurnal birds, since they possess a startling array of brightly coloured retinal oil droplets. The retinae of birds and turtles are remarkably similar, in that both taxa possess the same types of carotenoids responsible for the bright oil droplet colouration, and also have a similar spectral sensitivity of their visual pigments (Liebman & Granda, 1971; Liebman & Granda, 1975; Goldsmith *et al.*, 1984). The concentration of lipid-dissolved carotenoids in the coloured oil droplets of these species is thought to be sufficiently high to enable the droplets to act as intra-ocular cut-off filters (Liebman & Granda, 1975).

The primary determinant of the retinal oil droplet complement in any species appears to be the environmental constraints placed upon that species (**Table 2.0**). For instance, brightly coloured oil droplets are only found in those species that possess a diurnal lifestyle (e.g. diurnal birds and turtles), whereas those species that are nocturnal usually possess colourless or very pale droplets (e.g. crocodilians and nocturnal birds, such as the Tawny owl, *Strix aluco*) (Walls, 1942; Bowmaker & Martin, 1978).

2.1 CLASSIFICATION

In the earliest studies of avian retinal oil droplets, different types of droplet were distinguished using microscopy. Unfortunately, the uncorrected microscope objectives used at the time gave rise to the identification of spurious droplet types, such as blue and violet (Walls & Judd, 1933). A study of the domestic chicken, *Gallus gallus*, by Roaf (1929) provided the first objective measurements of avian retinal oil droplets. These heavily pigmented organelles were shown to act as long-pass cut-off filters, blocking wavelengths toward the short wavelength end of the spectrum.

Microspectrophotometry (MSP) is the foremost method by which the spectral absorbance of avian retinal oil droplets have been measured (Strother & Wolken, 1960; Strother, 1963; Goldsmith *et al.*, 1984; Partridge, 1989; Bowmaker, 1991).

MSP measurements have an added bonus, in that a specific oil droplet type can be correlated with its associated visual pigment within the photoreceptor, thus enabling identification of specific cell types (Bowmaker & Knowles, 1977; Bowmaker, 1977). However, microspectrophotometric measurements are not ideal for some of the more heavily pigmented oil droplets like those identified in many avian retinae. The problem lies with the high degree of light leakage that occurs around the edge of oil droplets that have a high absorbance (in excess of 1.0), since light that is not normally transmitted by-passes the oil droplet filter and makes a significant contribution to the estimate of transmittance (Liebman & Granda, 1975; Goldsmith *et al.*, 1984). A further problem in measuring avian retinal oil droplets is their small size, typically 2 to 4 μm in diameter. This increases the leakage of light around the droplet and further reduces the reliability of measurements at higher absorbances. The result is a typically 'flat-topped' spectra, especially when making spectral measurements of the highly pigmented R- and Y-type droplets.

Lipetz (1984) considered the brightly coloured oil droplets of birds and their role as cut-off filters, and introduced a new means of classifying oil droplets according to their 'cut-off' wavelength (λ_{cut}). It was shown that light by-passing the oil droplet in MSP measurements does not effect the λ_{cut} of that droplet. However, this method does assume that light which bypasses the droplet is constant at all wavelengths, which might not necessarily be true. λ_{cut} can be directly related to peak absorbance since it represents the wavelength below which there is no significant transmission, and as such it is now the standard means of classifying retinal oil droplets (Jane & Bowmaker, 1989; Maier, 1992; Bowmaker *et al.*, 1993; Bowmaker *et al.*, 1997; Das *et al.*, 1999).

Bowmaker, 1991, combined the oil droplet nomenclatures of a number of studies (Liebman & Granda, 1975; Bowmaker, 1977; Goldsmith *et al.*, 1984; Partridge, 1989) in order to produce a universal classification system for avian retinal oil droplets (**Table 2.1**). This system classifies oil droplets according to their λ_{cut} and also the λ_{max} of the visual pigment to which they are associated. The orange oil droplets located in the retinae of some birds, do not have their own category within this classification. They are thought to represent either an R-type droplet which has a

Table 2.1
Classification of avian retinal oil droplets based upon the terminology introduced by Bowmaker, 1991.

Cone Type	Pigment λ_{max} (nm) *	Appearance of Oil Droplet	Droplet λ_{cut} (nm) #	Oil Droplet Type
Single Cones:				
LWS	550- 570	Red	560- 570	R-type
MWS	500- 515	Yellow	500- 510	Y-type
SWS	440- 460	Colourless → Pale Yellow	420- 440	C-type
UV/VS	360- 420	Transparent	-	T-type
Double Cones:				
Principal	550- 570	Colourless → Yellow	480- 490	P-type
Accessory	550- 570	Colourless	-	A-type

Table adapted from Varela *et al.* ,1993

* λ_{max} values adapted from **Table 4.3**.

λ_{cut} values taken from Pekin robin, *Leiothrix lutea* (Maier & Bowmaker, 1993)

low carotenoid concentration due to a dietary deficiency (Bowmaker *et al.*, 1993) or a highly pigmented Y-type droplet (Partridge, 1989).

2.2 COMPOSITION

Neutral lipids, such as cholesterol, glycerol ester, mono-, di- and tri-glycerols, are the main constituent of oil droplets. These lipids are also enriched with polyunsaturated fatty acids (e.g. linoleic and arachidonic acids) which make a solvent mixture that is ideal for obtaining maximal solubility of oil droplet carotenoids (Johnston & Hudson, 1976). The carotenoids vary in colour between yellow and red, and comprise a subgroup of polyene pigments (carotenes) and their oxygenated derivatives (xanthophylls). They consist of eight isoprene units, configured to form either an aliphatic or alicyclic structure, with a series of conjugated double bonds constituting the chromophore (Meyer *et al.*, 1965).

Wald (1937) purified and crystallized three retinal extracts from chicken retina, and provided the first classification of oil droplet carotenoids (Wald & Zussman, 1937). The extracts were identified as red, orange and yellowish green in colour and designated respectively as astacene, xanthophyll and an unidentifiable hydrocarbon. Astacene was later found to be the readily formed oxidation product of astaxanthin, (a carotenoid that is found *in vivo* in the retina) and the yellowish-green extract was later identified as an alcohol and named galloxanthin (Kuhn *et al.*, 1939; Strother & Wolken, 1960).

The carotenoids isolated in the brightly coloured oil droplets of birds cannot be synthesised *de novo* and must be taken in the diet (Davies, 1979), and thus birds that are raised on a carotenoid-deprived diet develop transparent droplets e.g. Japanese quail, *Coturnix japonica* (Wallman, 1979; Bowmaker *et al.*, 1993). Naturally occurring transparent (T-type) droplets, which show no observable absorption between 320–750 nm, probably only contain neutral lipids and possess no carotenoid pigments. The carotenoids present in each avian oil droplet type are summarised in **Table 2.2.** The most varied droplet type are the P-type droplets located in the principal member of double cones. Under standard white light microscopy, P-type

Table 2.2
Summary of carotenoids in avian retinal oil droplets.

DROPLET TYPE	CAROTENOIDS IDENTIFIED
R-type	astaxanthin & some evidence for a second carotenoid*
Y-type	zeaxanthin and/or lutein
C-type	galloxanthin & 'fringillixanthin' #
T-type	none
P-type	galloxanthin & unidentified secondary carotenoid
A-type	low-concentration; triple-peaked

* suggested by Goldsmith *et al.*, (1984) and Bowmaker *et al.*, (1993).
It is thought that this secondary carotenoid absorbs at short wavelengths.

trivial name given to a carotenoid with 385 nm λ_{cut} (Goldsmith *et al.*, 1984)

droplets can vary in appearance from colourless to green or yellow. It is known that these droplets contain galloxanthin which is responsible for their variability in λ_{cut}, however, the identity and absorption profile of a second carotenoid is still unknown (Liebman & Granda, 1975; Goldsmith *et al.*, 1984).

2.3 EMBRYOLOGY

Within the inner segments of the LWS single cones of the pigeon retina, clusters of red microdroplets (diameter <0.5μm) have been observed in addition to the single large oil droplets (R-type) (Pedler & Boyle, 1969; Bowmaker, 1977). The function of these tiny droplets is unknown. Also, multiple droplets have been shown in the accessory member of the double cones of the Japanese quail (Hazlett *et al.*, 1974).

Before the cone outer segment membranes have fully formed, the oil droplets in the retina of the chicken have already developed to a microscopically observable size by the accumulation of lipids from the cellular cytoplasm (Meyer *et al.*, 1965; Meyer & Cooper, 1966). It is thought that the first droplets to appear are colourless, with the pigmentation developing after the droplet has formed (Meyer *et al.*, 1965). All adult colouration of droplets is discernible by seventeen days post-fertilisation, and since the chick has yet to hatch by this stage of development all the carotenoids must have been derived solely from stores within the egg yolk (Davies, 1985).

2.4 FUNCTION AS LONG-PASS CUT-OFF FILTERS

The histological studies of chicken retinae by Roaf (1929) suggested that the pigmented retinal oil droplets of birds act as long-pass cut-off filters. A comparison of the electroretinographic (e.r.g.) response produced by illuminating the retinae of another anseriform (the Japanese quail, *Coturnix japonica)*, either normally or from behind the retina provided evidence to support this theory (Kawamuro *et al.*, 1997). The spectral sensitivity curve produced by the retina is narrower and has its maximum displaced to longer wavelengths when the retina is illuminated normally, thus providing evidence for the filtering effect of the retinal oil droplets which are interposed between the incident light and the visual pigment in the outer segment.

The effect that oil droplets have on the spectral sensitivity of avian cones was summarised by Bowmaker (1977) when he concluded that the effect of an oil droplet in a given cone is:

- to displace the effective maximum sensitivity of the cone to a wavelength longer than the λ_{max} of the visual pigment.

- to reduce the bandwidth 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 λ_{max}.

The effects of oil droplets are exemplified by calculations of the relative spectral sensitivity of all the cone types found in the pigeon retina (Bowmaker, 1977). For instance, the heavily pigmented R-type droplets of the red sector effectively cut off all light below 560 nm, which displaces the maximum sensitivity of the LWS cone into longer wavelengths by up to 50 nm and reduces the bandwidth of the P567 visual pigment spectrum by about 30%. Pigmented oil droplets narrow the cone spectral sensitivities and reduce overlap between cone types that could cause a loss of spectral discrimination between narrow-band stimuli. It is thought that although the filtering action of oil droplets reduces overall spectral sensitivity, the benefits to avian vision of increased hue discrimination outweigh this loss (Bowmaker & Knowles, 1977; Govardovskii, 1983; Vorobeyev *et al.*, 1998).

Non-pigmented, transparent (T-type) oil droplets are found only in those cones containing pigments which functionally absorb at shorter wavelengths (UVS/VS cones). It has been proposed that the pigmented retinal oil droplets possessed by all the other cone photoreceptor types, protect them from the damaging effects of ultraviolet (UV) radiation (Kirschfeld, 1982). It is thought that the yellow lens and corneas of other vertebrates perform a similar function, preventing the harmful UV radiation from entering the eye and causing potential photochemical damage (Miki, 1991).

The absorption spectrum of a visual pigment possesses three main peaks: an α band (the absorption peak of the visual pigment), a β band (also known as *cis* peak and is the 11-cis isomer of retinal) and a γ band (that represents the protein component of the visual pigment) (Wolbarsht, 1976). The pre-retinal ocular media absorbs light below 300 nm (Govardovskii & Zueva, 1977; Emmerton *et al.*, 1980), therefore voiding any function for the γ band which absorbs in the far violet, near 280 nm (Palacios *et al.*, 1996). As the spectral location of the β band is in proportion to the spectral location of the α band, then the β band of UV/VS visual pigments is also below 300 nm and has no observable effect. However, the β band of LWS and MWS visual pigments is present at the short wavelength end of the spectrum and raises the problem that short wavelength light could be absorbed by the β band and generate a visual signal. The pigmented R- and Y-type oil droplets serve to prevent this from happening, by cutting out short wavelength light before it reaches the visual pigment (Wolbarhst, 1976).

2.5 OIL DROPLETS AS SECONDARY LENSES

C-type droplets which have a low carotenoid concentration and T-type droplets are ineffective filters, and as such are unlikely to have a significant effect on the spectral sensitivity of avian cones (Bowmaker & Knowles, 1977). Since the occurrence of 'colourless' droplets is so widespread across taxa, from teleost fishes to metatherian mammals (Walls, 1942), it has been suggested that oil droplets must function as more than just long-pass cut-off filters. The position of oil droplets, juxtaposed between the incident light and the visual pigment, suggests that they would be in the ideal position to act as a secondary lens, funnelling light into the outer segment (Roaf, 1929). By using a theoretical model based on wave optics, Young and Martin (1984) analysed the scattering properties of avian retinal oil droplets. It was deduced that oil droplets do significantly aid light collection in cones and therefore improve visual sensitivity.

2.6 DETECTION OF MAGNETIC FIELD

Edmonds (1996) suggested a possible role for oil droplets in the magnetoreception of birds. The proposed theory relies on the properties of the carotenoids endogenous to

avian oil droplets. These long chain molecules only absorb light when the direction of their electric field is along the long axis of the chromophore. Therefore, light of wavelengths absorbed resonantly by the carotenoid would only transmit through the oil droplet when the axis of the cone was parallel or anti-parallel to the direction of the earth's magnetic field. However, for this theory to work the carotenoid molecules would have to be arranged regularly in a liquid crystal formation and be associated with small magnetic crystals. In fact, avian oil droplets are generally regarded as homogenous structures, although the P-type droplets of the duck, *Anas paltyrhynchos* (Braekevelt, 1990) and the emu, *Dromaius novaehollandiae* (Braekevelt, 1998b) have been shown to be more heterogeneous than those found in the single cones. Also, no magnetic crystals have been located in avian oil droplets.

2.7 REDUCTION OF CHROMATIC ABERRATION

The eyes of most diurnal vertebrates are focused for the yellow or red end of the spectrum and any blue light will form a blurred retinal image (Wolbarsht, 1976). Chromatic aberrations are particularly pronounced in larger eyes. Pigmented retinal oil droplets absorb short wavelength light (especially R- and Y-type droplets), thus reducing the effect of chromatic aberration.

It has been observed that avian retinal oil droplets are stratified into separate layers, with a tendency for the larger droplets to be closer to the outer retinal margin (Mariani & Leure-Dupree, 1978; Jeffery & Williams, 1994). This promotes the possibility that stratification of the oil droplets might be a means of reducing chromatic aberration. The focal length of the pigeon eye has been measured as about 7.91 mm (Martin, 1985). The longitudinal chromatic aberration (between 442-633 nm) of the vertebrate eye (including birds), is on average about 4.6% of the total focal length (Kreuzer & Sivak, 1985). This means that the chromatic aberration of the pigeon eye is about 363 microns in axial separation between the 442 and 633 nm foci. The stratification of oil droplets in the pigeon retina is about 5 microns (Mariani & Leure-Dupree, 1978). This means that the separation between oil droplets is not near the amount necessary to correct for chromatic aberration.

2.8 MONOPIGMENT HYPOTHESIS

It was once thought that an avian colour vision system could function with just one cone pigment with a selection of oil droplets each with a specific absorption profile (Donner, 1958; King-Smith, 1969). This 'Monopigment Hypothesis' has since been proven to be invalid because microspectrophotometric studies have isolated more than one cone pigment in avian retinae. For example, the chicken has been shown to have at least four spectrally distinct cone pigments (Bowmaker & Knowles, 1977; Bowmaker *et al.*, 1997). Also, birds that are fed on a carotenoid-deprived diet develop colourless oil droplets but this does not deprive them of their colour vision (Meyer, 1971; Meyer *et al.*, 1971; Wallman, 1979; Bowmaker *et al.*, 1993).

VISUAL PIGMENTS

"The absorption of photons is an indispensable first step on the road to sight."

G.H. Jacobs (1998)

3.0 THE RECEPTOR MEMBRANE

Visual pigment molecules lie within the membrane of the lamellar disks in photoreceptor outer segments (see section 1.11). Each visual pigment molecule is oriented such that the long-axis of the chromophore is parallel to the lamellar membrane surface. Also, fluidity of the lamellar membrane allows rotation of the pigment molecules about an axis normal to the disk surface (Fein & Szuts, 1982). The electronic transition associated with absorption of light by visual pigment molecules is the initial event in vision. For efficient absorption of light to occur, light must approach the visual pigment molecule from the correct direction, that which efficiently couples the wave motion of the light with the electronic transition (Knowles & Dartnall, 1977). The orientation of pigment molecules in rod and cone lamellar membranes is designed to maximise the absorption of light passing down the long axis of the photoreceptor whatever the orientation of its plane of polarization. However, light of e-vector perpendicular to the long axis of the receptor cell is also strongly absorbed (Schmidt, 1938; Wright *et al.*, 1972). This dichroic nature of visual pigments is exploited in microspectrophotometric measurements of photoreceptor outer segments, where it is technically easier to measure the cells transversely rather than axially.

3.1 PHOTOTRANSDUCTION

In recent years, there has been a phenomenal growth in our understanding of phototransduction and a number of excellent reviews reflect this progress (Yau, 1994; McNaughton, 1995; Baylor, 1996; Pugh, 1999). A brief summary of phototransduction is given below.

The surface membrane of photoreceptor cells contains cGMP sensitive ion channels that are permeable to cations. During periods of darkness these channels are open and Na^+ and Ca^{2+} ions flow with the electrochemical gradient into the outer segment.

Simultaneously, Ca^{2+} and K^+ ions are extruded from the cell by ion-exchange pumps which help to maintain a high Na^+ level within the cell. This 'dark current' partially depolarizes the cell (around – 40 mV) and maintains a high continual release of neurotransmitter from the synaptic terminal.

Turning Phototransduction On

When a visual pigment absorbs a photon of light the 'kinked' (11-cis) isomer of retinal is converted to the 'straight' (all-trans) form, thus causing a conformational change in the associated opsin. The photoactivated opsin can then bind to and activate many G-proteins (transducins) which catalyse the exchange of GDP (bound to the α-subunit of transducin) for GTP. A transducin molecule comprises an α- and βγ-subunit, which dissociate following activation to release Tα- GTP subunits. These released subunits remove the inhibition from phosphodiesterase (PDE) molecules which convert cGMP into 5'GMP and protons, thus decreasing the concentration of cGMP within the cell. The cGMP sensitive cation channels of the dark current are shut-off, thus hyperpolarising the photoreceptor (around – 80 mV) and reducing transmitter release. The phototransduction cascade amplifies its response at each step, with a single photon resulting in the hydrolysis of many cGMP molecules and the closure of many outer segment membrane channels.

Turning Phototransduction Off

Despite the closure of the cation channels, the ion exchange pumps continue to extrude Ca^{2+} out of the cell causing a decrease in its intracellular concentration. The reduction in Ca^{2+} stimulates production of guanylate cyclase, which synthesises cGMP from GTP, increasing the cGMP concentration to levels high enough to reopen the cation channels and recommence the dark current. The activated opsin is turned off through phosphorylation by opsin kinase and the binding of arrestin. The G-protein turns off as GTP is hydrolysed to GDP, since when it is bound to GDP it can no longer disinhibit PDE.

3.2 CHROMOPHORES

Visual pigments comprise a membrane bound protein receptor (opsin) to which a chromophore (11-*cis* retinaldehyde or an 11-*cis* analogue) is covalently bound (Bownds, 1967). The chromophore embues the visual pigment with photosentivity, whilst the opsin governs the wavelength sensitivity and is also involved in the regulation of transduction. The visual pigments of most vertebrate groups (and some invertebrates), contain a chromophore which is a vitamin A_1 derivative (retinal) and are known as rhodopsins. Teleost fish, amphibia and some reptiles utilise 3,4-dehydroretinal (a vitamin A_2 derivative) as chromophore (Bridges, 1972; Knowles & Dartnall, 1977), and these pigments are known as porphyropsins. Another chromophore type, 3-hydroxyretinal, has been observed in some insects, most notably in butterflies, moths and some types of fly (Vogt, 1983, 1984, 1987; Vogt & Kirschfeld, 1984; Goldsmith *et al.*, 1986; Smith & Goldsmith, 1990). The 4-hydroxyretinal chromophore, however, has only been identified in a bioluminescent squid (firefly squid, *Watasenia scintillans*) in which retinal and dehydroretinal chromophores were also present (Matsui *et al.*, 1988). The absorbance of any visual pigment type is significantly altered according to the chromophore it contains. In comparison to rhodopsins, for instance, the absorbance spectra of porphyropsins have a broader α-band with a λ_{max} at longer wavelengths. This spectral shift is wavelength dependent and can be as little as 5-10 nm at shorter wavelengths or as great as 60 nm at longer wavelengths (Whitmore & Bowmaker, 1989; Bowmaker, 1995).

3.3 OPSIN STRUCTURE

The opsin component of a visual pigment is a G-protein receptor and is composed of a single polypeptide chain of 340-500 amino acids which loop through the photoreceptor membrane to form seven α-helical transmembrane (TM) domains. These TM domains are linked together via alternating cytoplasmic and extracellular straight chain loops. Each helix is made up of 26 predominantly hydrophobic amino acids with only a core 18 amino acids lying within the membrane (Dratz & Hargrave, 1983; Findlay & Pappin, 1986; Applebury & Hargrave, 1986). The retinal binding pocket is created by the seven helices forming a bundle within the

membrane, this creates a hollow cavity on the extracellular surface (Baldwin, 1993; Schertler *et al.*, 1993; Unger *et al.*, 1997; Krebs *et al.*, 1998). The site of attachment of 11-cis retinal is a lysine (K) located in the centre of helix VII (position 296 in bovine rod opsin) (Bownds, 1967; Wang *et al.*, 1980). A condensation reaction between the ε-amino group of the lysine residue and an aldehyde group on the chromophore binds the retinal covalently to the opsin via a carbon-nitrogen double bond known as a Schiff's base linkage. In most opsins the Schiff's base is protonated (Wang *et al.*, 1980) and so at the extracellular end of helix III is a glutamic acid (E) residue which acts as the counterion for the protonated Schiff's base (position 113 in bovine rod opsin) (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989; Nathans, 1990). Many other amino acid sites within the helices have been shown to have a crucial role in the functioning of the visual pigment. It is therefore not surprising that the helices are highly conserved, although the overall length of an opsin differs according to variations in the lengths of the non-helical regions, such as the connecting loops and carboxyl- and amino-terminals. (For a review of G-protein receptors see O'Dowd, 1989).

There are a number of ubiquitous features of opsin molecules:

- one or two asparagine residues form **glycosylation sites** near the amino or N-terminal (Applebury & Hargrave, 1986).
- one or two **palmitoylated cysteines** in the carboxyl or C-terminal anchor the tail and form a fourth cytoplasmic loop (Ovchinnikov *et al.*, 1988).
- a **disulphide bridge** is formed between the first and second extracellular loops via two highly conserved cysteine residues, thereby guaranteeing the correct folding and formation of the opsin molecule (Karnik *et al.*, 1988; Karnik & Khorana, 1990).
- **sites of phosphorylation** by opsin kinase, typically serines and threonines, are found on the C-terminal (Hargrave, 1982; Thompson & Findlay, 1984; Applebury & Hargrave, 1986).

- some of the residues of the **cytoplasmic loops** are thought to interact with cytoplasmic proteins including the membrane-bound G-protein, transducin (Hargrave, 1982; Applebury & Hargrave, 1986; König *et al.*, 1989; Franke *et al.*, 1992).
- numerous **proline residues** are present in five of the seven TM domains of bovine rhodopsin. These residues are capable of producing a 20° kink in the helices, and in doing so might be responsible for the formation of the retinal binding pocket (Dratz & Hargrave, 1983; Ostrer & Kazmi, 1997).

3.4 COMPONENTS OF SPECTRAL ABSORPTION

The conjugated chain of single and double bonds of retinal produces a π electron shell that is responsible for the absorption of light by the chromophore. It is the degree of delocalisation of the electrons in the π shell that determines the spectral sensitivity of retinal. The maximal absorbance of retinal is about 380 nm and a model retinylidene Schiff base linkage has a λ_{max} of about 440 nm (Knowles & Dartnall, 1977; Nakanishi, 1991). Those visual pigments identified in avian retinae (rhodopsins) have maximal sensitivities ranging from 355 to 570 nm (see **Table 4.3**). Therefore a shift in the peak spectral sensitivity of the Schiff base linkage to either longer (bathochromic shift) or shorter wavelengths (hypsochromic shift) must occur in order to create the individual absorption characteristics of each pigment type (Knowles & Dartnall, 1977; Applebury & Hargrave, 1986). Such spectral shifts are dependent on the interactions between the chromophore and the specific opsin to which it is bound. If the interaction between the chromophore and opsin increases the degree of delocalisation of the π electrons in the retinal, then the energy difference between the ground and excited states of the molecule is decreased thus shifting its spectral absorption to longer wavelengths. Conversely, shorter wavelength pigments are created by reducing the degree of delocalistion of the π electron shell and thus promoting a greater difference between the ground and excited states of retinal. In this way, the spectral sensitivity of each visual pigment is genetically determined by the amino acid complement of the opsin molecule it contains, particularly charged amino acids lining the retinal binding pocket.

The most notable amino acid within an opsin that influences the spectral absorbance of the visual pigment is the glutamic acid residue in helix III. Its negative charge stabilises the protonated Schiff's base and thus creates a bathochromic shift to a λ_{max} of around 500 nm (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989; Nathans, 1990). Substitution of the glutamic acid counterion with an uncharged residue, glutamine (E113Q), loses the stability inferred on the proton of the Schiff base and produces a dramatic hypsochromic shift to 380 nm. The acidity and chloride ion concentration of the environment of the Schiff base also influences its spectral absorbance. At acidic pHs, an unprotonated Schiff base (like that created by the E113Q mutation) will undergo a bathochromic shift to around 495 nm (Sakmar *et al.*, 1989), as is also the case in the presence of Cl^- ions which can act as surrogate counterions (Nathans, 1990).

3.5 CLASSIFICATION OF OPSINS

Bowmaker & Hunt (1999) in their review on the molecular biology of photoreceptor spectral sensitivity, provide a classification system for opsins which combines the two previous terminologies of Okano *et al.*, (1992) and Yokoyama (1994):

L = Long-wave sensitive cone pigments (λ_{max} 520 – 575 nm)

M_{Rd} = Rod pigments (λ_{max} 460 – 530 nm)

M_C = Middle-wave sensitive cone pigments (λ_{max} 460 – 530 nm)

S_B = Short-wave sensitive cone pigments (λ_{max} 430 – 470 nm)

S_V = Short-wave sensitive cone pigments (λ_{max} 350 – 440 nm)

Bird species typically possess a rod pigment (M_{Rd} - λmax 500 – 509 nm) and four cone pigments (L - λmax 543 – 570 nm; M_C - λmax 501 – 508 nm; S_B - λmax 430 – 463 nm; S_V - λmax 355 – 421 nm) as shown in **Table 4.3**. The amino acid sequences for the complete complement of opsins have only been elucidated in two avian species, the chicken, *Gallus gallus* (Takao *et al.*, 1988; Okano *et al.*, 1992) and the canary, *Serinus canaria* (Das *et al.*, 1999).

3.6 L OPSINS

The spectral sensitivity of the chicken LWS visual pigment is known to be sensitive to the presence of Cl^- (chloride) ions. When this pigment is extracted into solution in the absence of Cl^- the λ_{max} is hypsochromically shifted to about 520 nm, however, when Cl^- ions are added the λ_{max} is shifted back to 562 nm (Knowles, 1976, 1980; Slobodyanskaya *et al.*, 1980; Shichida *et al.*, 1990). It is not only avian LWS cone pigments that are chloride sensitive, for instance the MWS cone pigment (λ_{max} – 521 nm) of the Today gecko, *Gecko gecko*, undergoes a hypsochromic shift of around 20 nm in the absence of Cl^- ions (Crescitelli, 1977; Crescitelli & Karvaly, 1991). This is in contrast to the SWS and VS pigments extracted from chicken retinae (Fager & Fager, 1981) and the SWS of the gecko (Crescitelli & Karvaly, 1991) which have all been shown to be insensitive to Cl^- ions, suggesting that chloride sensitivity is common only to longer wavelength sensitive pigments. Kleinschmidt & Hárosi (1992) made *in situ* measurements of the chloride sensitivity of the LWS cone pigments from an amphibian and four species of fish. The results of their study corroborated the data obtained from chicken and gecko LWS pigments and through a comparison with the effects of other anions provided evidence that Br^- (bromide) ions have a similar effect to Cl^- ions on the spectral sensitivity of LWS pigments. It is suggested that anion sensitivity is ubiquitous to vertebrate LWS cone pigments.

By using site-directed mutagenesis, Wang *et al.* (1993) identified two positively charged amino acids in the chloride binding site of human 'red' and 'green' cone pigments. His-181 and Lys-184 (bovine rod opsin numbering) are generally conserved across all LWS pigments but are absent in SWS and rod pigments. These residues are located in the extracellular loop connecting transmembrane helices IV and V. His-181 is close to both Cys-110, which forms the disulphide bond that is essential for the correct formation of opsin (Karnik *et al.*, 1988) and the Schiff's base counterion which is involved in the determination of spectral sensitivity of an opsin (Sakmar *et al.*, 1989). The 'green' cone pigment of the mouse, *Mus musculus* (Sun *et al.*, 1997) and blind mole rat, *Spalax ehrenbergi* (David-Gray *et al.*, 1998a, 1998b) have been shown to possess a tyrosine rather than histidine residue at site 181. Mutation of human red cone pigment from histidine to tyrosine at this site (i.e. H197Y) produces a 28 nm blue-shift, which confirms the importance of this site for

the tuning of L opsins and partly explains why the λ_{max} of the rodent green cone pigments are blue-shifted (508nm and 528 nm in the mouse and blind mole rat respectively) (Sun *et al.*, 1997; David-Gray *et al.*, 1998).

The shifts in spectral sensitivity conferred by anions are likely to be due to either an indirect malformation of the opsin structure or they may act to stabilise the bond between chromophore and opsin (Yoshizawa *et al.*, 1991). However, the chloride binding site can only account for up 15-25 nm of the bathochromic shift (Wang *et al.*, 1993) and so there must be other residues that are essential for the spectral tuning of LWS opsins. Nathans *et al.* (1986) cloned and made a comparison between human red and green opsin sequences and discovered only 15 differences in amino acids with these two opsins showing a 98% homology. It is likely that at least some of these residues must be involved in determining the spectral difference between the red and green opsins. Indeed a similar study, this time comparing the opsin sequences of New and Old World primates (Neitz *et al.*, 1991), identified three sites (180, 277, 285) where a substitution between a non-polar residue with one bearing an hydroxyl side group produced bathochromic shifts of up to 15 nm. The three residues identified in primate 'green' opsins were also found in bovine rhodopsin, and upon substitution with hydroxyl bearing amino acids bathochromic spectral shifts similar to those for the primate study were observed (Chan *et al.*, 1992). The three polar substitutions found in the 'red' opsins of primates, are highly conserved across species and have been identified in several non-mammalian species (Yokoyama & Yokoyama, 1990; Johnson *et al.*, 1993; Kawamura & Yokoyama, 1993; Yokoyama, 1995) including the chicken (Kuwata *et al.*, 1990; Okano *et al.*, 1992) and canary (Das *et al.*, 1999). Asenjo *et al.* (1994) used both single and multiple point mutations and identified four further amino acids that are involved in spectrally tuning human red and green pigments, these were sites 116, 230, 233 and 309. These findings have been corroborated in several other studies (Winderickx *et al.*, 1992; Deeb *et al.*, 1992; Williams *et al.*, 1992; Merbs & Nathans, 1992, 1993; Kochendoerfer *et al.*, 1997; Yokoyama & Radlwimmer, 1998). Recent studies of the 'red' opsin of the bottle-nosed dolphin, *Tursiops truncatus* (Fasick & Robinson, 1998; Fasick *et al.*, 1998) and the 'green' opsin of mouse, *Mus musculus* (Sun *et al.*, 1997) suggest that site 292 is also an important tuning site. These dolphin and

mouse pigments are both blue-shifted L opsins with λ_{max}s of about 524 nm and 508 nm respectively, and both opsins possess a serine residue at site 292, which is at odds with all other L opsins which have an alanine residue. Site-directed mutagenesis of bovine rod opsin, A292S, resulted in a 10 nm blue-shift. Whilst the blind mole rat green opsin is highly homologous with that found in the mouse (91%), it lacks the S292A substitution and this might partly explain the 20 nm difference in the λ_{max} of these two pigments (David-Gray *et al.*, 1998a, 1998b).

3.7 M_{Rd} & M_C OPSINS

MSP analyses of the rod and MWS pigments of the budgerigar, *Melopsittacus undulatus*, and duck, *Anas platyrhynchos* (**Table 4.3**), reveal both a striking similarity in their λ_{max} values and a high degree of homology amongst the opsin sequences (Heath *et al.*, 1997). However, site 122 (numbered as per bovine rod opsin) has been shown to be different between the two opsin classes. Budgerigar and duck rod opsins possess the charged glutamic acid (E) residue at this position, which is replaced by an uncharged glutamine (Q) residue in the MWS opsins. This difference is significant, since it has been suggested that site 122 is important in determining rate of formation and stabilisation of metarhodopsin II (Weitz & Nathans, 1993). Indeed, the rate of regeneration and metarhodopsin II decay in rod opsins is transformed into that typically seen for cone opsins (which bleach and regenerate faster than rod opsins) when Glu-122 is replaced with Asn-122 (a similar residue to Gln) by site-directed mutagenesis (Imai *et al.*, 1997). Substitutions between charged and uncharged amino acids at site 122 may contribute to the differential kinetics of rod and MWS cone opsins, however, substitution of the glutamic acid residue with asparigine (E122N) produces a 15 to 20 nm hypsochromic shift in human and bovine rod opsin (Sakmar *et al.*, 1989; Nakayama & Khorana, 1990). In avian MWS opsins, therefore, a bathochromic shift in spectral absorbance is required, since these opsins should have maximal sensitivities similar to rod opsins. Positions 222 and 299 are possible tuning sites for this function, since they face the retinal binding pocket and have a serine in the MWS opsins that is not present in the rod opsins of duck, budgerigar, chicken and canary (Okano *et al.*, 1992; Heath *et al.*, 1997; Das *et al.*, 1999). Serine is an hydroxyl-bearing amino acid

and is known to cause bathochromic shifts when added to bovine rod opsin (Chan *et al.*, 1992).

Site 211 contains a His residue in all avian MWS and rod that is replaced by a Cys residue in other opsin pigments; this site is thought to be involved in stabilizing the metarhodopsin II conformation of bovine rod opsin (Weitz & Nathans, 1992).

3.8 S_B & S_V OPSINS

Substitutions from negative polar to non-polar amino acids at sites close to the protonated Schiff's base are thought to destabilise the chromophore and thus reduce the energy differential between ground and excited states, thereby creating a shift to longer wavelengths (Merbs & Nathans, 1993). Instability of the chromophore is also enhanced in L opsins by the presence of polar residues along the whole length of the conjugated chain (Chan *et al.*, 1992). Chang *et al.* (1995) investigated four putative spectral tuning sites that are possibly involved in the tuning of S_B opsins, these are sites 124, 289, 292 and 307 (bovine rod opsin numbering). Sites 289 and 307 are situated in Helix VII in the vicinity of the Schiff's base, and possess non-polar residues in LWS opsins that are replaced by negatively charged polar residues in S_V opsins. Also in close proximity is site 292, which has a non-polar residue in most vertebrates opsins but is a negatively charged polar residue in S_B opsins. In contrast site 124, which is close to the Schiff's base counterion in Helix III, has a non-polar residue in all vertebrate opsins except some of the S_V opsins where it is replaced by a polar residue. It is suggested that in S_B & S_V opsins, hydroxyl-bearing amino acids around the protonated Schiff's base increase the overall stability of the chromophore and therefore increase the energy differential between ground and excited states that produces the blue-shift in the opsin. Other studies have also suggested possible tuning sites involved in blue-shifting the spectral sensitivities of opsins (Nakayama & Khorana, 1991; Yokoyama, 1994, 1995). The λ_{max} of the chicken SWS pigment is around 11 nm red-shifted compared to other avian SWS pigments (Bowmaker & Knowles, 1977; Bowmaker *et al*, 1997). Sites 208 and 269 are sites at which a non-conservative substitution has occurred between the chicken SWS opsin and those for other avian opsins and have been suggested as possible tuning sites within the S_B opsins of birds (Das *et al.*, 1999).

In birds, the λ_{max} of visual pigments within the S_V opsin class can range from around 420 nm, as with the VS opsins of chicken and duck (Bowmaker & Knowles, 1977; Bowmaker *et al.*, 1997; Jane & Bowmaker, 1988), to around 365 nm as observed in the UVS opsins of budgerigar and canary (Wilkie *et al.*, 1998; Das *et al.*, 1999). Lin *et al.* (1998) identified possible spectral tuning sites for VS opsins by site-directed mutagenesis between human rod (λ_{max} – 500 nm) and violet (λ_{max} - 425 nm) opsin sequences. Nine sites were identified in human violet that were predicted to be responsible for around 80% of the required 75 nm hypsochromic shift from human rod.

Wilkie *et al.* (1998) made a comparison of the VS opsin sequence of chicken (Okano *et al.*, 1992) with the UVS opsin of the budgerigar, in order to elucidate the spectral tuning sites for the UVS pigments of birds. Five non-conservative amino acid substitutions were identified as possible tuning sites at positions 86, 93, 118, 119 and 298 (bovine opsin numbering). It was proposed that sites 86, 93 and 118 were the most likely to be involved in spectral tuning since they were located close to the Schiff's base counterion.

3.9 PHYLOGENY OF OPSINS

Comparison of the amino acid identity of chicken opsin sequences with those of other vertebrate visual pigments, provided evidence of the phylogeny of avian opsins (Okano *et al.*, 1992). It was suggested that the four cone opsin groups (L, M_C, S_B and S_V) were all evolved from one ancestral opsin, with the rod opsin class (M_{Rd}) having evolved later as a divergence from the M_C opsin class. This was later corroborated by the opsin sequences from canary, which also fitted into the same classes (Das *et al.*, 1999). The S_V opsin class has been shown to contain both UVS and VS cone opsins. For instance, the chicken VS opsin and budgerigar and canary UVS opsins all cluster together in this group (Das *et al.*, 1999).

AVIAN COLOUR VISION

"Diurnal neognathous birds probably have, at least at the retinal level, one of the most elaborate mechanisms for colour vision within the vertebrates."

J.K. Bowmaker (1996)

4.0 VISUAL PIGMENT EXTRACTION

In scientific research, the solution to any question is restricted by the empirical means available to determine the answer. Study of the visual pigments has been attempted by a variety of means. The first successful method was visual pigment extraction. This involved isolating the cone pigment from the retina into solution by use of detergents. Wald (1937) used digitonin to extract a pigment from chicken retinae and this was the first identified cone visual pigment, which he called iodopsin. Cone pigments are, however, particularly difficult to extract. Indeed, early extractions from avian retinae yielded only one cone visual pigment, similar to Wald's iodopsin (Wald *et al.*, 1955; Crescitelli *et al.*, 1964; Sillman, 1969). Also, extraction only identifies that a pigment is present, it gives no indication in which cell type it is located. The data derived from visual pigment extraction is summarised in **Table 4.0**. Most commonly rod, rather than cone, photoreceptors have been the subjects of extraction studies and only in the chicken have all the cone pigments been extracted. Therefore, extraction studies provide little data on avian colour vision.

4.1 ELECTRORETINOGRAPHY

A method which does give some indication of spectral specificity is performing an e.r.g. (electroretinogram). This provides the mass electrical output from the entire retina in response to specific wavelengths of light. However, such a response does not accurately represent the specific absorbance of a single cone visual pigment, since the e.r.g. is the electrophysiological response of the entire retina, which is made up of both rods and a variety of cone types (Rodieck, 1973; Govardovskii & Zueva, 1977). This technique can be combined with a flickering stimulus that only stimulates the cone visual pigments (flicker photometry) or the use of a criterion response method which uses a white background to saturate the rods whilst bright

TABLE 4.0
Visual pigment spectral sensitivities of birds, as obtained by extraction techniques.

	VS	SWS	MWS	LWS	ROD
Galliformes:					
Chicken (*Gallus gallus*)[1,2,3,4,5,6]	417-425	449-455	508	560-571	500-504
Domestic turkey (*Melagris gallopovo*)[7]	-	-	-	562	504
California quail (*Lophortyx californicus*)[8]	-	-	-	-	500
Bobwhite quail (*Colinus virginianus*)[8]	-	-	-	-	500
Anseriformes:					
Mallard duck (*Anas platyrhynchos*)[9]	-	-	-	-	502
Columbiformes:					
Pigeon (*Columba livia*)[8,9]	-	-	-	544	502
Mourning dove (*Zenaida macroura*)[8]	-	-	-	-	502
Ring dove (*Streptopelia risoria*)[8]	-	-	-	-	502
Passeriformes:					
Bengalese finch (*Lonchura striata*)[8]	-	-	-	-	502
Brown-headed cowbird (*Molothrus ater*)[8]	-	-	-	-	501
House finch (*Carpodacus mexicanus*)[8]	-	-	-	-	502
Brown towhee (*Pipilo fuscus*)[8]	-	-	-	-	500
Wh-c* sparrow (*Zonotrichia leucophrys*)[8]	-	-	-	-	502
Zebra finch (*Taeniopygia guttata*)[8]	-	-	-	-	502
Wh-b* sparrow-weaver (*Plocea mahali*)[8]	-	-	-	-	504
Bl-r* waxbill (*Estrilda troglodytes*)[8]	-	-	-	-	502
Common silverbill (*Lonchura malabaric*)[8]	-	-	-	-	502
Chestnut mannikin (*Lonchura malacca*)[8]	-	-	-	-	503
Psittaciformes:					
Budgerigar (*Melopsittacus undulatus*)[8]	-	-	-	-	505
Pelicaniformes:					
Brown pelican (Pelecanus occidentalis)[10]	-	-	-	-	502

(Continued on next page)

*Abbreviations used:
Wh-c → White-crowned
Wh-b → White-browed
Bl-r → Black-rumped

(Continued from previous page)

	VS	SWS	MWS	LWS	ROD
Strigiformes:					
Burrowing owl (*Speotyto aluco*)[8]	-	-	-	-	503
Screech Owl (*Otus asio*)[10]	-	-	-	-	505
Great horned owl (*Bubo virginianus*)[10]	-	-	-	-	502
Ciconiiformes:					
Roadside hawk (*Buteo magnirostris*)[8]	-	-	-	-	500
Western gull (*Larus occidentalis*)[10]	-	-	-	-	503
Caprimulgiformes:					
Poorwill (*Phaeleonoptilus nuttallii*)[8]	-	-	-	-	506

References:

1 – Bliss (1946)
2 – Wald (1937)
3 – Wald *et al.*, (1955)
4 – Fager & Fager (1982)
5 – Fager & Fager (1981)
6 – Okano *et al.*, (1989)
7 – Crescitelli *et al.*, (1964)
8 – Sillman (1969)
9 – Bridges (1962)
10 – Crescitelli (1958)

flashes of light stimulate the cones (Chen & Goldsmith, 1986). However, interactions between the photoreceptor classes cannot be ruled out and could markedly effect the effective absorption of each visual pigment type.

4.2 PIGEONS AND PENTACHROMACY

The avian visual system most studied using electroretinography is that of the pigeon, *Columba livia*. An early study characterised three peaks in the spectral sensitivity of the cones from this species, with maximal sensitivities measured by flicker photometry at 502 and 547 nm, and a further 605 nm peak elucidated by selective adaptation with a 547 nm background light (Ikeda, 1965). The 502 nm peak is likely to correspond to the pigeon MWS cone, as MSP has revealed the λ_{max} of this pigment to be around 507 nm (Bowmaker, 1977; Bowmaker *et al.*, 1997). No pigment with λ_{max} around 547 nm has been measured by MSP, however an earlier extraction study elucidated a P544 pigment (Bridges, 1962). The 605 nm peak is likely to represent a LWS cone pigment (λ_{max} 568 nm by MSP) that has been filtered by an R-type oil droplet.

Studies of the red and yellow sectors of the pigeon retina revealed differing spectral sensitivities for these two areas (Martin & Muntz, 1978; Wortel *et al.*, 1984). The peak sensitivity of these two fields was almost identical, however the photopic sensitivity of the red sector was much lower than that for the yellow sector. It has been shown that P-type droplets, contained within the principal member of double cones which are the most numerous cone type in pigeon retinae (Waelchli, 1883), have a λ_{cut} at longer wavelengths in the red sector than those found in the yellow sector (Bowmaker, 1977). This suggests that the double cones play a major role in determining the spectral sensitivity of avian retinae. The λ_{cut} of R- and Y-type droplets is also longer in the red sector (Bowmaker, 1977) and this must invariably create differences in the ability to discriminate hue between the two sectors (Martin & Muntz, 1978).

Criterion response retinography, involving the use of selective chromatic adapatation with long wavelength light, revealed the presence of two shorter wavelength pigments in the pigeon and also the chicken, *Gallus gallus* and jackdaw, *Corvus*

monedula (Graf & Norren, 1974; Norren, 1975). One of these pigments was maximally sensitive at 480 nm and probably represents a SWS cone pigment that is filtered by an associated pigmented C-type oil droplet. The other pigment (around 400 nm λ_{max}) is likely to be a VS cone pigment that is associated with a transparent, T-type droplet.

MSP evidence suggests that the pigeon has four distinct single cones classes with maximal absorbances of 409 nm (VS), 453 nm (SWS), 507 nm (MWS) and 568 nm (LWS) (Bowmaker, 1977; Bowmaker *et al.*, 1997) and the above studies corroborate these findings. Furthermore, MSP studies indicate that the fourth single cone class of birds is either VS or UVS (see **Table 4.3**). Again, this is corroborated by e.r.g. studies, for instance, the passerines have a peak in their photopic sensitivity at around 370 nm which represents an UV pigment coupled with a transparent, T-type droplet, and there is no evidence of a sensitivity peak for a VS cone class (Chen & Goldsmith, 1986). However, a disparity exists in other studies over the spectral position of the fourth single cone class in the pigeon retina since some e.r.g. measurements have indicated a sensitivity peak which is maximal at around 370 nm (Chen *et al.*, 1984; Wortel *et al.*, 1984; Chen & Goldsmith, 1986; Vos Hzn *et al.*, 1994). That both an UVS and VS cone mechanism has been observed (Vos Hzn *et al.*, 1994) raises the possibility of a fifth single cone class in the pigeon and that this species might possess pentachromatic colour vision (Vos Hzn *et al.*, 1994). This is supported by behavioural data that suggests that pigeons have a very high sensitivity between 325 and 360 nm (Kreithen & Eisner, 1978). The 370 nm peak in e.r.g. measurements of photopic sensitivity of the pigeon however, may not represent an UVS cone class. The β-band of a long-wave-sensitive visual pigment, of which avian retinae have an abundance, would be close to the observed 370 nm peak. Furthermore, in fish this β-band has been shown to absorb light and can undergo photoisomerisation with a high quantal efficency (Palacios *et al.*, 1996). Yokoyama *et al.* (1998) transfected vector cells with the pigeon VS opsin gene and then purified and regenerated the expression products. Spectrophotometry of these regenerated products gave a λ_{max} of 393 nm, which does not agree with any of the other studies. It is the author's opinion that MSP evidence is probably the most accurate (see section 4.4) and that the pigeon does not possess an UVS fifth cone class.

4.3 MICROSPECTROPHOTOMETRY (MSP)

To measure with accuracy the absorbance spectra of individual cone visual pigments, the best technique is now widely considered to be microspectrophotometry (MSP). A minute beam of light is passed transversely through the outer segment of the photoreceptor and the transmittance, and hence the absorbance, of the visual pigment within the cell is calculated for each specific wavelength in the spectrum. Thus MSP provides accurate measurements of the absorbance spectrum of each individual measured photoreceptor. It also has an added bonus, in that a specific cell-type can be correlated to a specific visual pigment. Despite the fact that MSP has been utilized for over thirty years, only a dozen or so avian species have had their visual pigments identified by this means, but the data collected represents by far the most accurate indication of the visual pigments present in these species (see **Table 4.3**).

4.4 DUAL -BEAM MICROSPECTROPHOTOMETER

MSP was originally developed as a technique for the measurement of the absorption of nucleic acids (Caspersson, 1940; as cited by Wolken *et al.*, 1968) and it was not until much later, that Hanaoka & Fujimoto (1957) applied this technique to visual pigments. Their study of the photoreceptors in the retinae of the carp, *Cyprinus carpio*, successfully characterised five pigment types, and showed that with MSP the spectral absorption of visual pigments could be measured *in situ* in individual photoreceptors. This represented a fundamental advantage over other techniques used to study visual pigments.

In making MSP measurements, two beams of light are employed; a sample beam (that passes through the photoreceptor outer segment or oil droplet being measured) and a reference beam (that is passed through an area of the slide that is devoid of tissue). The initial measurements of visual pigments using MSP, were made by instruments that produced separate sample and reference beams (Hanaoka & Fujimoto, 1957). Chance *et al.* (1959) made the first MSP measurements using a dual-beam microspectrophotometer, in which the beams were produced by a single monochromator but were separated spatially by 12 μm. This design was optimised for measuring visual pigments in photoreceptors by 'chopping' a single beam of

TABLE 4.3

	UVS	VS	SWS	MWS	LWS	ROD
Galliformes:						
Chicken *(Gallus gallus)*[1,2]	-	418	455	507	569	509
Japanese quail *(Coturnix japonica)*[3]	-	419	456	505	569	505
Turkey *(Meleagris gallopavo)*[4]	-	420	460	505	564	504
Peacock *(Pavo cristatus)*[5]	-	421	457	505	566	504
Anseriformes:						
Mallard duck *(Anas platyrhynchos)*[6]	-	420	452	502	570	505
Columbiformes:						
Pigeon *(Columba livia)*[2,7]	-	409	453	507	568	506
Passeriformes:						
Zebra Finch *(Taeniopygia guttata)*[2]	360-380	-	430	505	567	507
Pekin Robin *(Leiothrix lutea)*[8]	355	-	453	501	567	500
Canary *(Serinus canaria)*[9]	366	-	442	505	569	506
European Starling *(Sturnus vulgaris)*[10]	362	-	449	504	563	503
Blackbird *(Turdus merula)*[5]	373	-	454	504	557	504
Blue tit *(Parus caeruleus)*[5]	374	-	449	503	563	503
Psittaciformes:						
Budgerigar *(Melopsittacus undulatus)*[2]	371	-	444	508	564	509
Ciconiiformes:						
Penguin (*Spheniscus humboldti*)[11]	-	403	450	-	543	504
Manx shearwater *(Puffinus puffinus)*[2]	-	402	452	-	-	505
Strigiformes:						
Tawny Owl *(Strix aluco)*[12]	-	-	463	503	555	503

λ_{max} of visual pigments identified by microspectrophotometry (MSP) in neognathous birds. Only those species for which the majority of suspected visual pigments have been characterised are included:

1 - (Bowmaker & Knowles, 1977); 2 – (Bowmaker *et al.*, 1997); 3 - (Bowmaker *et al.*, 1993); 4 - (Hart *et al.*, 1999); 5 – (Hart, 1998); 6 - (Jane & Bowmaker, 1988); 7 - (Bowmaker, 1977); 8 - (Maier & Bowmaker, 1993); 9 - (Das *et al.*, 1999); 10 – (Hart *et al.*, 1998); 11 - (Bowmaker & Martin, 1985); 12 (Bowmaker & Martin, 1978).

monochromatic light so that it sequentially produced the sample and reference beams, and then passing these beams through a condenser objective lens in order to match the dimensions of the beams with those of the outer segments of the photoreceptors (Liebman, 1962; Liebman & Entine, 1964; Liebman & Entine, 1968).

During a scan, a monochromator scans through the spectrum and a photomultiplier sequentially measures the relative intensities of the two beams at each wavelength. The ratio of photocurrent measured gives a transmission value at each wavelength, from which the absorption can be calculated. Sample and reference beams are not identical as they originate from different points on the lamp filament or arc, traverse different parts of the optics and terminate at different locations on the photocathode. Feedback is important, since it ensures that throughout a scan the reference beam is held constant and therefore that any absorbance measured by the sample beam is not just an artefact of fluctuations in the output of the light source. The reference beam serves as a negative feedback loop, since its signal is used to control the high tension voltage of the photomultiplier. Separate baseline recordings are made, in which both beams are passed through an area devoid of tissue, and these measurements are subtracted from the sample scan.

Each photoreceptor outer segment is relatively small and so contains a low visual pigment concentration. This necessitates the use of high light exposure during MSP measurements in order to reduce relative noise fluctuations and so obtain a good spectral resolution from these weakly-absorbing cells (Liebman, 1972). Unfortunately, the more light the measured photoreceptor is exposed to, the more likely it is to undergo substantial bleaching that may significantly distort the resulting absorbance spectrum. The intensity of a measuring beam during a scan therefore, must be high enough to produce a high signal to noise ratio but low enough to prevent destruction of the visual pigment. Measurements of visual pigments extracted from many photoreceptors are not subject to such problems since the concentration of visual pigment molecules is high enough to prevent the effects of bleaching significantly altering the absorbance spectrum (Liebman & Entine, 1964).

4.5 TYPICAL AVIAN PHOTORECEPTOR COMPLEMENT

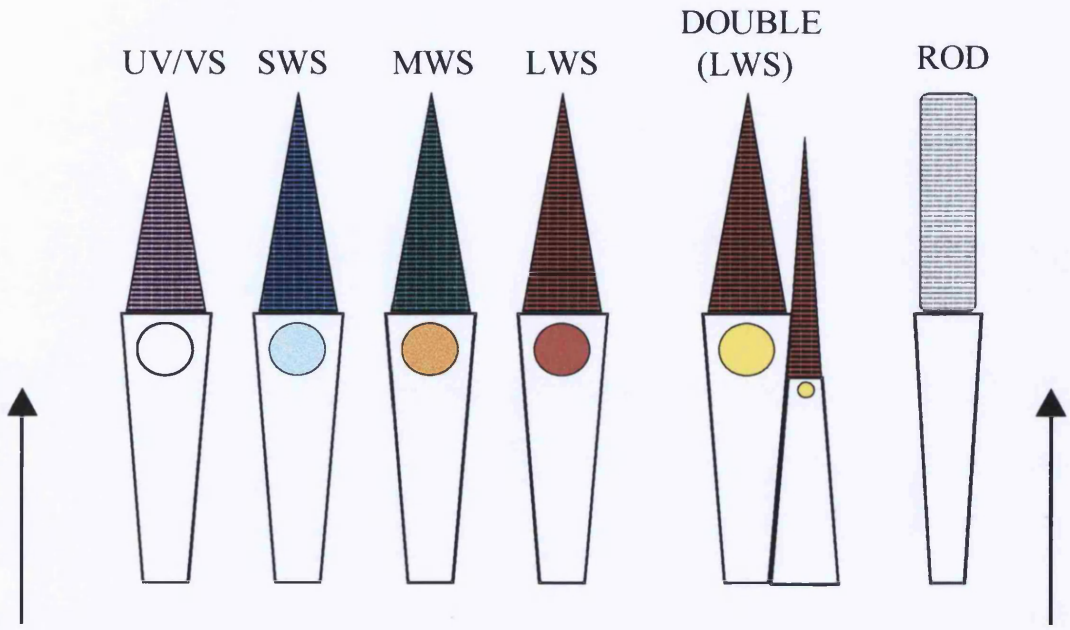


FIGURE 4.5
Schematic diagram of a typical avian photoreceptor complement. This arrangement within avian retinae has been observed by microspectrophotometric studies (references in **Table 4.2**) for most neognathous avian species. The arrows indicate the direction of incident light through the photoreceptors. The colour key to the oil droplets is: white = T-type droplets; pale blue = C-type droplets; orange = Y-type droplets; large yellow = P-type droplets; small yellow = A-type droplets. Partridge (1989) and Bowmaker (1991) offer good reviews of avian oil droplets. (Diagram originated by J.K.Bowmaker).

Microspectrophotometric analyses of avian retinae have revealed a typical complement of photoreceptor cells, as shown in **Figure 4.5** (for the purposes of this study, this suggested template will be known as the 'Bowmaker complement'). Most birds possess rods, four classes of single cone and one class of double cone. How closely each group within Class *Aves* fits the Bowmaker complement is discussed in the following sections. In general, most diurnal birds have cone-dominated retinae (Maier & Bowmaker, 1993) which comprise at least four distinct spectral classes of cone, suggesting that these birds have the potential for tetrachromatic colour vision (Chen & Goldsmith, 1986; Bowmaker, 1991;

Bowmaker *et al.*, 1997). The spectral sensitivity of the rod pigments are highly conserved across all avian species, with λ_{max} ranging from 500-509 nm (**Table 4.3**).

4.6 GALLIFORMES & ANSERIFORMES

MSP studies of several species selected from both of these groups confirms their similarity to the Bowmaker complement (Bowmaker & Knowles, 1977; Bowmaker *et al.*, 1997; Bowmaker *et al.*, 1993; Hart *et al.*, 1999; Hart, 1998; Jane & Bowmaker, 1988). The maximal sensitivities of the four single cone classes of these species are about 568 nm (LWS), 505 nm (MWS), 456 nm (SWS) and 420 nm (VS) and within each cone class is the typical accompanying oil droplet. The spectral sensitivities of the LWS, MWS and SWS pigments are similar to those observed in most diurnal birds. In each species the MWS pigment usually has a sensitivity similar to that characterised for rod pigments. The main distinguishing difference between avian retinae is in the spectral location of the fourth single cone class. Only in Anseriformes and Galliformes, does the λ_{max} of this pigment cluster around 420 nm. It is interesting that the visual systems of jungle fowl (Galliformes) and water fowl (Anseriformes) are highly similar, despite their disparate environments and lifestyles. Maybe this similarity is a reflection of the close phylogenetic relationship between these groups (reviews of avian phylogenies in Sibley & Ahlquist, 1990; Mindell *et al*, 1998).

4.7 COLUMBIFORMES

The photoreceptor complement of the pigeon has distinct differences from that observed in the Anseriformes and Galliformes. Firstly, the spectral location of the fourth violet- or ultraviolet-sensitive cone class in the pigeon, as measured by MSP, is around 409 nm (Bowmaker, 1977; Bowmaker *et al.*, 1997). As discussed earlier, there is a possibility that the pigeon may also possess an UVS cone class, see section 4.3. The other main difference is the lack of uniformity across the retina in the λ_{cut} of the R- and Y-type oil droplets. These droplet types have a cut-off at longer wavelengths if found in the dorso-temporal region (named 'red sector' by Muntz, 1972, due to preponderance of LWS cone types), than those located in the rest of the retina (yellow sector) (Bowmaker, 1977).

4.8 PASSERIFORMES & PSITTACIFORMES

As discussed above, the spectral sensitivities of the cone classes are similar in most avian species with the main difference occurring in the fourth cone class. For the purposes of this study, the Passeriformes and Psittaciformes have been grouped together since they all possess an UVS pigment with λ_{max} between 355nm and 374 nm (Table 4.2). In recent studies, specific behavioural functions have been attributed to this UV sensitivity (see section 4.15).

MSP measurements of the C-type droplets of Passeriformes and Psitacciformes reveal that they have a relatively low absorbance, with densities of around 0.1, for example, observed in the Pekin robin (*Leiothrix lutea)*, zebra finch (taeniopygia guttata), budgerigar (*Melopsittacus undulatus*), European starling (*Sturnus vulgaris*) and canary (*Serinus canaria*) (Maier & Bowmaker, 1993; Bowmaker *et a*l, 1997; Hart *et al.*, 1998; Das *et al.*, 1999). With such low absorbances, this droplet type is unlikely to have a significant filtering effect on the associated SWS visual pigment and at most produces a slight narrowing of the shortwave limb. This is in contrast to other avian groups, Galliformes and Anseriformes for instance, in which the effective maximal absorbance of the SWS cone type is shifted to longer wavelengths by the associated pigmented C-type oil droplet (Bowmaker & Knowles, 1977; Bowmaker *et al.*, 1997; Bowmaker *et al.*, 1993; Hart *et al.*, 1999; Hart, 1998; Jane & Bowmaker, 1988). C-type droplets that have a substantial filtering effect are only found in those bird species that possess a VS rather UVS fourth cone class. The spectral distance between the SWS cone class and the VS cones is effectively increased by the pigmented C-type droplets. However, a relatively large spectral 'gap' already exists between the absorbances of the UVS and SWS cones from the Passeriformes and Psittaciformes (**Table 4.3**) and so shifting the absorbance of the SWS cone class to longer wavelengths by filtering with a pigmented C-type droplet is unnecessary.

In an MSP study of the canary, some birds were identified that did not possess the typical Bowmaker complement since the LWS cones did not contain an R-type oil droplet (Das *et al.*, 1999). Instead, in 80% of those birds measured, the LWS pigment found in single cones was paired with an oil droplet spectrally similar to a

P-type droplet (a droplet type usually only found in the principal members of double cones). However, it was suggested that these P-type-like droplets were actually R-type droplets that lacked the astaxanthin carotenoid which is responsible for shifting the λ_{cut} of R-type droplets to longer wavelengths (Wald & Zussman, 1937; Strother & Wolken, 1960; Meyer *et al.*, 1965). The loss of astaxanthin in the R-type droplets is probably due to a genetic mutation, the occurrence of which is made more likely by inbreeding and notably the canaries used in the MSP study were all in-bred as a result of domestication (Das *et al.*, 1999).

Budgerigars are the only species among the psittaciformes to have their retinal photoreceptors characterised by MSP (Bowmaker *et a*l, 1997), and it appears that this species has noteworthy differences from the passeriformes. Firstly, two different types of Y-type droplet have been identified, with one having a cut-off at shorter wavelengths to the other, although no correlation has been made to retinal distribution as in the red and yellow sectors of the pigeon retina (Muntz, 1972; Bowmaker, 1977). Secondly, compared to the passeriformes, the rod photoreceptors had of budgerigars were found to have larger outer segments and be more abundant (Bowmaker *et al.*, 1997). This is probably due to their lifestyle, which is slightly more crepuscular.

4.9 CICONIIFORMES

Members of this group of birds dive into a sub-aquatic environment in order to acquire their food. Surface waters filter lower energy, longer wavelengths of light more than light of shorter wavelengths (Tyler & Smith, 1970; Lythgoe, 1979), therefore the spectral composition of light in a sub-aquatic environment is radically different from that above the surface with relatively little light above wavelengths of 575 nm. It is not surprising that the spectral sensitivity of the ciconiiformes has adapted to take into account this unique environment. MSP studies of the penguin (*Spheniscus humboldti*) reveals that the LWS cone class possesses a visual pigment that is shifted to shorter wavelengths compared to most avian species, with a λ_{max} of 543 nm (Bowmaker & Martin, 1985).

The arrangement of retinal photoreceptors in the penguin substantially differs from the Bowmaker complement, since no R-type droplets have been identified and there is no evidence at all of a distinct MWS cone class. A Y-type droplet is found associated with the P543 visual pigment and the other single cone pigments, P403 and P450, are paired with very pale or transparent oil droplets (Bowmaker & Martin, 1985). It seems that the penguin visual complement is adapted to utilizing as much sub-aquatic light as possible. The spectral sensitivity of the fourth single cone class of the penguin, P403, is similar to that identified in another ciconiiforme, the Manx shearwater (*Puffinus puffinus*) which has a P402 pigment (Bowmaker *et al.*, 1997). This suggests that this avian group possesses a VS cone class that is spectrally clustered close to the VS cones of the columbiformes with λ_{max} around 409nm (Bowmaker, 1977; Bowmaker *et al.*, 1997).

4.10 STRIGIFORMES

Overall, the visual ability of diurnal birds has evolved to maximize, wherever possible, the sensory input from the visual environment. The requirements of the visual system of a nocturnal avian species are somewhat different, with the ability to detect low intensity light being paramount. MSP measurements of the visual pigments and oil droplets of the tawny owl, *Strix aluco* (Bowmaker & Martin, 1978), reveal a retinal complement that is adapted for nocturnal vision with features including:

- 90% of the photoreceptor population are rods
- LWS visual pigment is 10-15 nm shorter in wavelength than found in most diurnal birds
- Apart from Y-type droplets, all the other retinal oil droplets have a reduced carotenoid concentration and do not act as cut-off filters

Another feature of Strigiforme retinae that makes them different from the Bowmaker complement is that there is no evidence as yet for a fourth cone class.

4.11 PALEOGNATHOUS PHOTORECEPTOR COMPLEMENT

There has been only one study utilizing MSP to investigate visual pigments in paleognathous birds (Sillman *et al.*, 1981). The visual pigments and oil droplets in

the retinae of the emu (*Dromiceius novae-hollandiae*), the Brushland tinamou (*Nothoprocta c. cinerascens*) and the Chilean tinamou (*Nothoprocta perdicaria sanborni*) were characterized. All three of these birds were shown to have a typical rhodopsin-like pigment in their rods with approx. 500nm λ_{max}. In the cones, there was only definite evidence for one visual pigment. This was found to be approx. 567nm λ_{max} (emu), 564nm λ_{max} (Brushland tinamou) or 566nm λ_{max}, (Chilean tinamou). Thus indicating a typical iodopsin-like pigment.

This does not imply, however, that paleognathous birds have only one cone visual pigment in their entire retina. Due to the random selection of cones for MSP measurements, less abundant cones, smaller cones or those confined to highly localized regions of the retina could easily be overlooked and thus not included in the data. Also, there were high levels of noise at short wavelengths in this study, which could have simply masked the presence of any shorter wavelength cones. In addition, the majority of neognathous birds have been shown to have four spectrally distinct cone visual pigments with associated oil droplets. It is likely therefore, that Sillman's results do not represent the complete complement of photoreceptors for the paleognathous birds.

An histological study of the retinae of the emu, *Dromiceius novae-hollandiae*, has identified rods, single cones and double cones (Braekevelt, 1998b). The single cones and accessory members of the double cones showed no evidence of oil droplets and instead possessed a large ellipsoid with several small lipid droplets. In fact, the principal member of the double cone contained the only observable oil droplet in the emu retina. However, preparation of the retinal tissue for histology must have destroyed some of the retinal oil droplet types since more than one type has been identified by MSP (Sillman *et al.*, 1981). Sillman's analysis of the oil droplets in the retinae of the emu and both species of tinamou revealed evidence of only two types. With λ_{T50} 568 and 508, these are likely to be R-type and Y-type droplets respectively. Sillman *et al.*, (1981) also suggested that there was a class of single cones that lacked oil droplets, a cone type not found in neognathous birds. This suggests that the retinal oil droplet complement of paleognathous birds may be far from typical of those found in most neognathous birds.

4.12 BEHAVIOURAL STUDIES

MSP studies provide evidence of the photoreceptor complement present in the retina of a given species and the data obtained suggest the visual ability of that animal. However, simply because an animal is capable of detecting certain wavelengths of light, does not provide evidence that it is behaviourally sensitive to those wavelengths. Behavioural studies assess visual capability by performing psychophysical and psychometric evaluations. With regard to avian colour vision, some recent research has focused on the ability of a variety of avian species to detect ultraviolet wavelengths and the possible functions for this sensitivity (section 4.14). The pigeon has been the most popular experimental model for behavioural studies, and a number of different methods have been employed to gauge its visual sensitivity. One study used a cardiac conditioning paradigm in which pigeons were conditioned to expect an electric shock following the presentation of a monochromatic stimulus, the intensity of stimulus being varied in order to obtain a sensitivity function (Kreithen & Eisner, 1978). Other studies utilized increment threshold operant techniques (Graf, 1969; Romeskie & Yager, 1976; Martin & Muntz, 1978), but Graf (1979) provided the most comprehensive study by expanding the former studies to include selective chromatic adaptation with background illuminants. Graf predicted that the pigeon possesses four cone mechanisms, with maximal sensitivities of about 400, 480, 560 and 615 nm. In further studies, pigeons were trained to distinguish between spectral lights and additive mixtures and the results indicated that colour vision in the pigeon may actively involve five different cone mechanisms (Palacios *et al.*, 1990; Palacios & Varela, 1992). For a review of pentachromacy in pigeons see section 4.2.

Other successful studies using operant techniques were on the Pekin robin, *Leiothrix lutea* (Maier, 1992, 1994). Four peaks of maximal sensitivity were identified at around 370, 460, 530 and 620 nm and these were later confirmed by the measurement of the photoreceptor complement of this species by MSP which revealed four single cone classes (Maier & Bowmaker, 1993). It is important that the results of behavioural studies are corroborated by other means, since they are inherently subject to the conditions of the test and are also effected by both retinal and higher visual processing.

4.13 ULTRAVIOLET SENSITIVITY IN BIRDS

Many bird species, like many other vertebrates (for review see Tovee, 1995), possess a cone mechanism that is maximally sensitive at ultraviolet wavelengths (for examples see Table 4.2). UV sensitivity was first observed in the humming bird, *Colibri serrirostris* (Huth & Burkhardt, 1972) and the pigeon, *Columba livia* (Wright, 1972), and now has been established for over thirty avian species (Bennett & Cuthill, 1994). The Pekin robin, *Leiothrix lutea*, has been shown to possess sensitivity that extends from 320 nm through to 680 nm with sensitivity being highest in the UV (Maier, 1992). This species possesses four distinct peaks of spectral sensitivity at around 620, 530, 460 and 370 nm. However, it could be suggested that the UV cone mechanism is not part of a colour discrimination channel and is therefore not involved in the colour vision system. Maier's study provides evidence to the contrary, as the narrower shapes of the actual UV and SWS cone sensitivity peaks as compared to the effective single cone sensitivities suggest the influence of neural interactions in shaping the overall spectral sensitivity function. Indeed, the 'tips ' and 'dips' typical of behavioural measurements of spectral sensitivity (Neumeyer, 1984; Neumeyer & Jäger, 1985) are clearly evident in the study of the Pekin robin. The 'tips' or 'peaks' are thought to represent the underlying cone mechanisms, whereas the 'dips' or 'troughs' may be interpreted as the anatagonistic processes between different receptor types (Neumeyer, 1984; Nuboer, 1986). This indicates that the UV cone mechanism is part of a colour opponent system and that these birds are tetrachromatic.

4.14 SPECIFIC FUNCTIONS FOR UV SENSITIVITY ?

Since man lacks ultraviolet sensitivity, its discovery in birds has been looked on with wonder and recently data has been produced that proposes a variety of possible functions for the UVS cone mechanism. Specific behavioural functions that have been attributed to UV sensitivity, include the regulation of circadian rhythms, orientation/navigation, foraging and inter- and intra-specific communication such as sexual displays (Bennett & Cuthill, 1994; Maier, 1994; Burkhardt, 1989; Burkhardt & Finger, 1991; Bennett *et al.*., 1996; Bennett *et al.*., 1997; Burkhardt, 1982; Cooper *et al.*., 1986). Some of these theories are reviewed briefly below.

Light passing through the atmosphere is scattered and plane polarized by small molecules (e.g. O_2 or N_2 molecules and dust particles) that it encounters, with the degree of scattering and polarization proportional to the inverse of the fourth power of the wavelength of light – known as the Rayleigh effect (Born & Wolf, 1970; Lythgoe, 1979). As a consequence, UV light is scattered to a greater degree than light of longer wavelengths. Some birds use the sun as a 'compass', by orientating themselves against the position of the sun in the sky (Schmidt-Koenig, 1979, 1990). The position of the sun could be determined even when only part of the sky can be observed, if the concentric rings (e-vectors) of polarized light created by light scattering could be detected (Brines & Gould, 1982; Waterman, 1984; Wehner, 1989). Some insects have been shown to possess UV receptors which are sensitive to e-vectors of polarized light (von Frisch, 1967; Wehner, 1989), and there have been suggestions that birds use sensitivity to polarized light in a similar way (Kreithen & Eisner, 1978; Burkhardt, 1982, 1989; Martin, 1991; Jacobs, 1992; Able & Able, 1993). The position of the sun could also be determined by detection of intensity and wavelength gradients across the sky (Nuboer, 1986; Coemans & Vos Hzn, 1992; Coemans *et al.*, 1994, 1995), with UV wavelengths possibly being most useful for this function as they produce a more even distribution of intensities across the sky than longer wavelength light.

It has been suggested that some birds can detect UV reflectance from food items such as seeds (Bennet & Cuthill, 1984), berries (Burkhardt, 1982; Cooper *et al.*, 1986) and some insects (Kevan, 1978, 1983; Silberglied, 1979). Also, some flowers possess UV reflectant markings that may guide avian pollinators (Goldsmith, 1980) and it has been suggested that reflectance from the urine and faeces of small mammals may aid hunting birds in tracking down their prey (Vittala *et al.*, 1995).

Recent interest in UV sensitivity has focused on UV reflectant patches on avian plumage and their possible function in intra- and interspecies communication. Evidence for UV-reflecting plumage has been ascertained for a number of avian species, particularly passerines (Burkhardt, 1982, 1989; Burkhardt & Finger, 1991; Hunt *et al.*, 1998; Andersson *et al.*, 1998). UV light has been shown to be used in mate choice in the Pekin robin, *Leiothrix lutea* (Maier, 1994), zebra finch,

Taeniopygia guttata (Bennett *et al.*, 1996) and blue tit, Parus caeruleus (Hunt *et al.*, 1998). However, it may be spurious to isolate the UV wavelengths of the spectrum when considering plumage reflectance governing mate choice. Most diurnal avian visual systems have evolved to detect a wide visible spectrum comprising four cone mechanisms in a tetrachromatic colour system, it is likely that birds utilise the entirety of this system when viewing the world. Therefore, patches on a birds' plumage that reflect long-, middle- and short-wavelengths of light should be as important to a prospective mate as UV-reflecting patches.

4.15 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) has been utilized to highlight the presence of specific opsins for the visual pigments because it gives information on the actual distribution of the cone types across the retina (Szél *et al.*, 1986: Cserháti *et al.*, 1989; Oishi *et al.*, 1990). However, there are not, as yet, specific antibodies for all the pigments and thus subtraction of data from one antibody from another is required to achieve some of these results. Via this method correlation of these opsins, and hence visual pigments, with the associated oil droplet is not therefore without some difficulty. IHC studies have provided some useful data on the distribution and ratio of various photoreceptor types.

4.16 HISTOLOGICAL STUDIES

Although oil droplets are easily measured by MSP and each specific droplet type can be correlated to the visual pigment of the cone they are contained within, this does not provide data on their distribution and ratio in the retina. This information is provided by histology. The whole retina can be placed on a microscope slide and the R-type and Y-type droplets easily identified via light microscopy. Identification of the other droplet types, however, requires the use of various coloured filters and flourescent microscopy (Ohtsuka, 1984). The ratio of oil droplet types has been elucidated for a number of neognathous avian species (**Table 4.16**). In these species, P-type droplets (double cones) comprise the largest percentage of oil droplet types, with R- and Y-type droplets (LWS & MWS cone classes) present in roughly equal numbers. The main difference between species is in the C- and T-type droplets (SWS & UV/VS cones), in that the chicken and duck possess a much smaller

percentage of these droplet types than is seen in the other birds. An histological study of the emu retina has revealed the ratio of rods, single cones and double cones to be 10:1:4 respectively (Braekevelt, 1998b), suggesting that the ratio of photoreceptor types in paleognathous birds may be markedly different from that observed for neognathous birds.

TABLE 4.16

Relative percentage of oil droplet types.

	P-type	R-type	Y-type	C-type	T-type
Chicken[1] *Gallus gallus*	50-60%	15-20%	15-20%	~10%	
Duck[2] *Anas platyrhynchos*	52.0%	22.0%	21.0%	~5%	
Budgerigar[3] *Melopsittacus undulatus*	40.0%	20.0%	21.0%	10.0%	9.0%
European Starling[4] *Sturnus vulgaris*	52-57%	15-20%	17-18%	4-10%	3-7%

1 - Bowmaker & Knowles, 1977; 2 - Jane & Bowmaker, 1988; 3 - Wilkie *et al.*, 1998 4 - Hart *et al.*, 1998.

4.17 AIMS OF STUDY

This present study aims to clarify and expand our knowledge of the visual pigments and oil droplets of paleognathous birds by studying the retinae of the ostrich (*Struthio camelus*) and rhea (*Rhea americana*). The main technique used will be microspectrophotometry (MSP), from which measurements can be made of both the visual pigments and oil droplets within the outer and inner segments of isolated intact photoreceptors. In addition, light microscope sections of the retinae will be prepared to elucidate the relative numbers of oil droplets and their distribution across the retina. The data collected should provide some indication of the visual ability of paleognathous birds. Also, by comparison with the photoreceptor complements of neognathous birds, it should be possible to elucidate whether paleognathous visual systems are similar or distinct from those of other avian families.

This study also aims to sequence all of the ostrich and rhea opsin genes, in particular the seven helical regions. The genes coding for UV/VS opsins are of particular interest. Comparison of these sequences with those from other avian species (and other vertebrate classes) may help to clarify which amino acids within the opsins are responsible for spectral tuning in this spectral region. Further, since these opsins are the least conserved of the visual pigment opsins, they may provide valuable data on the evolutionary position of the visual systems of paleognathous birds with respect to other species.

METHODS AND MATERIALS

MICROSPECTROPHOTOMETRY & HISTOLOGY

5.0 SUBJECTS

Ostrich (*Struthio camelus*) and rhea (*Rhea americana*) chicks, aged between two and fourteen days, were obtained from commercial farms in England and Wales, where the birds are reared for their meat and, in the case of ostriches, also for oil, feathers and leather. The chicks were housed in environmentally controlled conditions, with a 12-hr light/dark ratio and a standard diet. Individuals were dark adapted for at least one hour prior to being sacrificed. The sacrifice and all following procedures were carried out under dim red illumination (Kodak Safelight No. 2). Both eyes were dissected out, with one being wrapped in foil and stored in a light-tight box at 4°C until the following day. The other eye was hemisected, and the anterior half and vitreous humour discarded. The resulting eyecup was placed in a solution of chilled calcium-free saline solution (154 mM NaCl; 5mM KCl; 17.5mM Na_2HPO_4; 7.5 mM NaH_2PO_4; 10 mM glucose; pH 7.1). A small piece of retinal tissue (approx. $1mm^2$) was taken and prepared for microspectrophotometric examination as described previously (Mollon *et al.*, 1984).

5.1 MICROSPECTROPHOTOMETRIC MEASUREMENTS

Measurements from individual photoreceptors were made using a modified Liebman dual-beam microspectrophotometer under computer control (Liebman & Entine, 1964; Mollon *et al.*, 1984; Bowmaker *et al.*, 1991). By using an infra-red converter, the measuring beam (approx. $2\mu m^2$ cross-section) was aligned to pass transversely through a given structure, either an outer segment or an oil droplet, while the reference beam passed through a clear space adjacent to the photoreceptor. The beams were polarized so that the e-vector of the beams was perpendicular to the long axis of the outer segment, thus utilizing the dichroism of the visual pigment molecules. The monochromator of the microspectrophotometer scanned between 750 nm to 350 nm in 2 nm steps, instantly then scanning from 351 nm to 749 nm at the interleaving wavelengths. To minimise the effects of bleaching, each sample

was normally only measured once. However, SWS and UV/VS photoreceptor cells are less prone to bleaching and are often few in number, thus these cell-types were usually measured up to three times. In addition, two independent estimates of the baseline absorbance spectrum for all cell types were usually obtained by arranging both beams to pass outside the cell.

All putative outer segments were bleached by exposure to white light from the monochromator passing through the measuring beams of the microspectrophotometer. The exposure time was varied according to the visual pigment type. Rod, LWS and MWS pigments were exposed for 2 to 3 minutes, whereas SWS and UV/VS pigments were exposed for 5 and 10 minutes respectively. The increased bleaching times for shorter wavelength pigments was to compensate for the tungsten light source which has a decreased output at shorter wavelengths. After bleaching, the outer segments are recorded again using exactly the same protocol as before. This equalises the weighting of data for pre- and post-bleach spectra for subsequent averaging and calculation of difference spectra.

5.2 ANALYSIS OF VISUAL PIGMENT SPECTRA

The wavelength of maximum absorbance (λ_{max}) for each outer segment was estimated using a standardized computer program. For each cell, the two spectra were averaged and then a 2nm averaged curve of the outward and return traces was created. These mean curves were analysed by fitting an A_1 visual pigment template (Knowles & Dartnall, 1977) to the data. This template is expressed on an abscissal scale of log frequency, since visual pigment absorbance curves have an almost identical shape when expressed in this way (Mansfield, 1985; Bowmaker *et al.*, 1991). The λ_{max} of each absorbance spectra was calculated using two methods. Firstly, each of the 20 absorbance values on the long wavelength limb of a curve was referred to the A1 template, thus providing an estimate of the λ_{max} for that curve. A second estimate was obtained by fitting each of the 25 consecutive absorbance points from either side of the centre of the absorbance spectrum, 50 points in total, to the same template curve (see Mollon *et al.*, 1984; Bowmaker *et al.*, 1991). The average of these two estimates provided the calculated λ_{max} value for each measured absorbance curve.

At shorter wavelengths, there is distortion of the data due to wavelength-dependent light scattering and formation of photoproducts (MacNichol *et al.*, 1983). However, since SWS and VS cells were few in number, it was decided to keep all recorded cells for further analysis. However, for all other photoreceptor types stringent criteria were applied in order to determine which cells would be selected. As a result of these criteria (as detailed in **Table 5.1**), around 40% of measured LWS and MWS cells were selected. The λ_{max} for each spectral class was determined from the mean spectrum for each class of cell derived from the selected data. A second estimate of λ_{max} for each cell type was also determined from the mean of individual λ_{max} for all the selected cells.

Table 5.1
The selection criteria implemented for each spectral class of photoreceptor.

	Transverse density at the λ_{max}	Standard deviation from right hand limb estimate of λ_{max}	Difference between two estimates of λ_{max}
RODS	> 0.016	< 5 nm	< 5 nm
LWS	> 0.009	< 7 nm	< 5 nm
MWS	> 0.009	< 7 nm	< 5 nm
SWS/VS	All cells selected for analysis		

5.3 ANALYSIS OF OIL DROPLET SPECTRA

The oil droplets in avian cones have diameters of about 2 - 4 μm and may contain high concentrations of carotenoids (Liebman & Granda, 1975; Goldsmith *et al.*, 1984). As a consequence, light leakage around the droplet becomes significant during spectral measurements and accounts for the 'flat-topped' spectra, especially of the R- and Y-types. Essentially, the droplets act as long-pass cut-off filters and

were classified according to their 'cut-off' wavelength (λ_{cut}) calculated using the method suggested by Lipetz (1984).

5.4 MEASUREMENT OF OCULAR MEDIA

The anterior segment of the eye (cornea and lens) was dissected from the intact eye and immediately placed in avian saline solution. A central strip was cut out and placed in a 10mm black-walled cuvette, such that the measuring beam of the spectrometer (Perkin Elmer Lambda 2) passed through the cornea and lens. Vitreous humour was collected and measured separately. Three independent measurements were taken and averaged to produce an estimate of absorbance for the ocular media.

5.5 ANALYSIS OF OIL DROPLET RATIOS

Eyes from both ostrich and rhea were hemisected and placed in chilled calcium-free saline solution. The retinae were cut into four quadrants and immediately flat mounted onto slides under saline, with the photoreceptor layer uppermost. These preparations were viewed with a light microscope under standard illumination and UV light (340-380 nm with a 430 nm cut-off). Most oil droplet types are easily classified according to their colour under natural light, for instance R- and Y-type oil droplets can be clearly observed as red and yellow respectively. C- and T-type droplets show no observable colouration under natural light, however under UV illumination C-type droplets exhibit a bright fluorescence which makes them easily discernible from T-type droplets which lack fluorescence (Ohtsuka, 1984). Photomicrographs were taken of the preparations and counts were made of the overall numbers of oil droplets, and hence cone photoreceptor classes. Obviously, as rod photoreceptors have no oil droplets they cannot be counted by this technique.

5.6 DETERMINATION OF RELATIVE SENSITIVITIES

In order to calculate the relative spectral sensitivity of each cone class, the outer segment sensitivity (OSS) needed to be calculated and the filtering effect of the associated oil droplet also had to be subtracted. Manipulation of both the visual pigment and oil droplet data was performed with the Axum™ graphics software. Mean visual pigment absorbance values were smoothed (using the Lowess function; a noise reduction smoothing algorithm), converted to 'end on' absorptance and then

normalised. The log of these absorptance values provides the OSS for that cone class. Once oil droplet absorbances had been converted to transmission values, normalised and then converted back to absorbance, they could be directly subtracted from the OSS values. This procedure provides the relative spectral sensitivity spectra shown in **Figure 6.5**.

METHODS AND MATERIALS

MOLECULAR GENETICS

5.7 EXTRACTION OF RETINAL mRNA

mRNA extractions were performed using the 'QuickPrep Micro mRNA Purification Kit' (27-9255-01: Pharmacia P-L Biochemicals):

Retinal tissue was dissected out of the eye of an ostrich or rhea and immediately homogenised in 1.2 ml of Extraction Buffer until a uniform suspension was achieved. With the addition of 2.4 ml of Elution buffer the sample was briefly homogenised again, before centrifugation at 16,000 rpm for 60 seconds. The supernatant was then transferred to a pre-prepared microcentrifuge tube containing a pellet of Oligo (dT)-cellulose. This tube was then manually inverted for 3 minutes in order to resuspend the Oligo (dT)-cellulose in the extract. After a brief 10 second centrifugation, the supernatant was discarded. The resulting pellet then underwent five consecutive washes with 1 ml aliquots of High-Salt Buffer and Low-Salt Buffer. After the final wash, the pellet was resuspended in 0.3 ml of Low-Salt Buffer creating a slurry. This slurry was transferred to a Microspin Column within a microcentrifuge tube. The column was washed three times by adding 0.5 ml aliquots of Low-Salt Buffer and centrifuging for five seconds. Finally, the polyadenylated mRNA was eluted with two 0.2 ml applications of pre-heated (65°C) of Elution Buffer.

20 µl of Glycogen Solution and 40 µl of K Acetate solution were added to 400 µl of the sample eluate. This mix was then added to 1 ml of pre-chilled (-20°C) 95% ethanol and placed at –80°C for at least 30 minutes. The mRNA was pelleted from the solution by centrifugation of the microcentrifuge tube at 13,000 rpm for 5 minutes at 4°C. The supernatant was decanted off and discarded, and the mRNA pellet was redissolved in 40 µl of DEPC-treated sterile water. A typical yield of poly $(A)^+$ RNA obtained by this method is approx. 100 µg/ml.

5.8 SYNTHESIS OF cDNA

The 3'/5' RACE (Rapid Amplification of cDNA Ends) kit (1734792: BOEHRINGER MANNHEIM) was used for first strand cDNA synthesis from poly $(A)^+$ RNA. Briefly, the following components were pipetted into a sterile microcentrifuge tube on ice:

Components	Amount
cDNA synthesis buffer	4 µl
deoxynucleotide mix	2 µl
oligo dT-anchor primer	1 µl
poly $(A)^+$ RNA	10 µl (0.5 – 2.0 µg)
AMV reverse transcriptase	1 µl
DEPC-treated water	2 µl

The reaction mixture was spun down in a centrifuge for 5 – 10 seconds and then incubated for 60 minutes at 55°C. The mix was then further incubated for another 10 minutes at 65°C before being briefly spun down again. This cDNA mix was then stored at -20°C.

5.9 PCR AMPLIFICATION

DNA amplification via Polymerase Chain Reaction (PCR) has become an increasingly important tool in molecular biology. In this study, it was utilized to amplify fragments of opsin genes from isolated retinal cDNA. Either a standard protocol, applicable to most PCRs or a specific protocol, applicable to a specific pair of primers was used. When PCR amplification failed to generate products or produced spurious bands, the conditions were optimised by varying the reaction conditions. Likewise, the cycling conditions were also varied. All the necessary information is provided in the sections below. For more detailed accounts of PCR amplification protocols and applications refer to Saiki *et al.*, 1985; Innis *et al.*, 1988.

5.10 DESIGN OF PRIMERS

Primers were designed in the range of 17 – 25 nucleotides in length. Non-repetitious template DNA sequence was used to design the primers, which reduces mispriming, unless in some circumstances where it was unavoidable. Primers were designed such that the percentage GC content was within the range of 50 to 60%, but in the case of G+C rich template DNA sequence, the G+C content of the primers was higher. Primers were also designed preferably with a 'C' or 'G' base at the 3' end of the primer, since this provides a stronger attachment to the template. The melting temperature (Tm) of the primers was calculated as follows: 2°C was assigned for A or T nucleotide base and 4°C for a G or C nucleotide base, and added together (Thein & Wallace, 1986). The primers used in this study (**Table 5.10**) were designed by Dr. L.A.Heath, Dr. S.E.Wilkie and Dr. D.Das, except the paleognathous primers which were designed by M.W.Wright.

5.11 STANDARD PCR PROTOCOL

The standard condition will amplify most target sequences. These conditions were initially used to amplify a target region specified by a primer pair; if the reaction failed to generate products of interest then the conditions were modified. The standard composition of a PCR reaction consisted of:

Components	**Amount**
NH_4 buffer	5 µl
$MgCl_2$	2.5 µl
dNTP (2.5 mM)	2 µl
Forward primer (25 µM)	1 µl
Reverse primer (25 µM)	1 µl
Bio*Taq* DNA Polymerase (d=5)	1 µl (BIOLINE)
SDW (Sterile distilled water)	37.5 µl

The total volume of each reaction was 50 µl.

The reaction mixture (without the enzyme) was first heated to 94°C for 10 seconds denaturation before the thermostable enzyme, Bio*Taq* DNA Polymerase, was added to the mix. These 'hot starts' are performed in order to eliminate non-specific priming in the reaction and are followed by a second denaturation step at 94°C for three minutes. The reaction then proceeded with 35 cycles repeated cycles of denaturation (94 °C for 20 seconds), annealing (Tm for 20 seconds) and extension (72 °C for 40 seconds). The annealing temperature (Tm) selected was usually the lowest figure of Tm for the primer pair.

5.12 OPTIMISATION OF PCR REACTON

Mg^{2+} can effect the enzyme fidelity and activity, primer-template specificity and strand dissociation temperature. If the standard protocol was unsuccessful in elucidating a target gene, then various magnesium titrations were carried out with the problem primer pair to establish the optimal magnesium concentration for that specific primer pair. Likewise, all other factors which effect the PCR process (such as annealing temperature, DNA template concentration and dNTP concentration) are optimised for a specific primer pair. For review, refer to Innis & Gelfand , 1990.

Table 5.10
A list of the specific primers used to amplify ostrich and rhea opsin genes

Primer	Sequence (5'...3')	Fwd/Rev	GC %	Tm (°C)	Opsin Designed for...
Cρ15^{+}	AGG CCA AGA CTT CTA CGT	Forward	50	56	Chicken Rod
Bρ337^{+}	GAA GGC TTC TTT GCT ACC CT	Forward	50	60	Budgerigar Rod
Oρ744^{+}	GAA GTG ACC CGC ATG GTC AT	Forward	55	62	Ostrich Rod
Cρ764^{-}	ATC ACC ATG CGG GTC ACT TC	Reverse	55	62	Chicken Rod
ρ1040^{-}	TGG CTG GWG GAS ACR GAG GA	Reverse	62	64	Chicken Rod
r70^{+}	TCT TCA CCT ACA CCA ACA GC	Forward	50	60	Canary LWS
r544^{+}	CCV CCC ATC TTY GGB TGG AGC	Forward	67	69	Chicken LWS
r571^{-}	ACC TGC TCC AGC CGA AGA TG	Reverse	67	64	Canary LWS
r978^{-}	RAA CTG YCG GTT CAT RAA GAC	Reverse	45	56	Chicken LWS
Cg34^{+}	CCT ATG TCC AAC AAG ACA GG	Forward	50	60	Chicken MWS
Og287^{+}	GTC ACY TTC TAC ACV GCC T	Forward	53	59	Ostrich MWS
Og319^{+}	CCC RTT GGC TGT GCW GTG	Forward	64	59	Ostrich MWS
Og1020^{-}	GAC YTC AGT CTT GCT CTG G	Reverse	55	59	Ostrich MWS
Ob82^{+}	CTS AGC CCS TTC CTG GT	Forward	56	64	Ostrich SWS
Ob988^{-}	GAG AAC ASS AGC TTS AG	Reverse	52	53	Ostrich SWS
Ov15^{+}	GAS TTY TAC CTS TTC AAG AAC	Forward	41	59	Ostrich VS
Bv141^{+}	CCC VYT SAA CGC CRT SGT	Forward	65	56	Budgerigar UVS
Ov552^{+}	CCG ACT GGT ACA CGG TG	Forward	65	56	Ostrich VS
Rv826^{+}	CAC GGC CTC GAC CTT CG	Forward	70	58	Rhea VS
Ov932^{+}	GCA TCA TGG AGA TGG TCT G	Forward	53	58	Ostrich VS
Ov570^{-}	CCC ACC GTG TAC CAG TC	Reverse	55	56	Ostrich VS

5.13 VISUALISATION OF PCR PRODUCTS

12 µl of each PCR sample (10 µl PCR product, 2 µl orange loading dye) was loaded onto a 1.5 % low melting point agarose gel and electrophoresed in 1x TAE buffer (pH 8.3) at 90-120 v depending on the size of the gel. A Hae III ϕX174 DNA size marker was used to size any PCR products (for size of marker, see below).

Hae III ϕX174

1.358
1.078
0.872
0.602
0.310
0.281
0.271
0.234
0.194
0.118

Gels were stained in ethidium bromide (0.5 µg/ml) 1x TAE buffer for 5-10 minutes and then visualized on a long-wave UV trans-illuminator. The gel was viewed for as short a time as possible, as longer exposure would introduce nicks in the double-stranded DNA. Using sterile scalpel blades, any bands which appeared to be close to the expected size of the product were excised and placed in a 1.5ml Eppendorf with 50 µl of SDW overnight. The resulting eluate could then be used for either ligation (see below) or direct sequencing (section 5.15).

With weak bands, the eluate was concentrated by using a Centricon®-100 Micro-concentrator column (Amicon, Perkin-Elmer P/N N930-2119). The 50 µl eluate was aliquoted onto the filter of a Centricon-100 column and 2ml of SDW added. The column was centrifuged in a bench centrifuge with a fixed angle rotor (MSE Mistral 3000I) at 2200 rpm for 10 minutes, then the waste receptacle was emptied. The column was then inverted and centrifuged for a further 2 minutes at 1400 rpm. This time the flow-through was collected as it contains the concentrated sample DNA (typically 5-15 µl).

5.14 CLONING IN PLASMIDS

Ligation

The ligation of PCR fragments and subsequent cloning was performed using the pGEM®-T Easy Vector System (PROMEGA). The pGEM®-T Easy plasmid is used to clone PCR generated products with 5'-adenosine (A) nucleotide overhangs, which are added on by Bio*Taq* DNA polymerases. The ligation was set up using the following ingredients:

Components	Amount
T4 DNA Ligase Buffer	1 µl
pGEM®-T Easy Vector	1 µl
PCR product	1 µl
T4 DNA Ligase	1 µl
SDW (Sterile distilled water)	6 µl

This mix was then incubated at 4°C overnight.

Transformation

The competent cells **(JM109)** provided were used with the pGEM®-T Easy Vector System. The competent cells were removed form storage at –80°C and allowed to thaw. 1 µl of the ligation mix was added to 25 µl of competent cells, and left to incubate on ice for thirty minutes. The cells were then heat-shocked at 42 °C for 45 seconds and placed back on ice. After a couple of minutes recovery time, 125 µl of SOC was added to the 26 µl of cells. This transformation culture was shaken in a rotary shaker incubator for 1-1 ½ hours at 37 °C.

Prior to the transformation, LB agar plates were prepared which contained IPTG (24mg/ml), X-Gal (10mg/ml) and the antibiotic Ampicillin (50 mg/ml). Across these plates the transformation culture was spread evenly with a spreader. The plates were inverted and left overnight at 37 °C. It was common practice to keep the plates at 4°C

for two hours following incubation, in order to intensify the colour of the blue colonies. Transformant colonies were identified by their white colouration (rather than blue).

5.15 COLONY PCR

A colony PCR was performed for each white colony to ascertain whether the plasmid contained an insert and if so, that it was the correct size. Each colony to be screened was dabbed with a sterile toothpick, which was then dabbed into a 0.5 ml eppendorf tube containing the following 50 µl mix:

Components	**Amount**
NH_4 buffer	5 µl
$MgCl_2$	3 µl
dNTP (2.5 mM)	2 µl
pTAg SEQ 5' (3.2 pmol)	1 µl
pTAg SEQ 3' (3.2 pmol)	1 µl
Bio*Taq* DNA Polymerase (d=10)	1 µl (BIOLINE)
SDW (Sterile distilled water)	37 µl

For colony PCR the following thermocycling parameters were employed. The first step was a one minute denaturation at 94 °C, followed by 35 cycles of 1second at 94 °C, 1 second at 50 °C and 20 seconds at 72 °C and finished with a final one minute extension at 72 °C. When the reaction was complete, 3 µl of the mix was visualised on an agarose gel. The pTAg primers anneal approximately one hundred base pairs of the vector either side of the insert, thus non-recombinants are visualised on a gel as a band of 200 bp. Whereas, successful recombinants should be approximately 200bp plus the size of the insert. Correctly sized recombinants were inoculated into 10 ml of LB broth (containing 50 µg/ml ampicillin) and left in a rotary shaker overnight at 37 °C.

5.16 PLASMID DNA PURIFICATION

A permanent stock of the cultured cells was commonly made by adding 1ml of the culture to 1ml of glycerol in a vial, which was then gently mixed and stored at –80 °C. For sequencing, 5 ml of the overnight culture was pelleted by centrifugation (13,000 rpm for 10 minutes) and resuspended in 200 µl of a resuspension solution (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A). The solution was then transferred to a microcentrifuge tube and 200 µl of Cell Lysis Solution (0.2 M NaOH; 1% SDS) was added, with the tube then being gently inverted several times until the solution was seen to be clear. 200 µl of Neutralization Solution (2.55 M potassium acetate, pH 4.8) was added and the tube inverted a few times to mix the solution before centrifugation at 13,000 rpm for 5 minutes. The supernatant was decanted off and thoroughly mixed with 1 ml of DNA Purification Resin. A vacuum manifold was employed to pull the DNA/resin mix into a Minicolumn, which was then washed using 2 ml of Column Wash (200 mM NaCl; 20 mM Tris-HCl, pH 7.5; 5 mM EDTA; diluted 1:1 with 95% ethanol). The wash was performed under vacuum, which was continued for a further 2 – 3 minutes after washing to dry the resin. Residual Column Wash was removed from the Minicolumn by centrifugation inside a 1.5 ml microcentrifuge tube for 20 seconds at 13,000 rpm. 50 µl of deionized water at 65-70 °C was soaked into the Minicolumn for 1-2 minutes and then centrifuged for eluted into a fresh microcentrifuge tube by centrifugation at 13,000 rpm for 30 seconds. The eluate (plasmid DNA) was then stored at -20 °C. Typically, 1 – 10 µl of plasmid DNA is yielded by this means.

5.17 ABI PRISM™ BIGDYE TERMINATOR CYCLE SEQUENCING

The Ready Reaction Kit containing **AmpliTaq® DNA polymerase,** was used for sequencing. This enzyme is a mutant form of *Taq* DNA polymerase which does not have a 5' to 3' nuclease activity, which gives it the advantage of low levels of discrimination against incorporation of dideoxynucleotides. AmpliTaq® DNA polymerase has been specifically designed for use with both dye-labelled primers and terminators in fluorescent cycle sequencing. A further advantage to a sequencing reaction is that much lower levels of dye-labelled terminators can be used and removal of unincorporated terminators is made easier. The dNTP mix includes

dITP (deoxy inosine triphosphate) in place of dGTP to minimise band compressions. Plasmid DNA was cleaned using S400 Micro-Spin™ columns (Pharmacia Biotech) packaged with Sephacryl® HR resin which when centrifuged retains small oligonucleotides while allowing large DNA fragments to pass through the column.

Cycle Sequencing

The following mix was added to a 0.2 ml microcentrifuge tube (CAMLAB): 4 µl ABI BigDye terminator ready reaction mix (A-dye terminator labelled with dichloro [R6G], G-dye terminator labelled with dichloro [ROX], C-dye terminator labelled with dichloro [R110], T-dye terminator labelled with dichloro [TAMRA], dATP, dITP, dCTP, dUTP, Tris-HCl buffer, pH9.0, $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq® DNA polymerase), 3.2 pmol of pTAg sequencing primer, 0.2 – 0.5 µg of cloned DNA template and made up to a total volume of 10 µl with SDW. The PCR cycling condition used was as follows: 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes. The tubes were then held at 4 °C. Perkin Elmer Cetus systems 9600 and 2400 were used for the cycling reactions.

Direct Sequencing

As discussed earlier, bands of PCR products visualised on agarose gels are dissected out and eluted into solution. 5.5 µl of the eluates of strong distinct bands and concentrated eluates of weak bands can be substituted in place of the DNA plasmid and SDW in sequencing reactions. This mix undergoes the same sequencing reaction and subsequent purification as that for plasmid DNA, except instead of pTAg primers one of the specific 5' or 3' primers used to amplify the PCR fragment are used in the reaction mix.

Purification of Extension Products

Excess terminators were removed from the extension products by ethanol precipitation as follows: 50 µl of ice cold absolute ethanol was added to the extension products in a 1.5 ml microcentrifuge tube and mixed gently before leaving on ice for 20 minutes. The contents were then centrifuged at 13,000 rpm for 15 minutes, after which, the supernatant was discarded and the DNA pellet was washed with 75 µl of ice cold 70 % ethanol. The tubes were then centrifuged at top speed

for 10 minutes. The ethanol was then carefully removed using a stretch pasteur pipette, making sure that all visible ethanol was removed from the tube. The pellet was then air dried in a fume cupboard for one hour to evaporate any residual ethanol. The dried pellet can be stored at -20 °C for up to a week. Just prior to loading, the DNA pellet is resuspended in 3 µl of loading buffer (deionized formamide; 25 mM EDTA, pH 8.0, containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran), denatured for 2 minutes at 94 °C and cooled on ice. 1.5 µl of the sample was then loaded into one of the 64 lane on the ABI sequencing gel, with odd number lanes being loaded first and electrophoresed for 5 minutes before the rest of the samples are loaded.

Preparation of Sequencing Gels

The plates are cleaned by scrubbing gently with hot water. They are then rinsed in de-ionised water and assembled as specified by the manufacturer. The gel mix was prepared as follows: 40 ml Sequagel™ 6% sequencing gel solution (#EC-836, National Diagnostics), 10 ml Sequagel™ complete buffer reagent (#EC-841, National Diagnostics) and 0.04 g powdered APS. The gel components were mixed well and poured carefully (using a 50 ml syringe) into the assembled plates so as not to create bubbles in the gel. Shark tooth combs were used to make the wells in the gel which was then left to set for one hour. After the gel had set, the plate were checked for the presence of any dirt by performing a pre-scan . Once the plates were clean, the gel was pre-run in 1x TBE (made with distilled water and ultra pure 10 x TBE) for a minimum of 15 minutes. The 373a DNA sequencer was set up and run in accordance with the manufacture's instructions. A sequencing gel would typically electrophorese for 10 – 12 hours.

5.18 ANALYSIS OF SEQUENCE DATA

An Apple Macintosh computer linked to the sequencer was programmed to analyse the data. The data is presented as a text file and an analysis file which contains an electropherogram. It is the electropherogram which represents the raw data and is analysed to produce the sequence of the DNA sample.

RESULTS & DISCUSSION

MICROSPECTROPHOTOMETRY & HISTOLOGY

RESULTS

6.0 *Visual Pigments*

A rod and four spectrally distinct classes of cone visual pigment were characterised. The rods had λ_{max} at about 505 nm, whereas three cone pigments were recorded with λ_{max} at about 570, 505 and 445 nm. The 570-nm pigment is located in both members of the double cones and in a class of single cone containing an R-type oil droplet. The 510-nm and 445-nm cone pigments were found in populations of single cones containing Y- and C-type oil droplets respectively. The pigment contained in a fourth single cone class, which to date has only been characterised in the ostrich, was violet-sensitive with a λ_{max} around 405 nm and containing a T-type droplet. The mean absorbance and difference spectra and spectral distribution for all the visual pigments are presented in **Figures 6.0a – 6.0f** (ostrich) & **6.0g – 6.0k** (rhea). **Table 6.0** represents a summary of the analysis of all the spectra.

6.1 *Oil Droplets*

Five oil droplet types were characterised in both the ostrich and rhea: red (R-type), yellow (Y-type), pale (P-type), clear (C-type) and transparent (T-type) (Bowmaker, 1991). The R-, Y- and C-type droplets were found in single cones, whereas the P-type droplets were located in the principal member of double cones. T-type droplets were found in single cones in the ostrich, however only isolated T-type droplets were measured in the rhea. The mean absorbance spectra of each oil droplet type are presented in **Figure 6.1** and the λ_{cut} for the droplets are summarised in **Table 6.1**.

The data from the two birds were almost identical. The R-, Y- and C-type oil droplets had mean λ_{cut} around 555 nm, 505 and 417 nm respectively (**Table 6.1**). The P-type droplets, characterised by a shoulder or peak close to 480 nm, could be divided into two populations in both species according to the relative absorbance of the 480-nm shoulder. In the ostrich, the droplets had either a clear peak at 480 nm or a 70% shoulder. In the rhea, the difference was more significant with a clear peak or

a shoulder of only 20% (**Fig. 6.1**), although the latter population was only found in two of the birds studied. The P-type droplets typically had a maximum absorbance of around 0.2, except for the 20% shoulder P-type droplets which had a low absorbance (around 0.1). The mean λ_{cut} for the 100% and 70% shoulder P-type droplets (assuming these droplets act as cut-off filters) were around 498 nm and 488 nm respectively. The absorbance was too low in the 20% shoulder P-type droplets for a λ_{Tcut} to be meaningful. T-type droplets showed no significant absorbance over the measured range. The accessory members of the double cones contained a small A-type droplet with a triple peaked absorbance spectrum (not shown) that had very low absorbance, indicating low levels of carotenoid in the droplet. These droplets are typical of other avian species (Bowmaker & Knowles, 1977; Jane & Bowmaker, 1988; Maier & Bowmaker, 1993; Bowmaker *et al.*, 1997; Hart *et al.*, 1998).

6.2 *Ocular Media*

The spectral absorbance of the lens and cornea and vitreous humour from the ostrich are shown in **Figure 6.2**. The lens and cornea are virtually transparent at longer wavelengths, with a small increase in absorbance apparent below 450 nm and a substantial increase below 380 nm. The vitreous humour is transparent throughout the measured spectral range. For comparison, the absorbance spectra of the lens from the canary (*Serinus canaria*) (Das *et al.*, 1999), Mallard duck (*Anas platyrhynchos*) (Jane & Bowmaker, 1988) and human (Wyszecki & Stiles, 1982) are also shown (**Figure 6.2**).

6.3 *Histology*

Figures 6.3a & 6.3b present photomicrographs from fluorescent and light microscopy of retinal whole tissue mounts and highlights the individual oil droplet types. The R-and Y-type droplets occur in approximately equal numbers, each accounting for about 24% of the total cone population (**Fig. 6.3c**). The C- and T-type droplets account for a further 11% and 2 % respectively, and the P-type droplets form the remaining 40%. These data are similar to those for chicken (*Gallus gallus*) (Bowmaker & Knowles, 1977), Mallard duck (*Anas platyrhynchos*) (Jane & Bowmaker, 1988), budgerigar (*Melopsittacus undulatus*) (Wilkie *et al.*, 1998) and

starling (*Sturnus vulgaris*) (Hart *et al.*., 1998) (**Table 6.3**), though the percentage of T-type droplets in the paleognathous birds is somewhat lower.

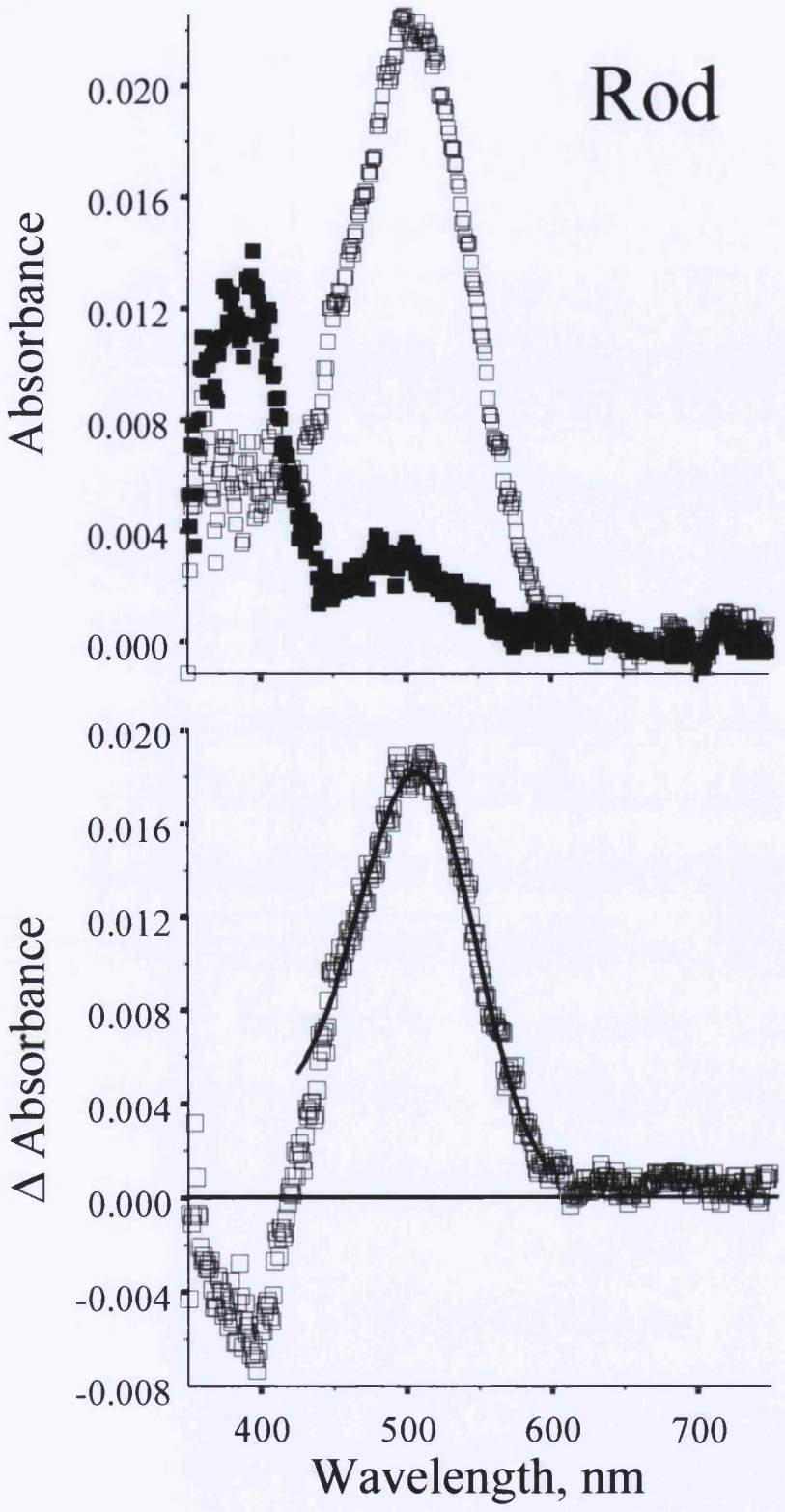


FIGURE 6.0a
Mean absorbance spectra of the rod visual pigment from the ostrich (*Struthio camelus*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 506 nm.

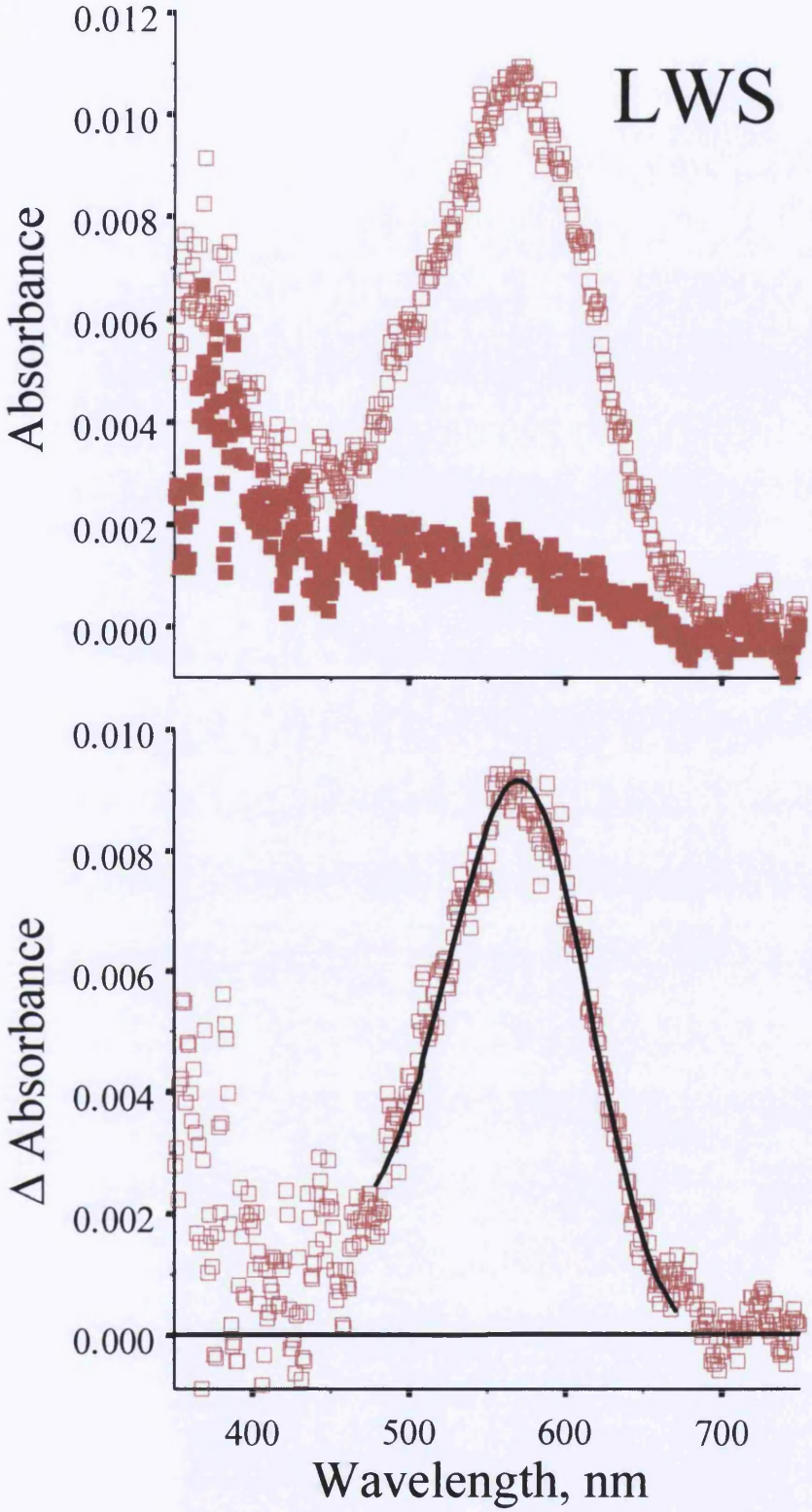


FIGURE 6.0b
Mean absorbance spectra of the LWS visual pigment from the ostrich (*Struthio camelus*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 571 nm.

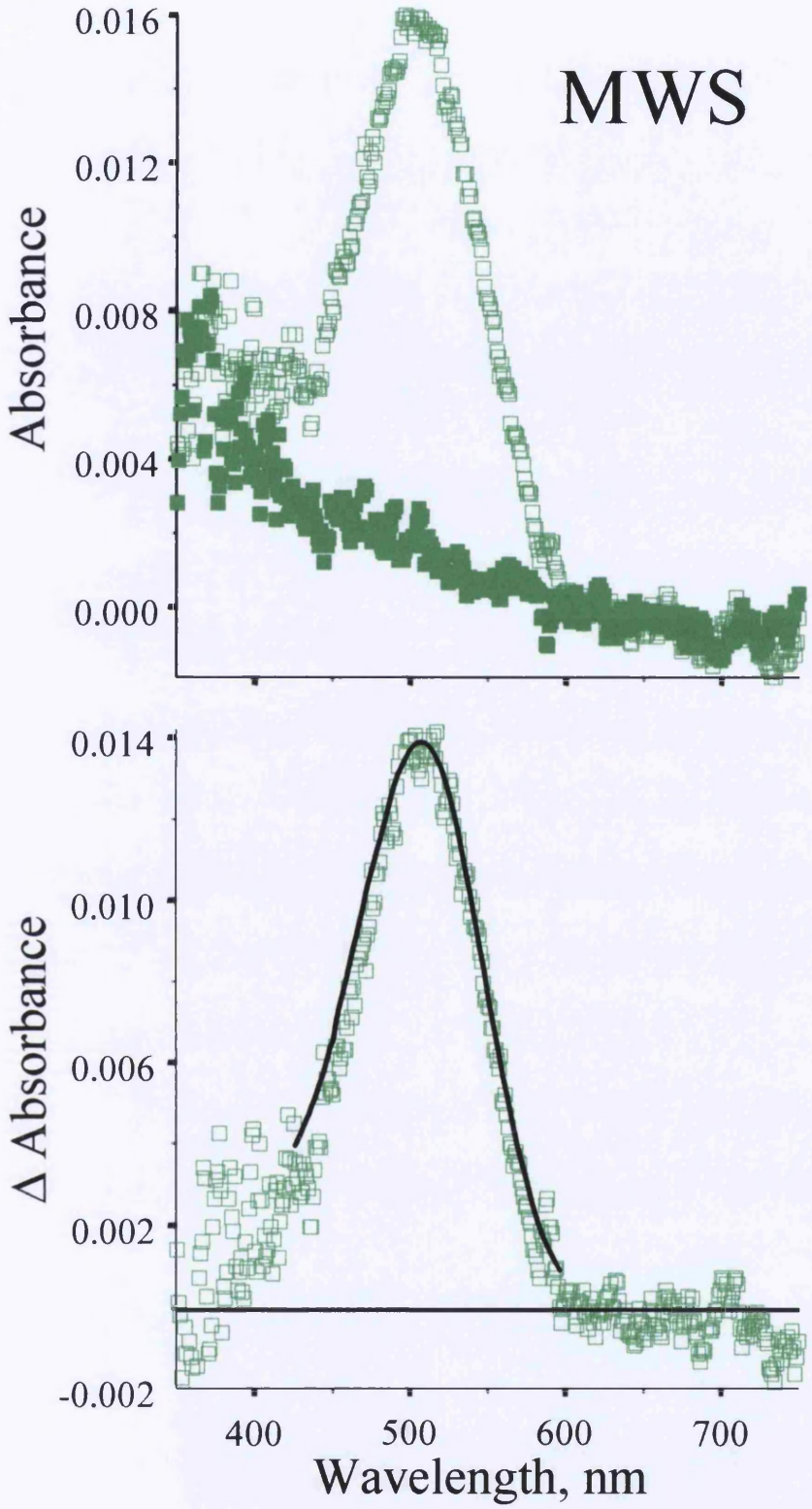


FIGURE 6.0c

Mean absorbance spectra of the MWS visual pigment from the ostrich (*Struthio camelus*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 507 nm.

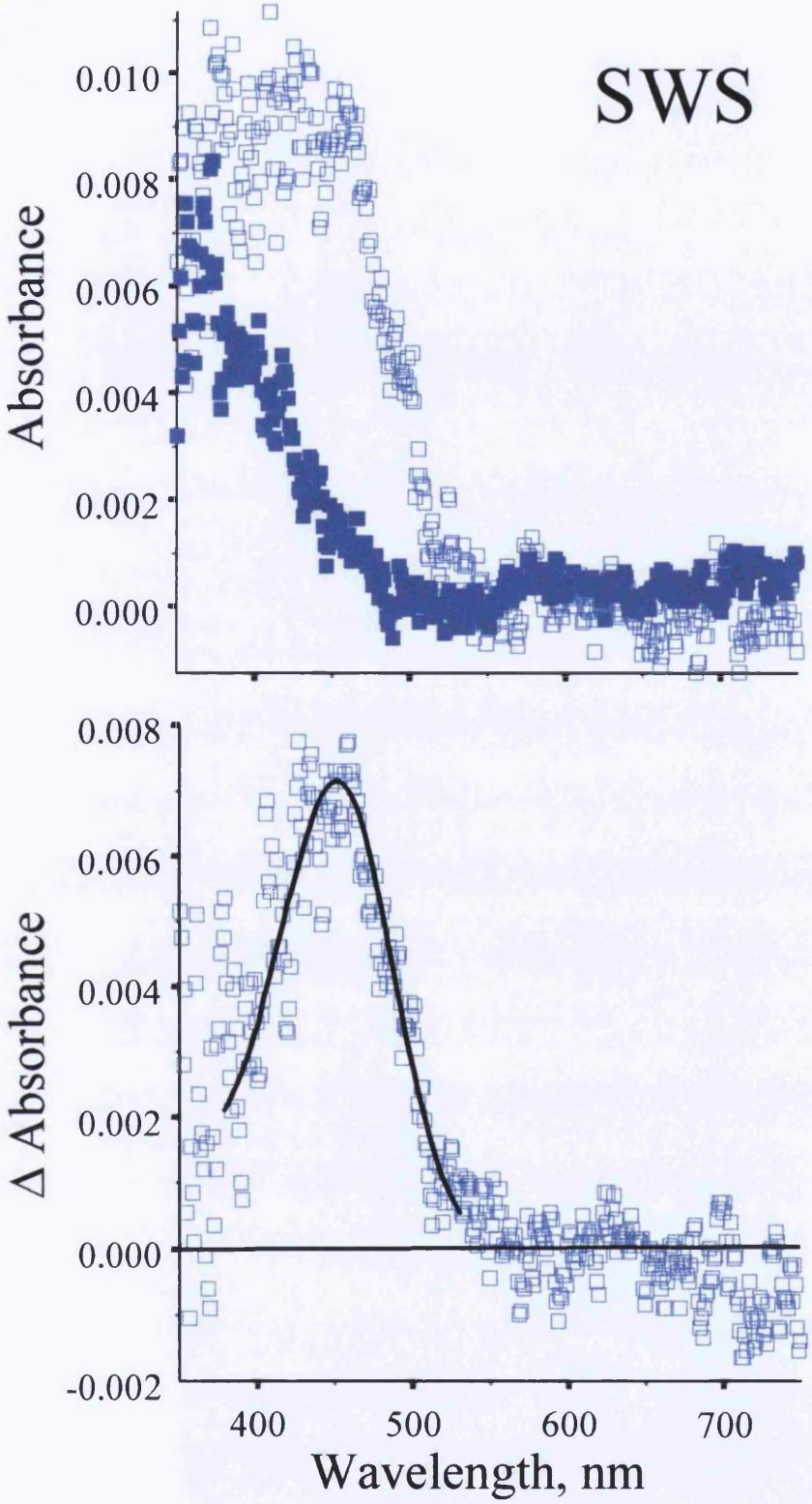


FIGURE 6.0d
Mean absorbance spectra of the SWS visual pigment from the ostrich (*Struthio camelus*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 451 nm.

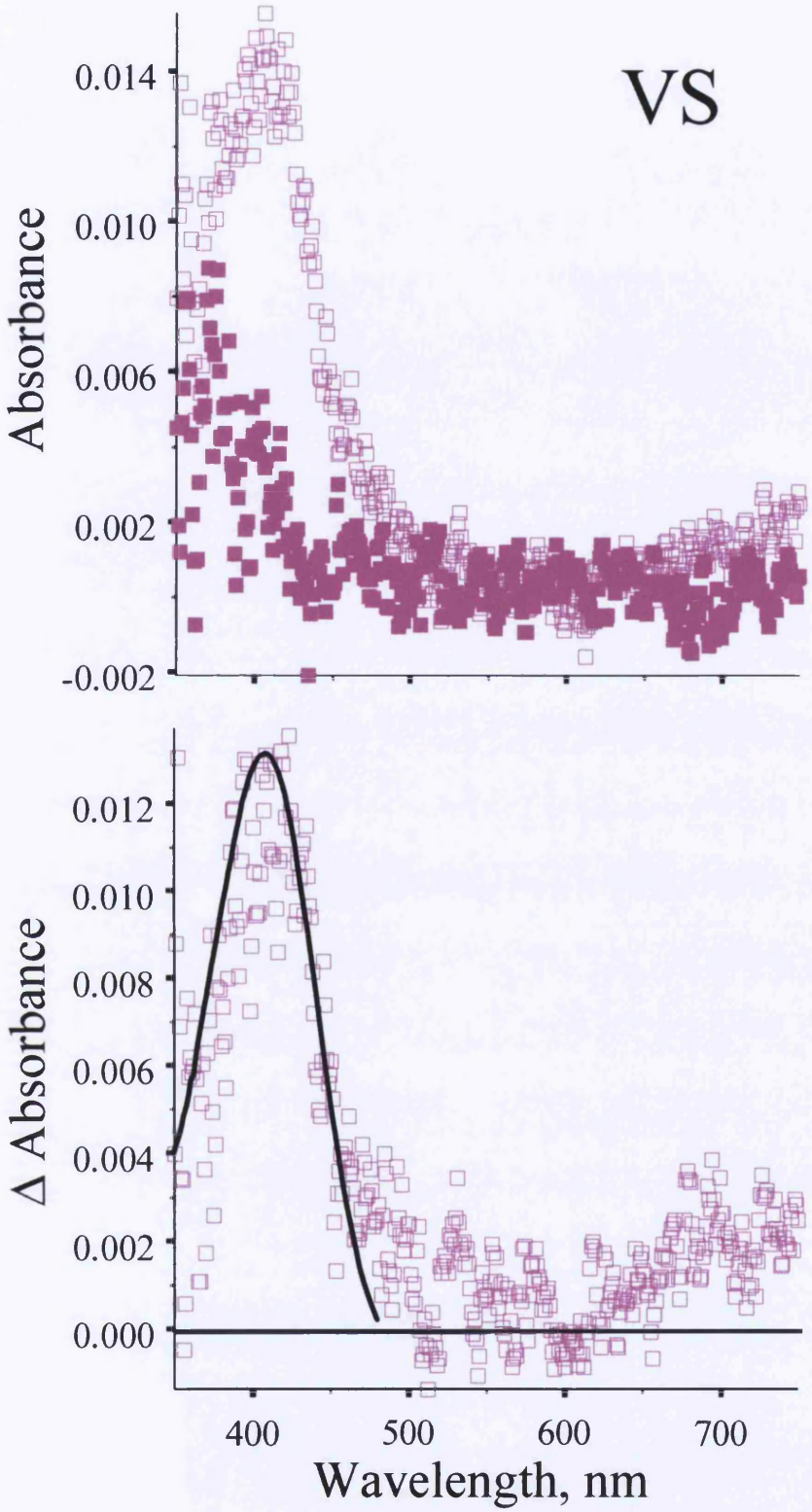


FIGURE 6.0e
Mean absorbance spectra of the VS visual pigment from the ostrich (*Struthio camelus*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 408 nm.

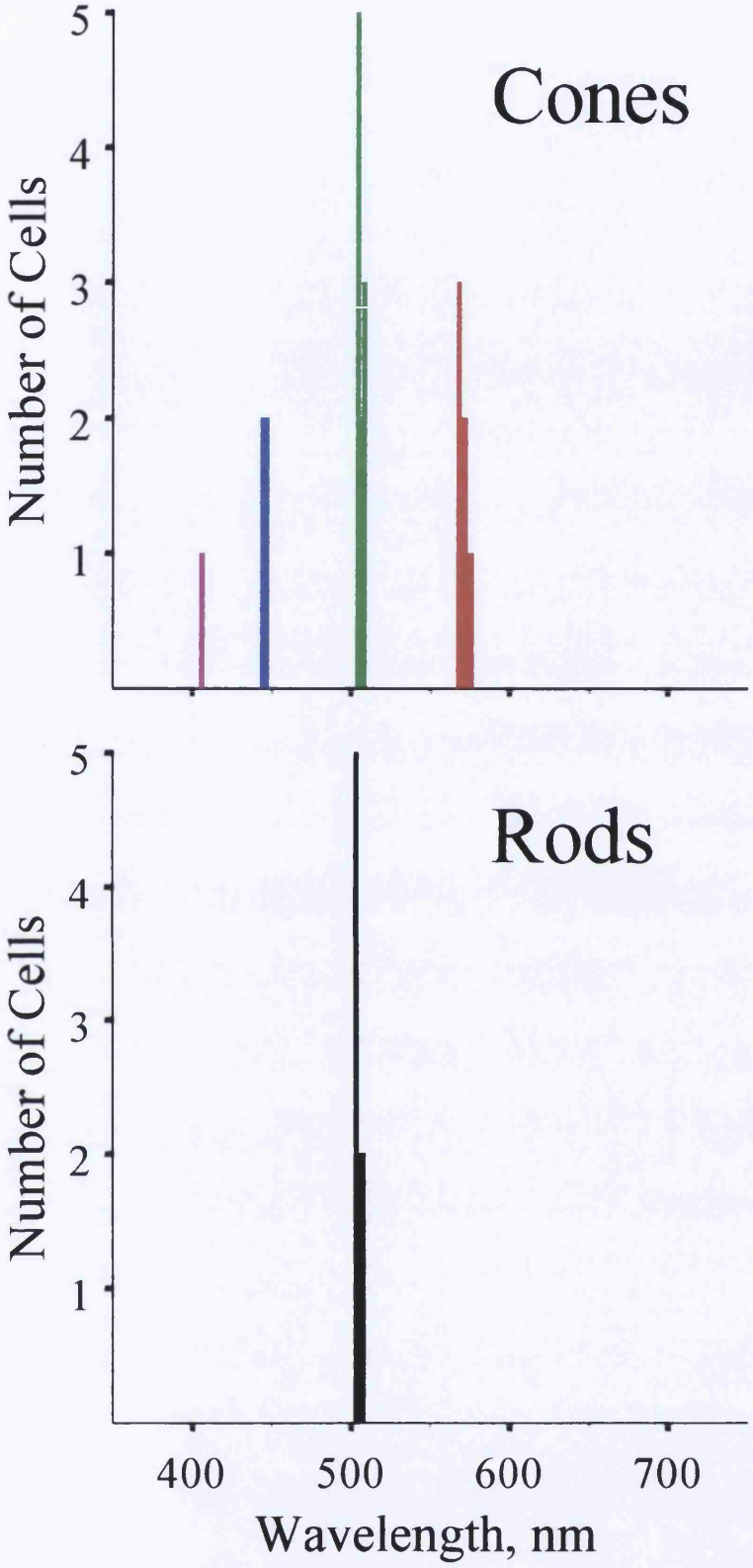


FIGURE 6.0f
Histograms to show the spectral distribution of the λmax of individual rods (lower panel) and cones (upper panel) of the ostrich (*Struthio camelus*).

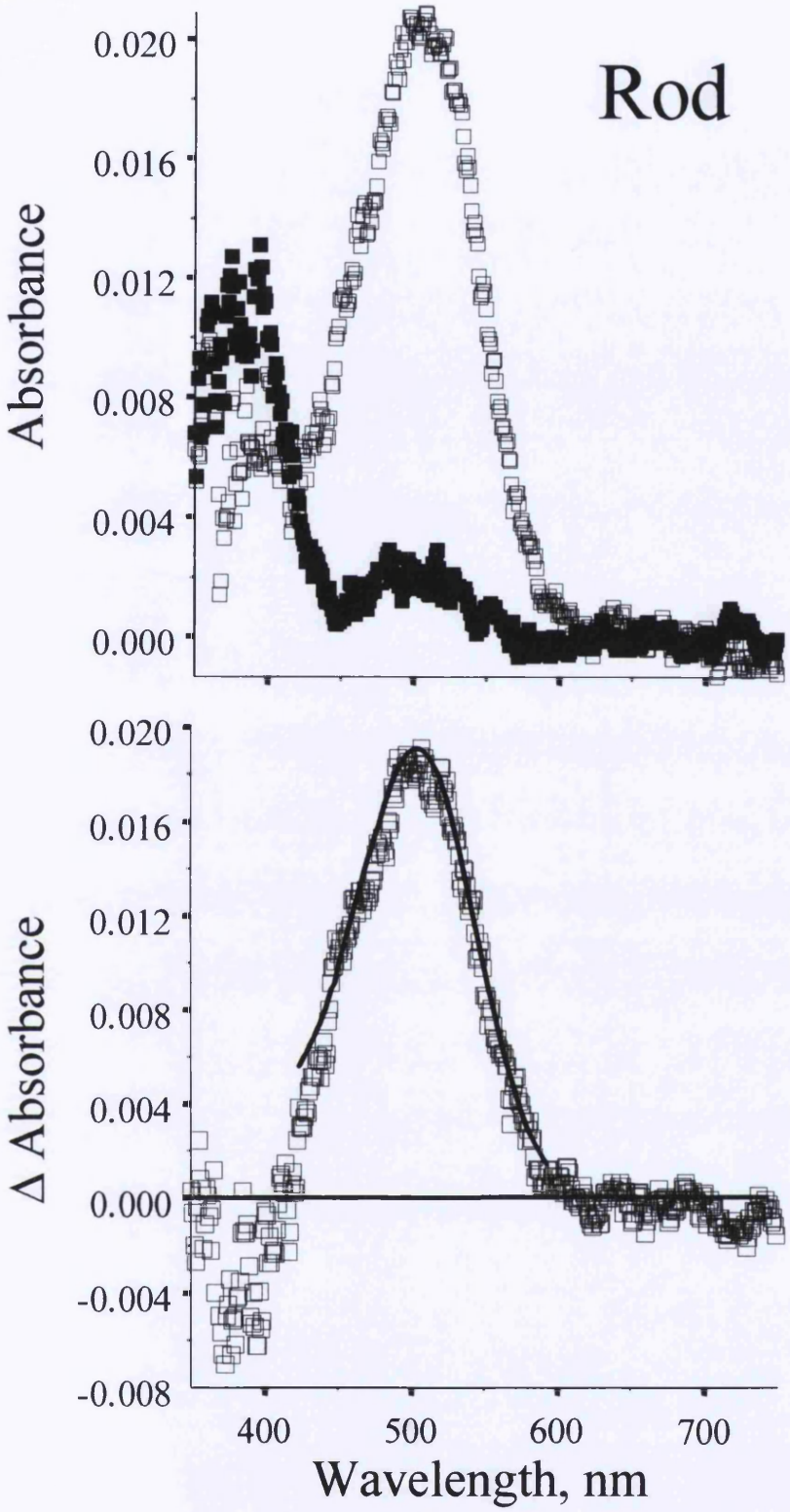


FIGURE 6.0g

Mean absorbance spectra of the rod visual pigment from the rhea (*Rhea americana*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 506 nm.

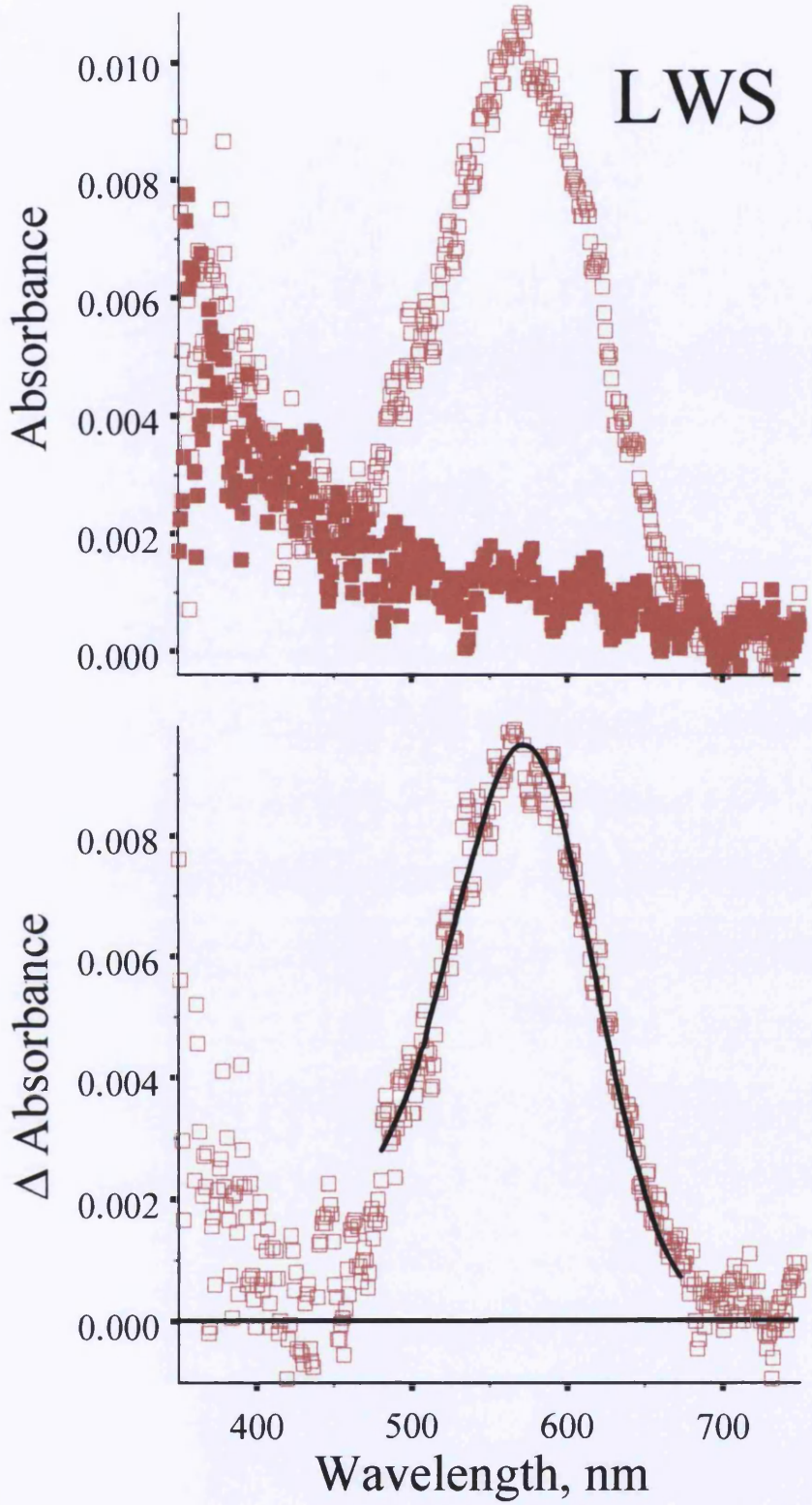


FIGURE 6.0h

Mean absorbance spectra of the LWS visual pigment from the rhea (*Rhea americana*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 570 nm.

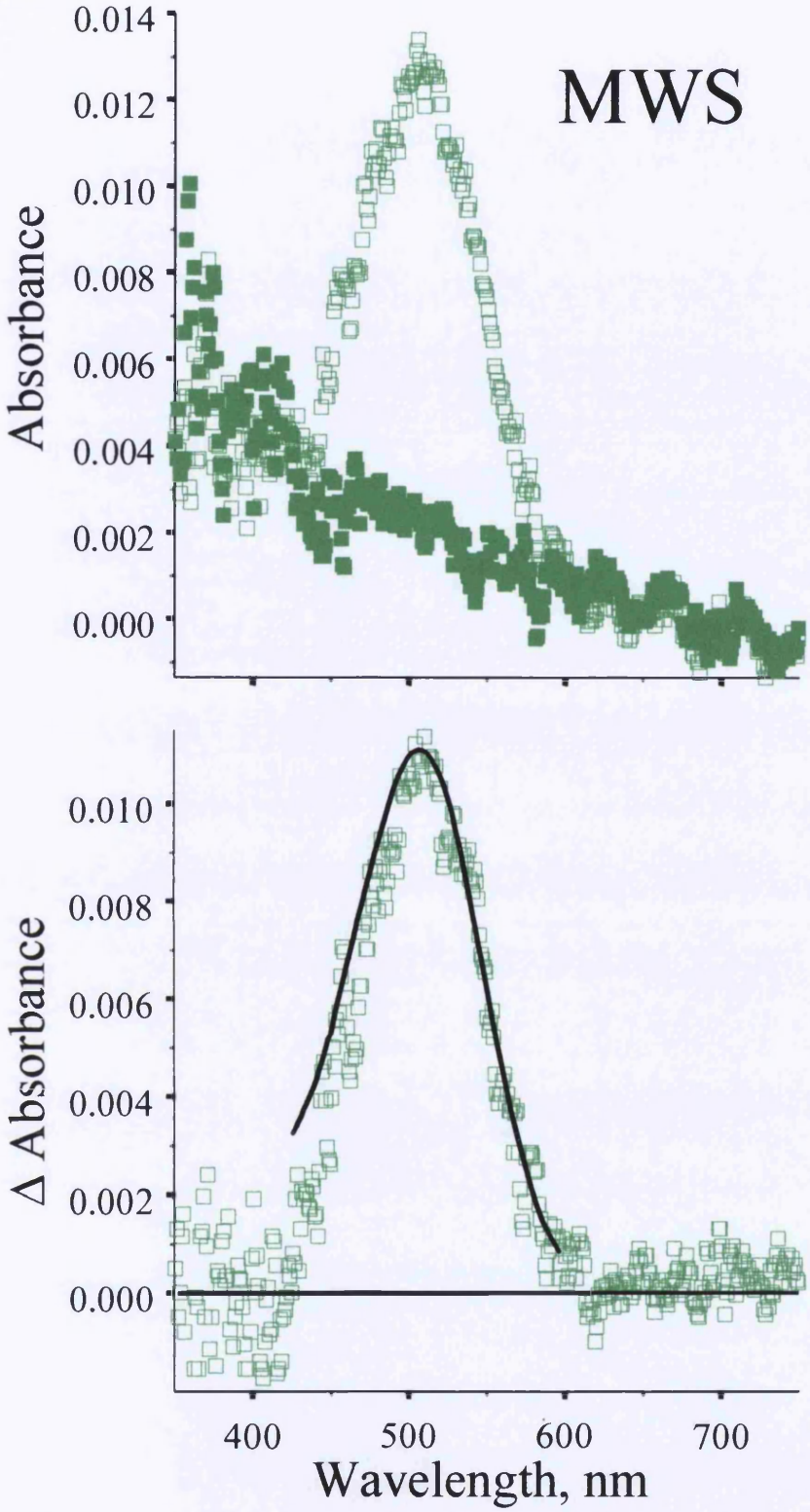


FIGURE 6.0i

Mean absorbance spectra of the MWS visual pigment from the rhea (*Rhea americana*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 507 nm.

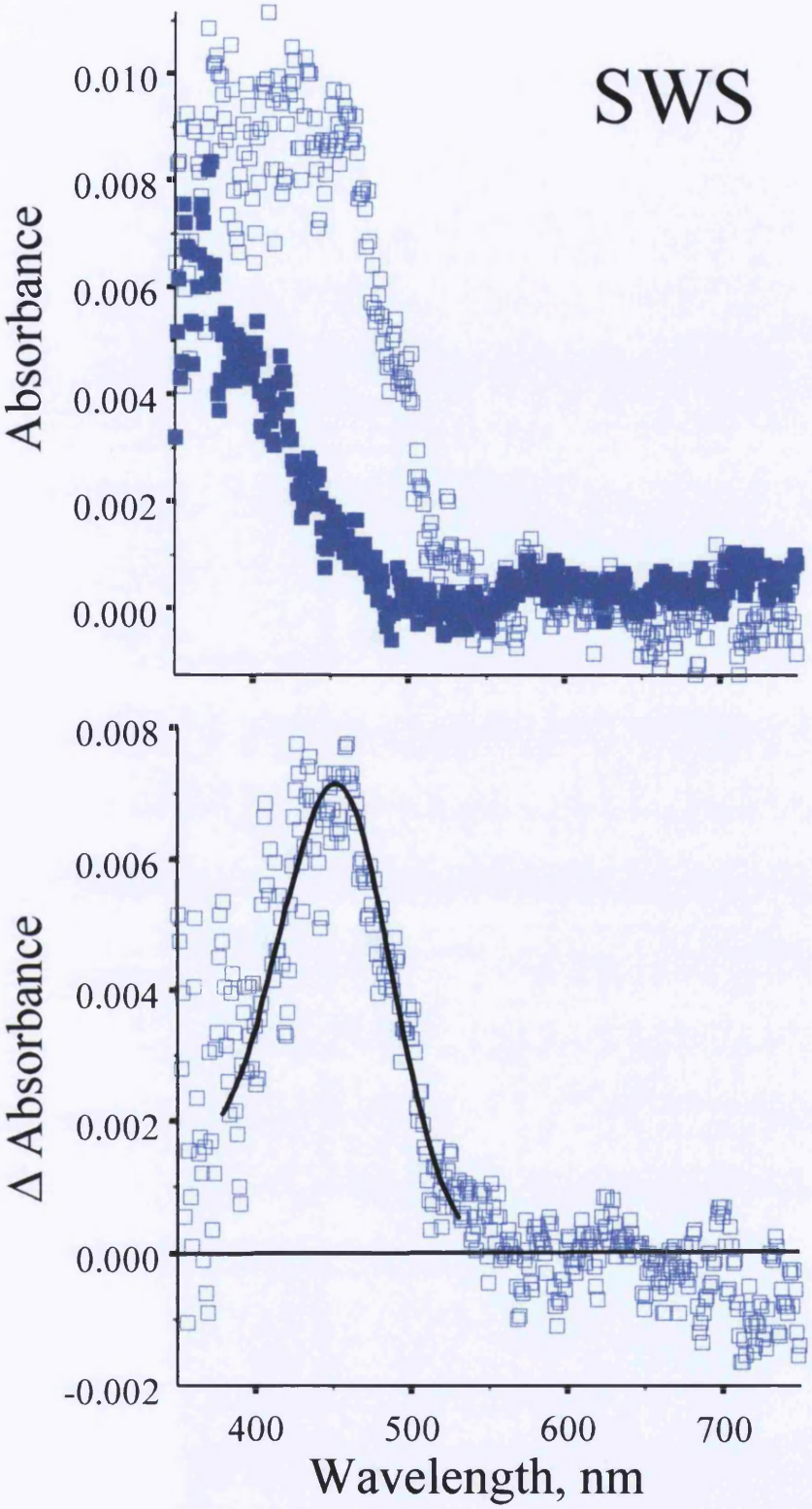


FIGURE 6.0j

Mean absorbance spectra of the SWS visual pigment from the rhea (*Rhea americana*). The upper panel represents the mean pre- and post-bleach spectra with open symbols, before bleaching; closed symbols after exposure to white light. The lower panel is the difference spectrum and is fitted with an A_1 visual pigment nomogram (Knowles & Dartnall, 1977) with a λmax at 456 nm.

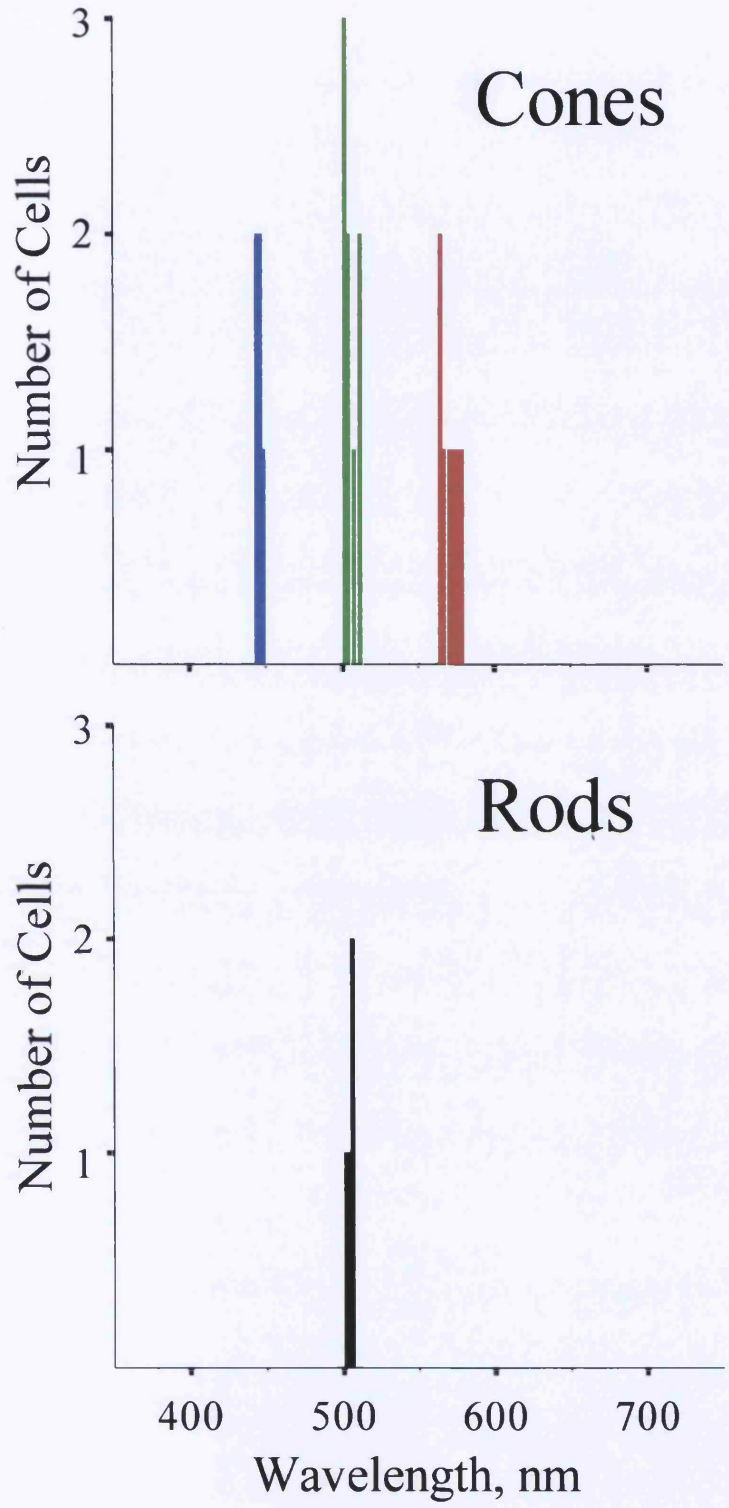


FIGURE 6.0k
Histograms to show the spectral distribution of the λmax of individual rods (lower panel) and cones (upper panel) of the rhea (*Rhea americana*).

TABLE 6.0

λ_{max} of visual pigments for the ostrich, *Struthio camelus*, and rhea, *Rhea americana*.

	Struthio camelus	*Rhea americana*
RODS		
Mean of λ_{max}, nm	504.3 ± 3.2	503.9 ± 2.2
λ_{max}, of difference spectrum, nm	505.6 ± 1.6	505.0 ± 1.8
λ_{max}, of mean spectrum, nm	504.2 ± 1.2	504.4 ± 1.8
Maximum absorbance	0.02/0.018	0.02/0.017
Number of cells	9	4
LWS CONES		
Mean of λ_{max}, nm	569.9 ± 2.9	570.7 ± 5.5
λ_{max}, of difference spectrum, nm	570.6 ± 2.9	570.1 ± 2.0
λ_{max}, of mean spectrum, nm	569.9 ± 1.8	569.9 ± 2.1
Maximum absorbance	0.01/0.009	0.01/0.009
Number of cells	9	8
MWS CONES		
Mean of λ_{max}, nm	505.5 ± 1.8	505.7± 4.4
λ_{max}, of difference spectrum, nm	506.7 ± 3.4	506.9 ± 1.8
λ_{max}, of mean spectrum, nm	505.2 ± 2.1	505.7 ± 0.9
Maximum absorbance	0.011/0.012	0.015/0.013
Number of cells	10	8
SWS CONES		
Mean of λ_{max}, nm	445.0 ± 2.2	446.7 ± 2.1
λ_{max}, of difference spectrum, nm	451.0 ± 4.3	456.4 ± 5.8
λ_{max}, of mean spectrum, nm	444.4 ± 2.9	445.5 ± 3.5
Maximum absorbance	0.008/0.007	0.009/0.007
Number of cells	4	5
VS CONES		
Mean of λ_{max}, nm	404.9	-
λ_{max}, of difference spectrum, nm	408.0 ± 6.5	-
λ_{max}, of mean spectrum, nm	404.9 ± 4.2	-
Maximum absorbance	0.013/0.01	-
Number of cells	1	-

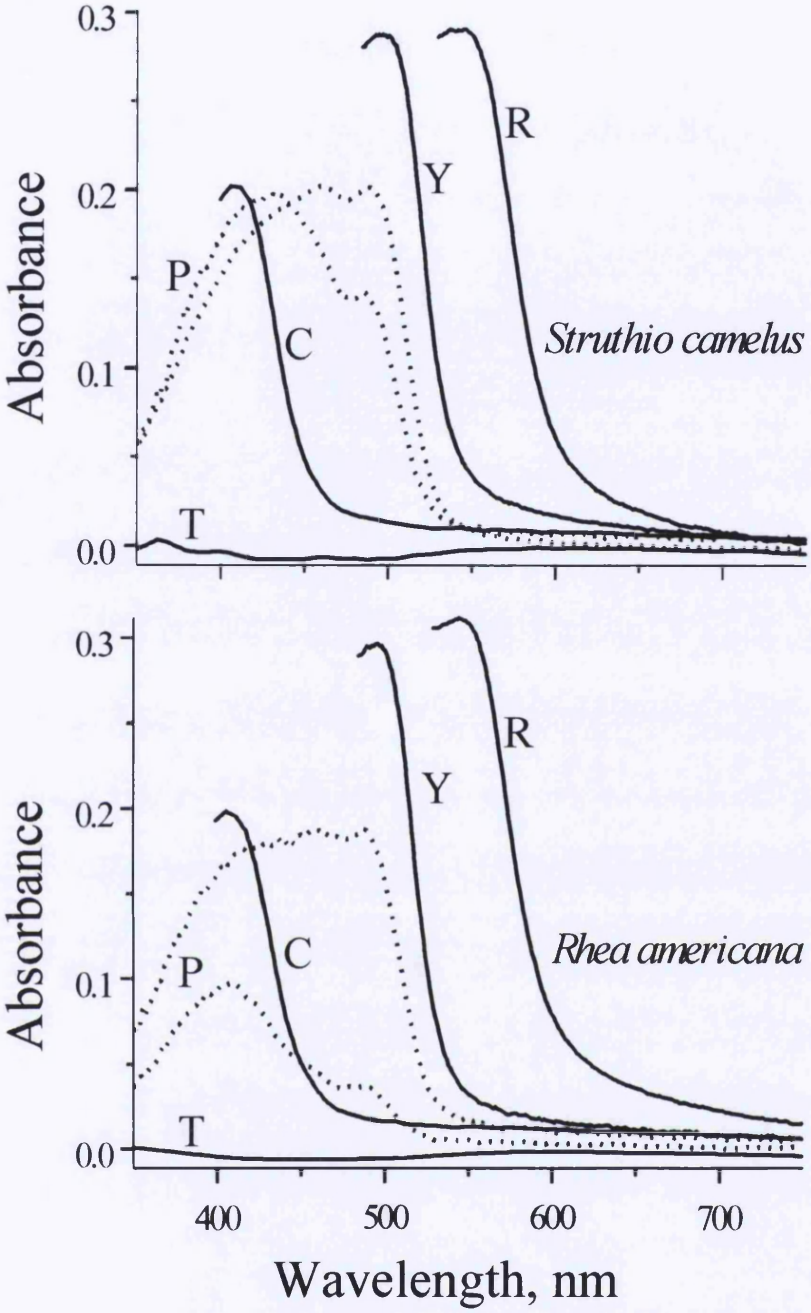


FIGURE 6.1
The mean absorbance spectra of the cone oil droplets from ostrich (upper panel) and rhea (lower panel). Letters indicate droplet type. **R**ed, **Y**ellow, **C**lear and **T**ransparent are all found in single cones (with the exception of rhea T-types droplets, which were measured in isolation). **P**ale droplets were located in the principal members of double cones and were split into two populations in both birds according to the relative size of the 480-nm shoulder on the absorbance spectra. In the ostrich, all P-type droplets could be classified as having a 100% or 70% shoulder. In the rhea, the difference was more significant with 100% and 20% shoulders, although the latter population were only found in the two of the birds studied.

TABLE 6.1

λ_{cut} of oil droplet spectra for the ostrich, *Struthio camelus*, and rhea, *Rhea americana*. For each droplet type, the left-hand figure is the λ_{cut} of the mean spectrum and the right-hand figure the mean of λ_{cut}, nm. The figure in brackets is the number of each droplet type measured. The P-type droplets are separated according to the relative size of the 480 nm shoulder, either 100% (both birds), 70 % (ostrich) or 20% (rhea).

	Struthio camelus		*Rhea americana*	
OIL DROPLET				
R-type	555.3	555.2 ± 3.1 (16)	554.5	555.0 ± 3.4 (16)
Y-type	507.1	506.4 ± 2.4 (16)	503.8	504.8 ± 3.6 (16)
C-type	417.1	416.5 ± 3.4 (10)	416.7	417.6 ± 2.9 (10)
P-type (100%)	498.1	497.9 ± 2.5 (14)	496.4	498.1 ± 4.5 (6)
P-type (70%/20%)	487.4	488.9 ± 4.7 (3)	-	- (4)

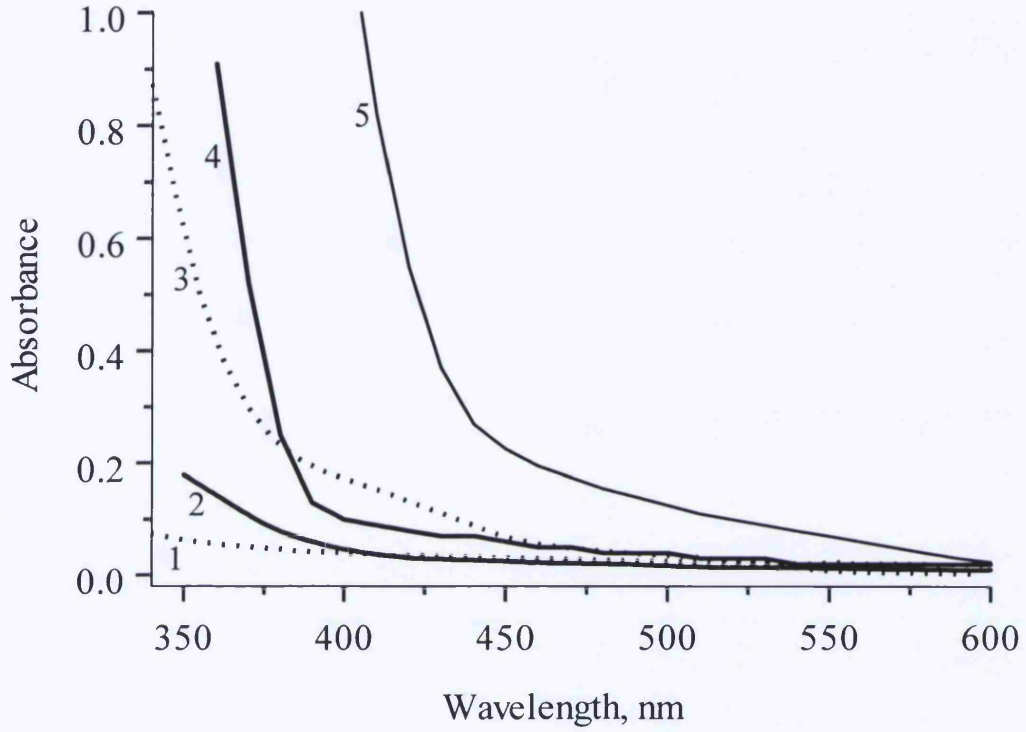


FIGURE 6.2
Absorbance spectra of ocular media. (1) vitreous humour from ostrich (*Struthio camelus*); (2) lens of canary (*Serinus canaria*) (data from Das *et al.*, 1999); (3) lens and cornea of ostrich (*Struthio camelus*); (4) lens of duck (*Anas platyrhynchos*) (data from Jane & Bowmaker, 1988); (5) lens of human (Wyszecki & Stiles, 1982).

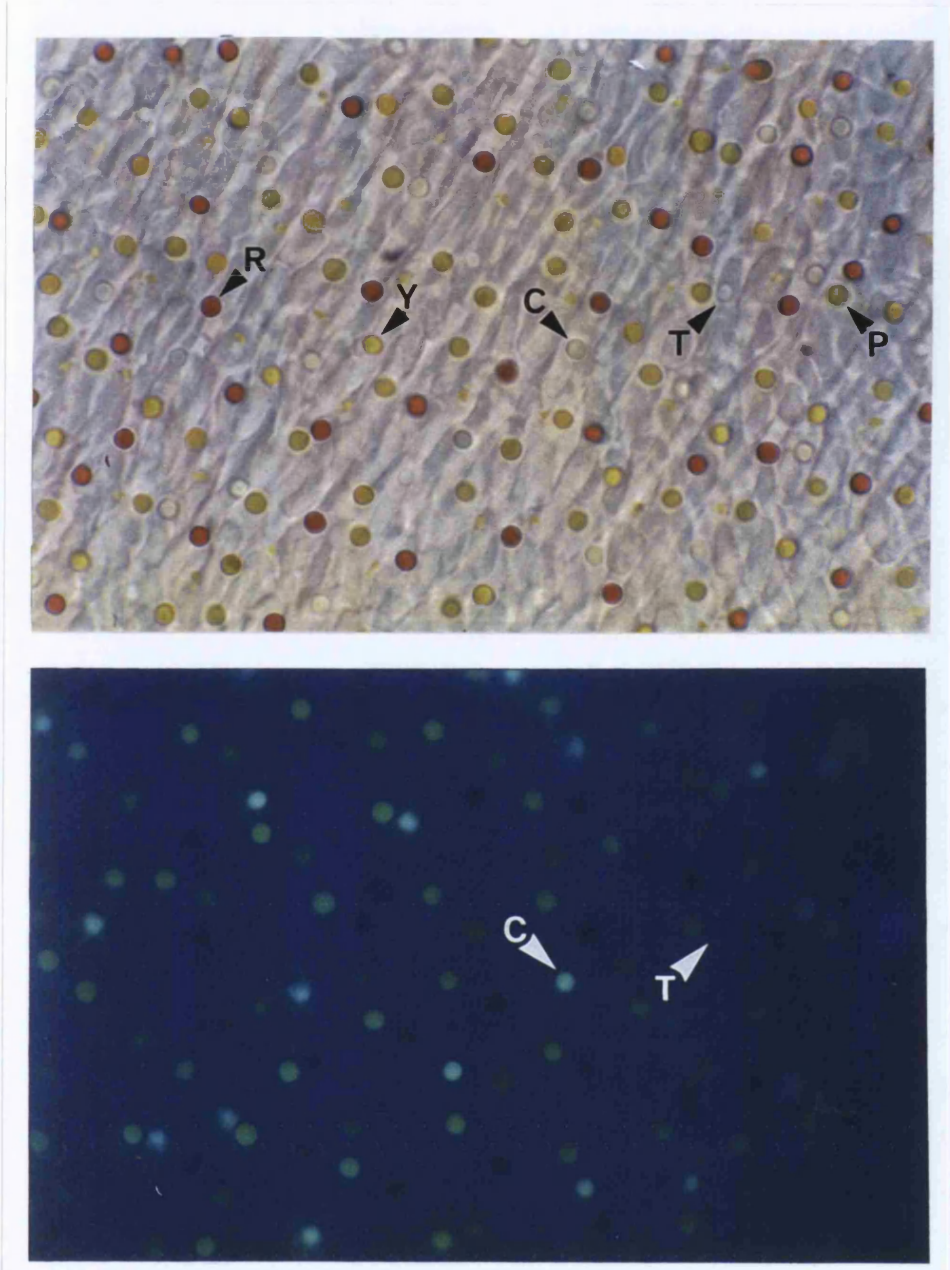


FIGURE 6.3a
Photomicrographs of sections of retina from ostrich (*Struthio camelus*) taken under visible light (upper panel) and UV illumination (lower panel). The R- and Y-type (labelled **R** and **Y**) oil droplets are clearly seen as red and yellow under natural light, whereas the P-type droplets (**P**) appear yellowish green and the C- and T-type (labelled **C** and **T**) are colourless. However, under UV illumination, C-type droplets exhibit a bright fluorescence that makes them easily discernible from T-type droplets that do not fluoresce.

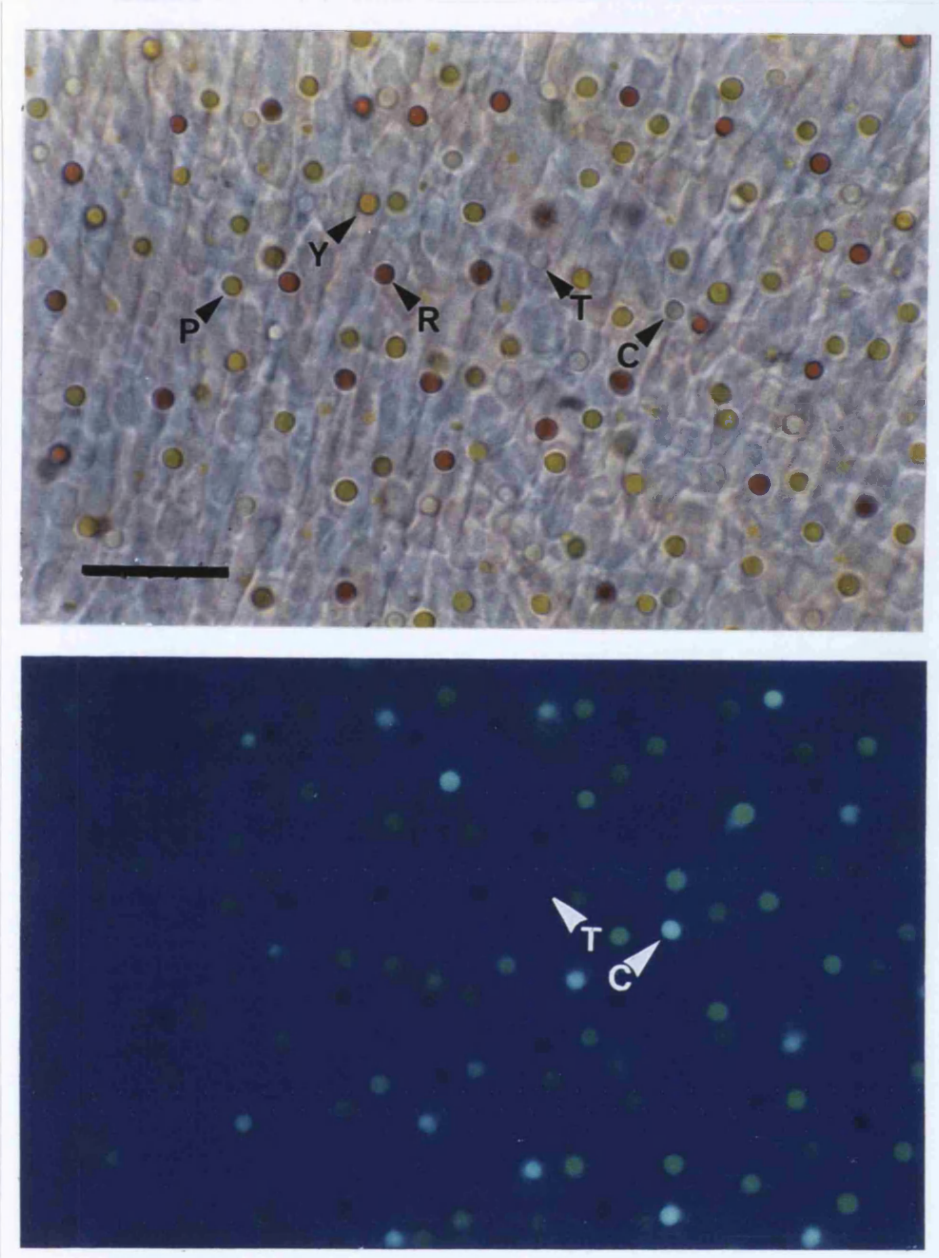


FIGURE 6.3b
Photomicrographs of sections of retina from rhea (*Rhea americana*) taken under visible light (upper panel) and UV illumination (lower panel). The R- and Y-type (labelled **R** and **Y**) oil droplets are clearly seen as red and yellow under natural light, whereas the P-type droplets (**P**) appear yellowish green and the C- and T-type (labelled **C** and **T**) are colourless. However, under UV illumination, C-type droplets exhibit a bright fluorescence that makes them easily discernible from T-type droplets that do not fluoresce.

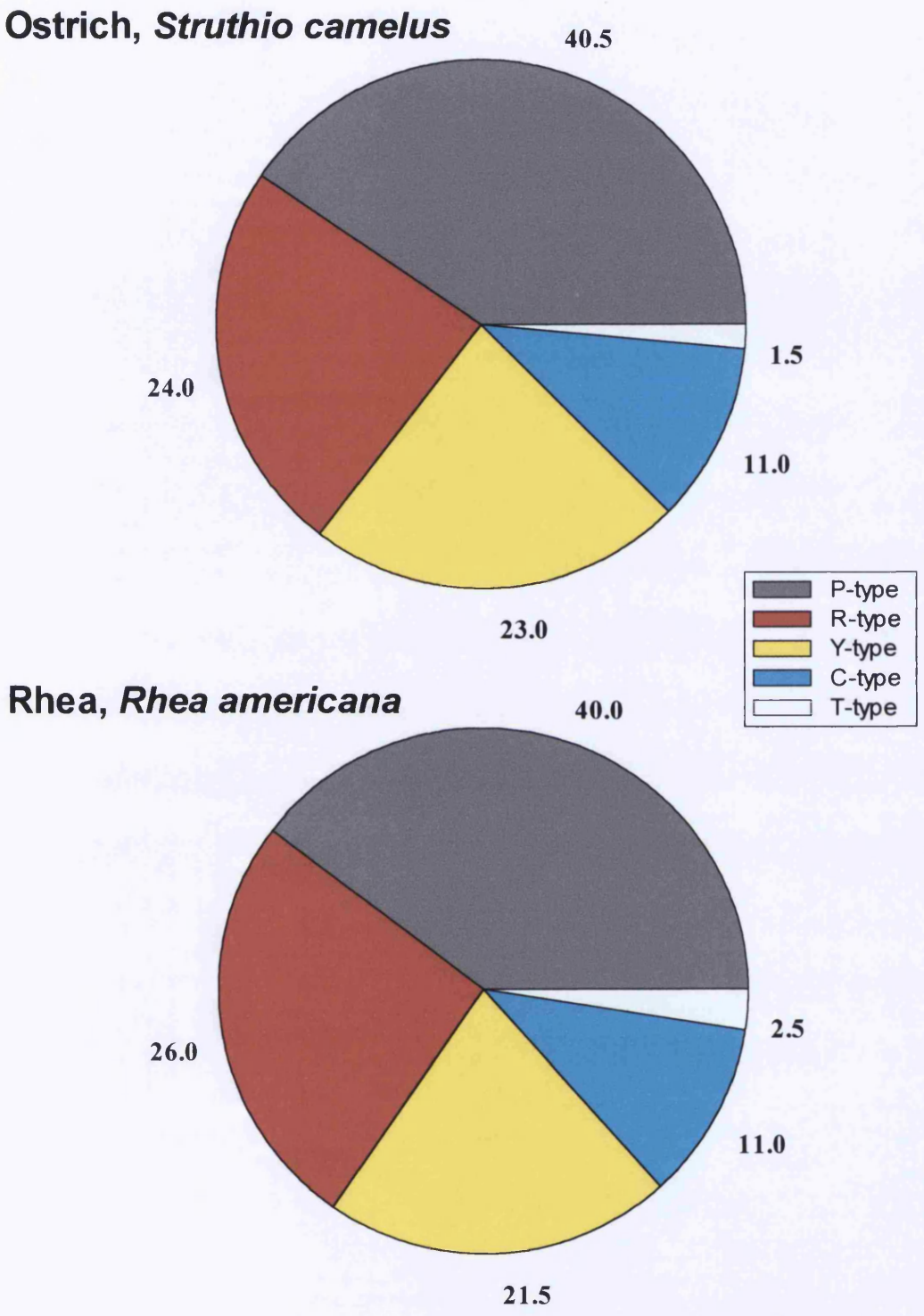


FIGURE 6.3c
Relative ratio of oil droplet types from the retinae of ostrich (upper chart) and rhea (lower chart). Figures next to each segment indicate the percentage of that droplet type.

TABLE 6.3
Comparison of relative percentage of oil droplet types.

	P-type	R-type	Y-type	C-type	T-type
Ostrich Struthio camelus	40.5% (291)	24.0% (172)	23.0% (164)	11.0% (81)	1.5% (12)
Rhea Rhea americana	39.0% (91)	26.0% (61)	21.5% (50)	11.0% (26)	2.5% (6)
Chicken Gallus gallus	50-60%	15-20%	15-20%	~10%	
Duck Anas platyrhynchos	52.0%	22.0%	21.0%	~5%	
Budgerigar Melopsittacus undulatus	40.0%	20.0%	21.0%	10.0%	9.0%
European Starling Sturnus vulgaris	52-57%	15-20%	17-18%	4-10%	3-7%

Figures in brackets indicate the number of each droplet type counted. For comparison, the some neognathous birds are included, namely chicken, *Gallus gallus* (Bowmaker & Knowles, 1977); duck, *Anas platyrhynchos* (Jane & Bowmaker, 1988); budgerigar, *Melopsittacus undulatus*, (Wilkie *et al.*, 1998) and European Starling, *Sturnus vulgaris* (Hart *et al.*, 1998).

DISCUSSION

6.4 *Visual Pigments*

The absorbance spectra of all the visual pigments could be fitted with an A_1 template (ostrich, **Figures 6.0a – 6.0e**; rhea, **Figures 6.0f – 6.0j**), implying that the visual pigments of paleognathous birds are, like their neognathous cousins, typically rhodopsins rather than porphyropsins. This is not an idle observation, since although porphyropsins are normally found only in freshwater species, some terrestrial reptiles are known to possess them (Provencio *et al.*, 1992). Characterisation of the visual systems of various neognathous avian species has revealed that they possess a highly conserved complement of retinal photoreceptors consisting of rods, one class of double cone and four classes of single cone (Bowmaker *et al.*, 1997). This typical avian complement is present in both paleognathous species observed in this study.

Analysis of the MSP measurements for each pigment class reveals the presence of more than just the absorbance peak in each trace. The absorbance spectra of rod, LWS and MWS pigments, in addition to a striking absorbance peak (α-band), possess a small peak around 380 nm (**Figures 6.0a – 6.0c & 6.0g –6.0i**). This is probably the cis-peak (β-band) which represents the 11-*cis* isomer of retinal (Wolbarsht, 1976). Whilst the cis-peak is observed in MSP measurements of LWS and MWS cones, it would not occur naturally in these photoreceptors as they possess oil droplets (R- and Y-type) that would most likely filter short-wave light before it reached the visual pigment.

Light is scattered to a greater degree at shorter wavelengths and this increased scatter could create problems in MSP measurements of SWS and UV/VS photoreceptor outer segments due to the close proximity of the retinal oil droplets. Since UV/VS pigments are paired with transparent droplets (T-types) this is not a problem, however, the SWS pigment of some birds are paired with pigmented C-type droplets (such as those found in the ostrich and rhea, see section 6.5) and this can make the left-hand limb of SWS pigment absorbance spectra particularly noisy (**Figures 6.0d & 6.0j**).

The post-bleach spectrum of the VS pigment possesses a small peak around 405 nm which probably represents incomplete bleaching of this pigment and a larger peak around 470 nm which may reflect the presence of a photostable substance that has leaked into the outer segment from the inner segment.

The λ_{max} of the visual pigments from the ostrich and rhea (**Table 6.0**) are almost identical and probably reflect the close phylogenetic relationship of the two species. Recent studies of mitochondrial DNA indicate that the ostrich belongs to a closely related sister group of the rhea (Härlid *et al.*, 1997; Härlid *et al.*, 1998; Härlid & Arnason, 1999).

Furthermore, the λ_{max} of the visual pigments of the rod, LWS, MWS and SWS cones of the paleognathous birds are strikingly similar to those of many neognathous birds (**Table 6.4**) and appear to be highly conserved across avian species. MSP studies of the penguin (*Spheniscus humboldti*) and tawny owl (*Strix aluco*) reveals that the LWS cone class of these birds possess a visual pigment that is shifted to shorter wavelengths compared to most avian species (**Table 6.4**), however these probably reflect adaptations to their specific environments. The spectral sensitivity of the visual pigments isolated to the rods and MWS cones are similar for all studied birds, including the ostrich and rhea. Whereas, SWS pigments appear to be more variable with λ_{max} ranging from 430 nm to 460 nm.

In contrast, notable differences are found in the spectral sensitivity of the fourth class of single cone that possesses a T-type oil droplet. The visual pigment of these cells is usually either violet-sensitive (VS) or ultraviolet-sensitive (UVS) with λ_{max} close to either 420, 405 or 370 nm (**Table 6.4**). In this study, the only cell of this class measured by MSP from the ostrich contained a visual pigment with a λ_{max} around 405 nm.

The reasons for the possible 'clustering' of the VS and UVS cone pigments are not immediately apparent. UVS cones with λ_{max} close to 370 nm are found in passeriforms, psittaciforms(see **Table 6.4**) and humming birds are also ultraviolet-

TABLE 6.4

	UVS	VS	SWS	MWS	LWS	ROD
Paleognathae:						
Ostrich *(Struthio camelus)*	-	**405**	**444**	**505**	**570**	**504**
Rhea *(Rhea americana)*	-	-	**445**	**506**	**570**	**504**
Galliformes:						
Chicken *(Gallus gallus)*	-	418	455	507	569	509
Japanese quail *(Coturnix japonica)*	-	419	456	505	569	505
Turkey *(Meleagris gallopavo)*	-	420	460	505	564	504
Anseriformes:						
Mallard duck *(Anas platyrhynchos)*	-	420	452	502	570	505
Columbiformes:						
Pigeon *(Columba livia)*	-	409	453	507	568	506
Passeriformes:						
Zebra Finch *(Taeniopygia guttata)*	360-380	-	430	505	567	507
Pekin Robin *(Leiothrix lutea)*	355	-	453	501	567	500
Canary *(Serinus canaria)*	366	-	442	505	569	506
European Starling *(Sturnus vulgaris)*	362	-	449	504	563	503
Psittaciformes:						
Budgerigar *(Melopsittacus undulatus)*	371	-	444	508	564	509
Ciconiiformes:						
Penguin (*Spheniscus humboldti*)	-	403	450	-	543	504
Strigiformes:						
Tawny Owl *(Strix aluco)*	-	-	463	503	555	503

Comparison of λ_{max} of visual pigments for the ostrich, *Struthio camelus*, and rhea, *Rhea americana* with some of those identified by microspectrophotometry (MSP) in neognathous birds. Data for comparison includes: chicken, *Gallus gallus* (Bowmaker & Knowles, 1977; Bowmaker *et al.*, 1997), pigeon, *Columba livia* (Bowmaker, 1977; Bowmaker *et al,* 1997), tawny owl, *Strix aluco* (Bowmaker & Martin, 1978), duck, *Anas platyrhynchos* (Jane & Bowmaker, 1988), penguin, *Spheniscus humboldti* (Bowmaker & Martin, 1985), Peking robin, *Leiothrix lutea* (Maier & Bowmaker, 1992), Japanese quail, *Coturnix japonica* (Bowmaker *et al.*, 1993), budgerigar, *Melopsittacus undulatus*, (Bowmaker *et al.*, 1997), zebra finch, *Taeniopygia guttata* (Bowmaker *et al*, 1997), European Starling, *Sturnus vulgaris* (Hart *et al.*, 1998), canary, *Serinus canaria*, (Das *et al.*, 1999) and the domestic turkey, *Meleagris gallopovo*, (Hart *et al.*, 1999).

sensitive (Goldsmith, 1980). Specific behavioural functions have been attributed to this sensitivity, including the regulation of circadian rhythms, orientation/navigation, foraging and inter- and intra-specific communication such as sexual displays (Bennett & Cuthill, 1994; Maier, 1994; Burkhardt, 1989; Burkhardt & Finger, 1991; Bennett *et al.*, 1996; Bennett *et al.*, 1997; Burkhardt, 1982; Cooper *et al.*, 1986). However, it may be misleading to consider regions of spectral sensitivity in isolation, since these visual functions may equally be applied to the LWS, MWS and SWS cones, though of course tetrachromacy will enhance chromatic discrimination at short wavelengths.

In contrast to the 'truly' ultraviolet-sensitive birds, the ostrich and rhea, as well as the galliforms and anseriforms, are less sensitive to such short wavelengths, with their fourth spectral cone class having maximum sensitivity above 400 nm. Why some birds, such as the ostrich, lack high sensitivity in the ultraviolet remains unanswered. It is notable however, that those birds which have been shown to possess a VS fourth cone class procure their food by foraging on the ground. An exception, of course, is the penguin, *Spheniscus humboldti*, which actively hunts underwater.

Whilst molecular studies may disagree with one another on the basal divergence of *Paleognathae* and *Neognathae*, almost all agree that there is a clustering between galliforms and paleognathous birds (Sibley & Ahlquist, 1990; Caspers *et al.*, 1997; Härlid *et al.*, 1997; Härlid *et al.*, 1998; Härlid & Arnason, 1999). Thus the similarity in the visual range (lack of ultraviolet sensitivity) in both galliforms and paleognaths may reflect a shared ancestral divergence from other avian species.

6.5 *The Oil Droplets*

The oil droplet complement (**Fig. 6.12**) and the λ_{cut} for each specific droplet type in the ostrich and rhea (**Table 6.2**) are remarkably similar to each other and are typical of those seen in most neognathous birds. The relatively high absorbances of the R-, Y-, and C-type droplets suggest that they act as long-pass cut-off filters, whereas this is probably not the case with the P-type droplets that have a relatively low absorbance at longer wavelengths.

Heavily pigmented R-type oil droplets have been isolated in the LWS cones of most birds studied by MSP. However, no R-type droplets were found in the retinae of the tawny owl (Bowmaker & Martin, 1978) and penguin (Bowmaker & Martin, 1985), and some canaries (Das *et al.*, 1999) possessed this retinal droplet type but they were weakly pigmented. As the LWS pigments of the penguin and tawny owl are blue-shifted compared to other birds the lack of long-wavelength filtering R-type droplets is a functional necessity. The weakly-pigmented (low-carotenoid concentration) R-type droplets of the canary are thought to be due to a genetic mutation derived from inbreeding of a domestic population. The heavily pigmented droplets identified in ostrich and rhea retinae will shift the spectral sensitivity of the LWS cone classes to longer wavelengths (**Figure 6.5**).

Pigmented Y-type droplets are found associated with the MWS cone class of most avian species and also the blue-shifted LWS cones of the penguin (Bowmaker & Martin, 1985). The λ_{max}s of MWS pigments are similar to those seen for rod pigments however, Y-type droplets probably act to red-shift the sensitivities of these pigments by up to 40 nm. In the ostrich and rhea for example, the λ_{max} is likely to be shifted from around 505 nm (**Table 6.4**) to about 540 nm (**Figure 6.5**). Interestingly, there is a spectral separation between the rods and MWS cones of mammals and some teleosts of up to 40 nm (Jacobs, 1993; Bowmaker, 1995). Pigmented retinal oil droplets narrow the spectral sensitivity of associated visual pigments and so increase hue discrimination, perhaps in addition to this primary function they also serve to maintain the spectral sensitivities typically seen for other vertebrates.

Although the C-type droplets appear to act as cut-off filters in the ostrich and rhea, this is not the case in all avian species. It has been suggested that the level of absorbance of these droplets is related to the λ_{max} of the UVS/VS cones and the spectral separation between the UVS/VS visual pigment and the SWS pigment (Bowmaker *et al.*., 1997). In species with UVS cones, where the separation is great (about 70 nm), the C-type droplets generally have low absorbance, but in species with VS cones, where the spectral separation is small (only about 30 - 40 nm), the droplets have high absorbance and probably act as cut-off filters. This is the most likely situation in the two paleognathous species, although only a single VS cone

outer segment was recorded in the ostrich with a P405. The high absorbance of the C-type droplets is possibly designed to increase the spectral separation between the maximum sensitivities of the SWS and VS cones. The cut-off of the C-type droplets associated with SWS pigments will cause a narrowing of the bandwidth of the spectral sensitivity of the cone and displace the maximum sensitivity to longer wavelengths to around 470 nm and maintain a more even spread of sensitivity functions throughout the spectrum (**Fig. 6.5**).

The P-type droplets in both ostrich and rhea fall into two distinct populations according to the relative absorbance of the 480-nm shoulder. Variation in the absorbance of this droplet type has also been reported in most neognathous birds (Bowmaker *et al.*, 1997). An extensive study on variations of P-type droplets in the European starling (Hart *et al.*, 1998) indicated a dorso-ventral gradient in the absorbance of the 480-nm shoulder. It is possible that a similar gradient is present in the ostrich and rhea, but the data are not available. The accessory droplet (A-type) contained within the accessory member of the double cones of the ostrich and rhea was very small, which is similar to those identified in the chicken (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) both of which are galliforms (Morris & Shorey, 1967; Bowmaker & Knowles, 1977; Bowmaker *et al.*, 1993). In contrast, other species (e.g. canary, *Serinus canaria*, Das *et al.*, 1999) contain no A-type droplet but low concentrations of carotenoids have been recorded in the distal tip of the inner segments of the accessory members.

Sillman *et al.* (1981) reported single cones without oil droplets in the emu and tinamou, but these were not observed in the ostrich and rhea. However, the accessory member of the double cones does not contain a droplet and these often become separated from the principal member in the preparation of retinal tissue for microspectrophotometry.

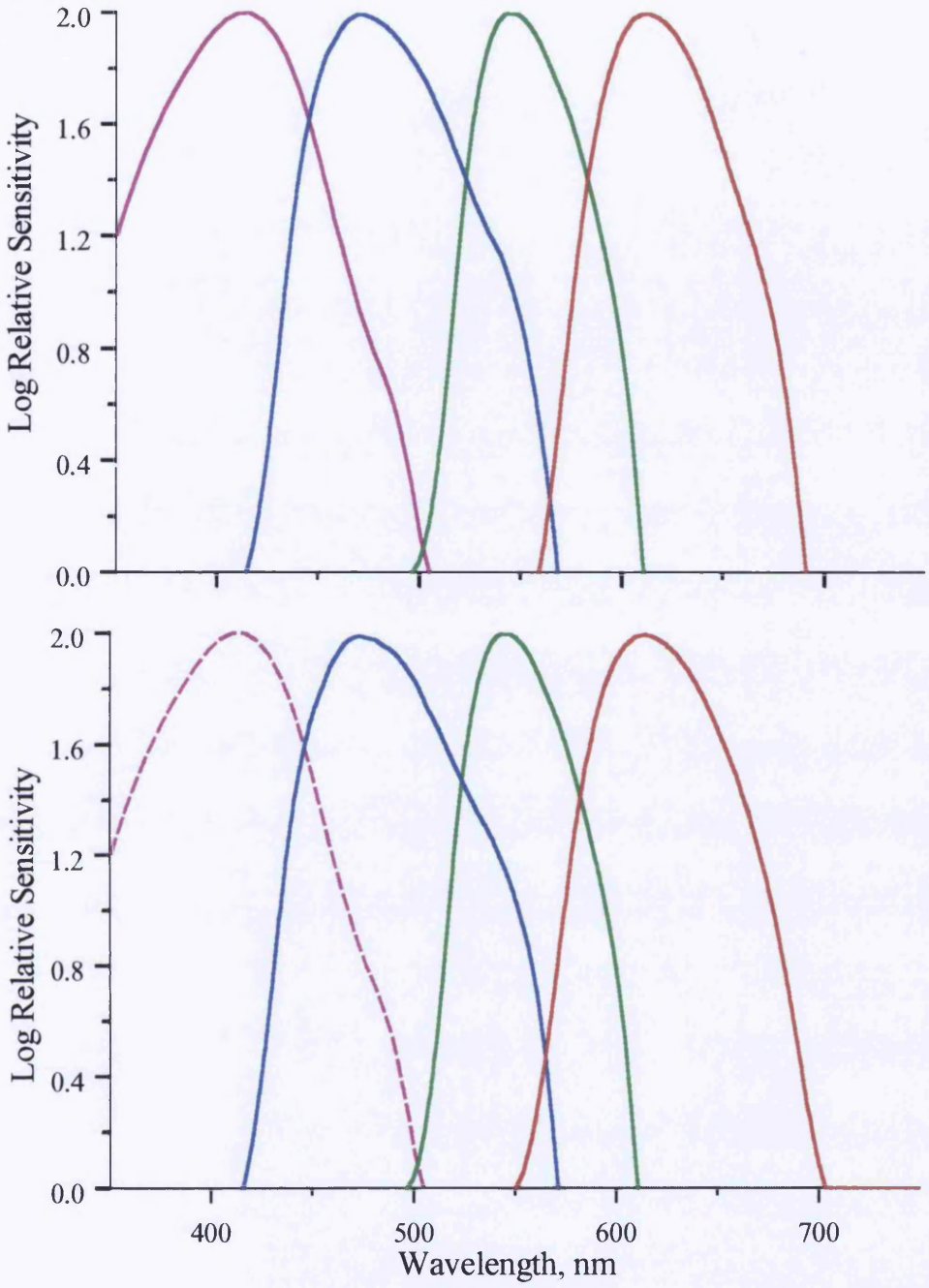


FIGURE 6.5
Relative spectral sensitivities of the four different cone classes from ostrich, *Struthio camelus* (upper panel) and rhea, *Rhea americana* (lower panel). Sensitivities for the four classes of single cone (LWS, MWS, SWS and VS) assuming that R-, Y- and C-type droplets act as cut-off filters. The approximate λ_{max} of the four classes are 615, 545, 470 and 408 nm. As there is no data available from rhea for a fourth single cone class, the relative sensitivity for this class has been added by using data from the ostrich (dotted line).

6.6 *Ocular Media*

The cornea and lens of the ostrich (**Fig. 6.13**) are relatively transparent, but show increased absorbance below about 450 nm with a steep rise below about 370 nm. The absorbance characteristics are more similar to that for the lens of the duck (*Anas platyrhynchos*) (Jane & Bowmaker, 1988) than to the more transparent lens of the canary (Das *et al.*, 1999) and starling (Hart *et al.*, 1998). The marked increase in absorbance of the lens in the near ultraviolet supports the conclusion that the ostrich (and rhea) has a fourth class of single cone that is violet-sensitive and not ultraviolet-sensitive.

6.7 *Cone Type Ratios*

There is a high similarity between the ratios of the different cone types in the ostrich and rhea (**Table 6.3**). As in a number of birds, the LWS and MWS single cones are in roughly equal numbers (between 15% and 25% each), with the double cones comprising the highest percentage of cones (upwards of 50%). The VS/UVS cones in the ostrich and rhea account for only about 2% of the total which is lower than reported in neognathous birds, being as high as 9% in the budgerigar and 3 – 7% in the starling. (**Table 6.3**). Given the extreme scarcity of VS cones in the paleognathous birds, it is not surprising that they were so difficult to locate by MSP. It appears that when the fourth cone class of a bird is VS, rather than UVS, then the cones in this class tend to make up a lower percentage of the total photoreceptor population. These results are in contrast with the previous study by Braekevelt (1998c) on the emu (*Dromaius novaehollandiae*) in which the ratio of rods, single cones and double cones was found to be 10:1:4 respectively, suggesting that the emu has a different photoreceptor complement to other paleognathous birds. However, no retinal oil droplets were identified in the emu by histology but some have been identified by MSP (Sillman *et al.*, 1981). Histological preparation of the retina may have destroyed the oil droplets and therefore altered the morphology of these cells thus making identification of the different cone types very difficult. It is more likely that all paleognathous birds possess a similar photoreceptor complement.

RESULTS & DISCUSSION
MOLECULAR GENETICS

RESULTS

7.0 *Opsin Sequences*

A number of opsin sequences have been successfully isolated, cloned and sequenced from retinal cDNA of both ostrich, *Struthio camelus* and rhea, *Rhea americana*. For each of the sequences that have been isolated all seven transmembrane helical domains have been identified. The table below (**Table 7.0**) illustrates how much of each of the opsins has been sequenced:

TABLE 7.0
The type and length of the seven opsin sequences deduced in this study.

	ROD	LWS	MWS	SWS	VS
Struthio camelus	**1,026 bp**	**899 bp**	**904 bp**	**862 bp**	**1,117 bp**
Rhea americana	**1,026 bp**	**899 bp**	-	-	-

The following pages illustrate the nucleotide and deduced amino acid sequences for rod opsin (**Figure 7.0a**) and the four cone opsins (**Figures 7.0c, 7.0e, 7.0g & 7.0i**). Since data are available from both bird species for the rod and LWS opsins, the nucleotide sequences of these genes are shown in comparison (**Figures 7.0a & 7.0c**). A two-dimensional schematic model for each of the ostrich opsin amino acid sequences are also presented (**Figures 7.0b, 7.0d, 7.0f, 7.0h, 7.0j**).

7.1 *Sequence Comparison*

A list of the abbreviations used to construct the phylogenetic tree and subsequent tables of comparison is detailed in **Table 7.1a**. The data from this study are included in a phylogenetic tree of vertebrate opsins (**Figure 7.1b**) which indicates the similarity of the ostrich and rhea opsin sequences with those of other vertebrates. The identity of the ostrich opsins in comparison to those from all avian opsins that have been sequenced are presented in **Table 7.1b**.

7.2 *Tuning Sites*

The specific residues contained within the seven transmembrane helical regions of ostrich and rhea opsins are shown in comparison to those found in the same helices of other vertebrate opsins (**Table 7.2a**). This table was used for reference purposes in constructing tables of conserved sites and proposed tuning sites. Conserved differences between rod and MWS cone opsins are shown in **Table 7.2b** and proposed tuning sites for the LWS, SWS and UV/VS opsins have also been tabulated (**Tables 7.2c – 7.2f**). An approximation of the positions of the helices relative to eachother are shown in helical opsin diagrams (**Figures 7.2a – 7.2e**) which include the relative positions of those sites suggested by **Tables 7.2c – 7.2f.**

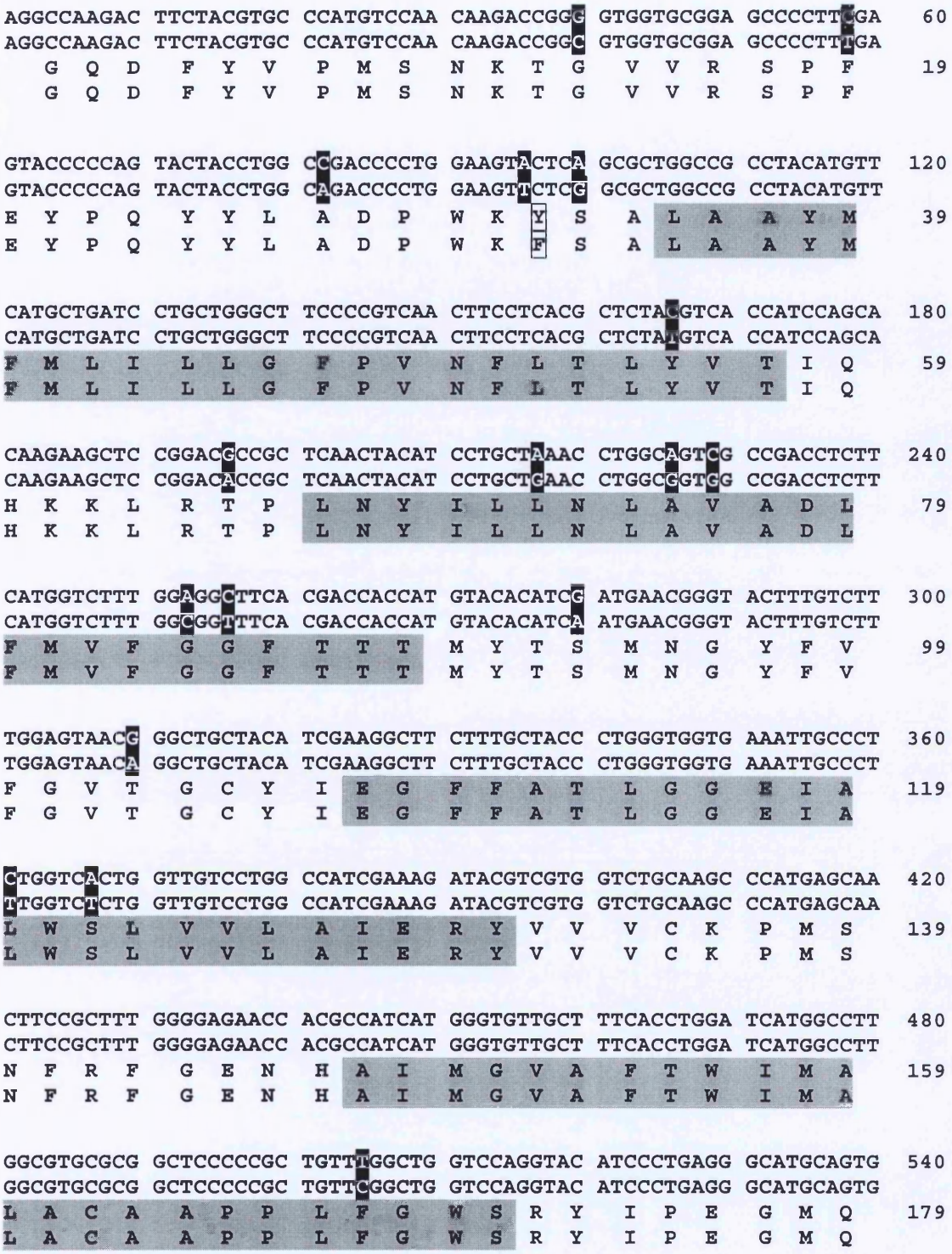


FIGURE 7.0a

Partial cDNA sequences and predicted amino acid translations of the rod opsin gene from ostrich, *Struthio camelus* (upper lines) and rhea, *Rhea americana* (lower lines). Nucleotide substitutions between the two sequences are highlighted in black, amino acid substitutions are boxed and transmembrane helical regions are highlighted in grey. (Continued on next page)

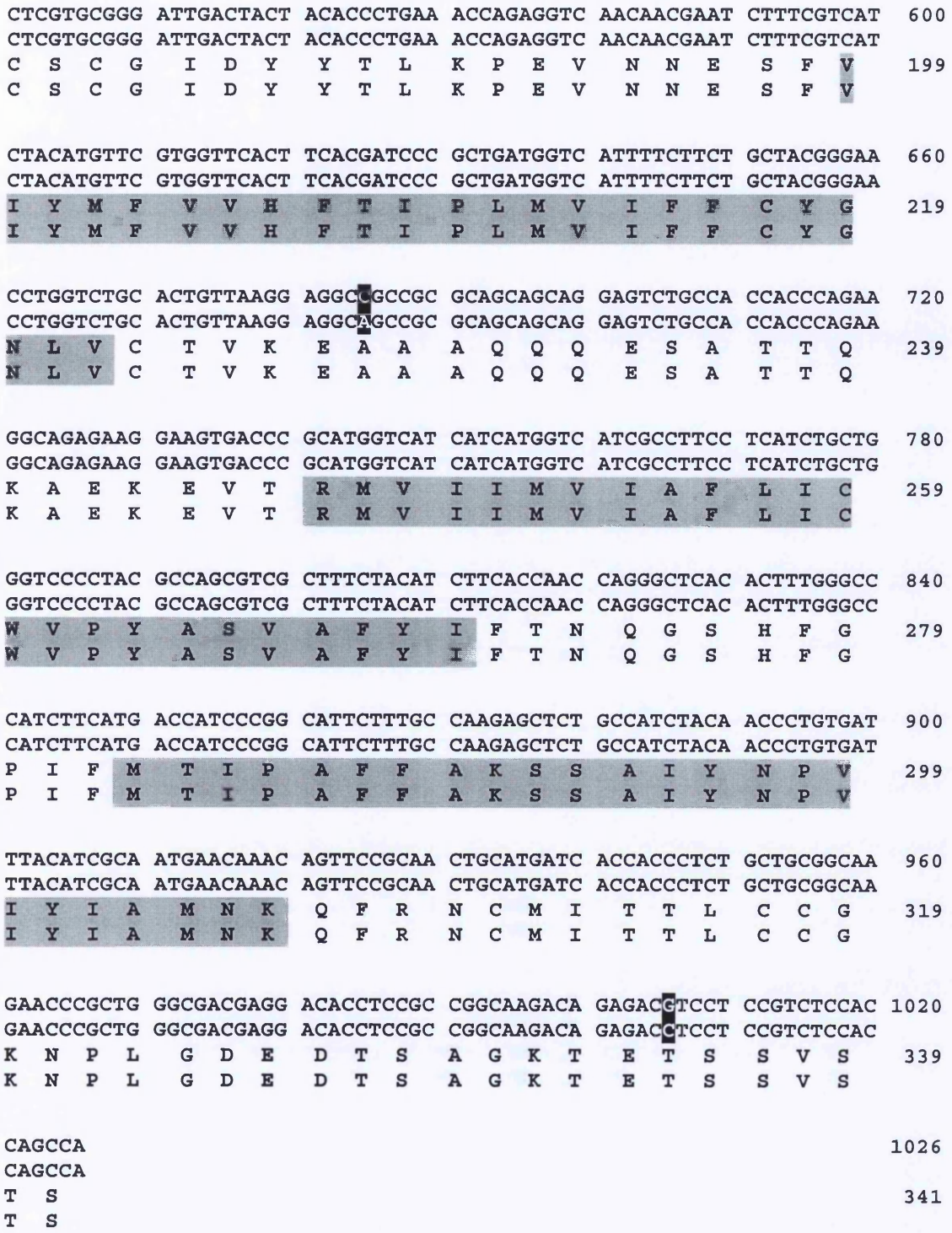
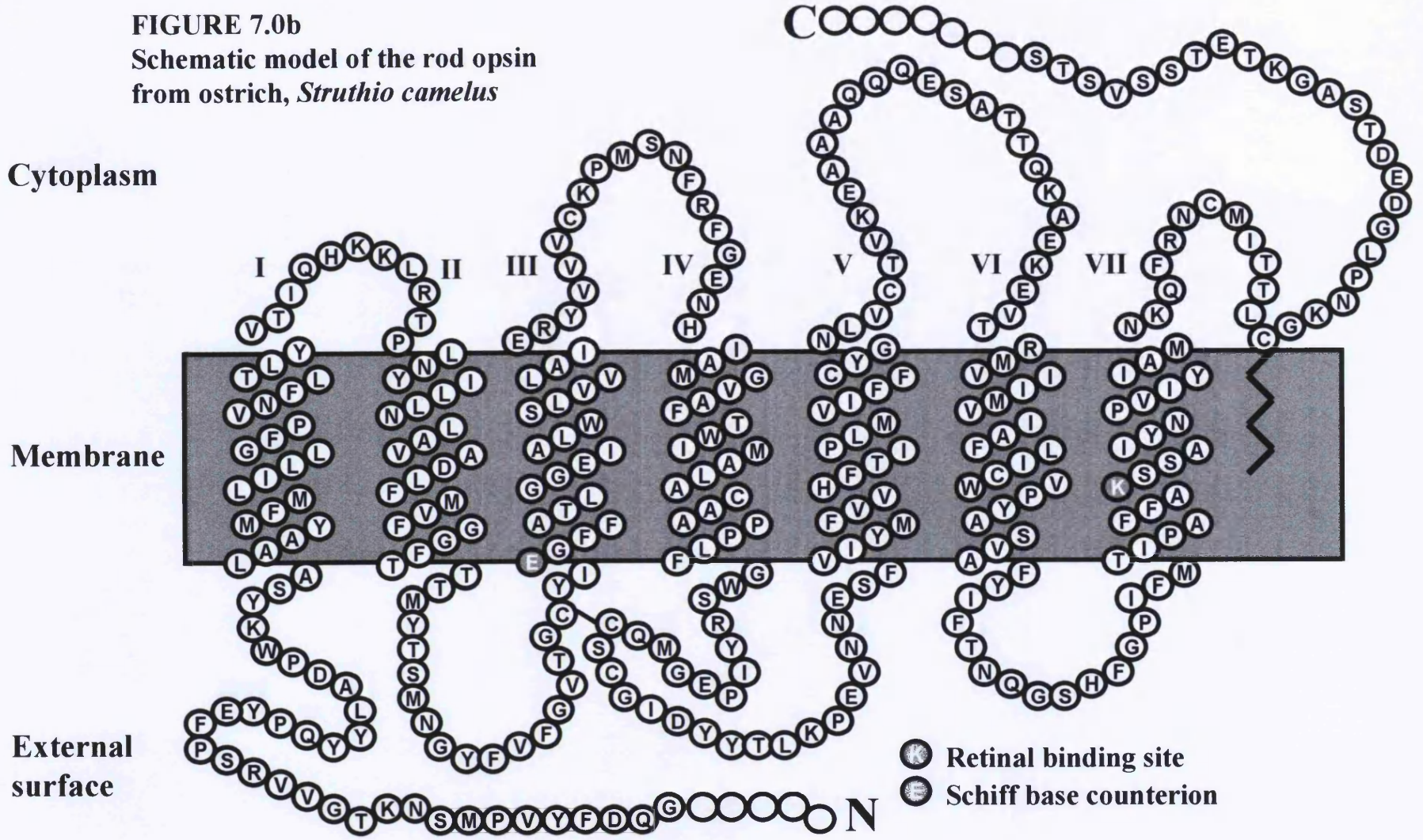


FIGURE 7.0a

Partial cDNA sequences and predicted amino acid translations of the rod opsin gene from ostrich, *Struthio camelus* (upper lines) and rhea, *Rhea americana* (lower lines). Nucleotide substitutions between the two sequences are highlighted in black, amino acid substitutions are boxed and transmembrane helical regions are highlighted in grey. (Continued from previous page)

FIGURE 7.0b
Schematic model of the rod opsin from ostrich, *Struthio camelus*



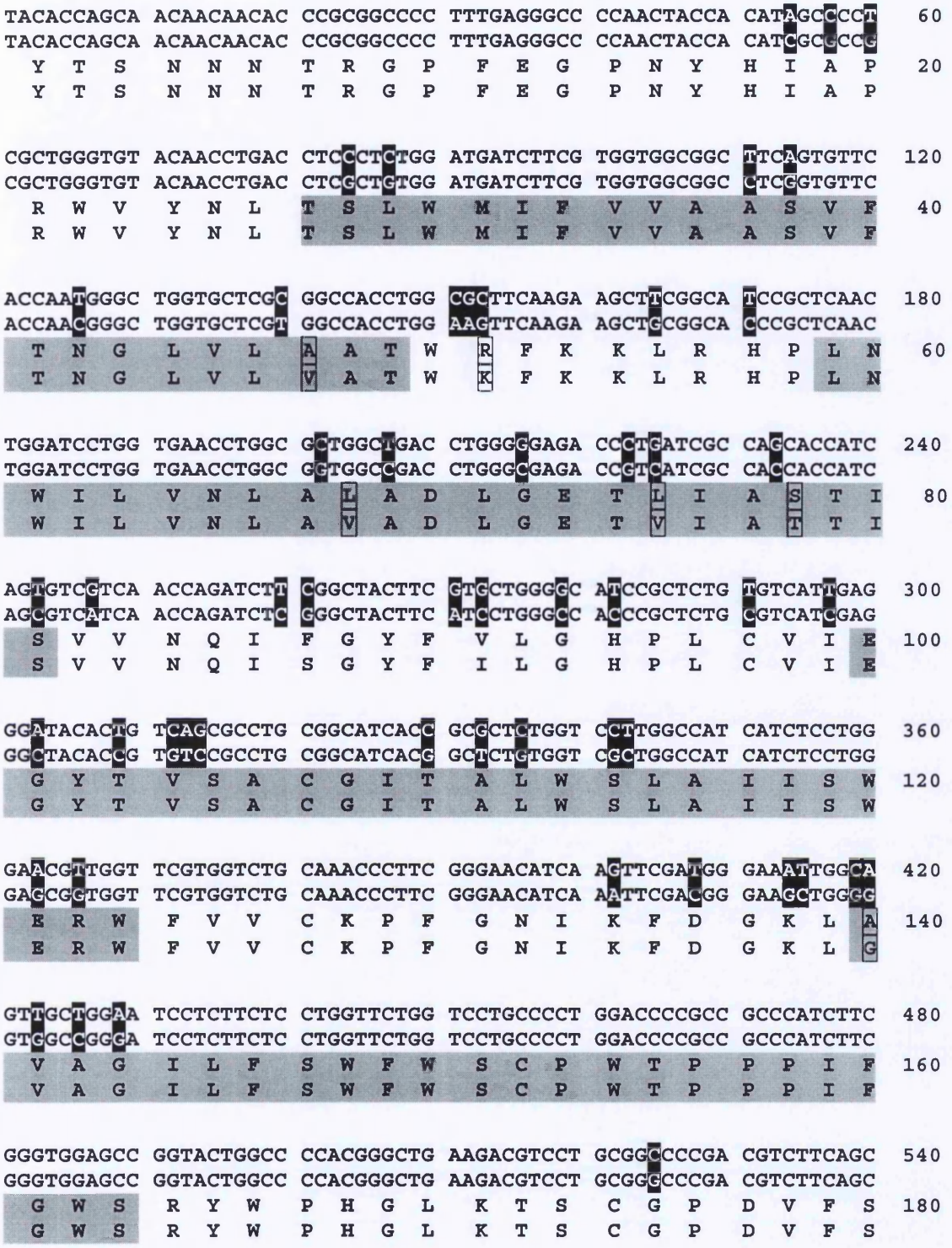


FIGURE 7.0c

Partial cDNA sequences and predicted amino acid translations of the LWS cone opsin gene from ostrich, *Struthio camelus* (upper lines) and rhea, *Rhea americana* (lower lines). Nucleotide substitutions between the two sequences are highlighted in black, amino acid substitutions are boxed and transmembrane helical regions are highlighted in grey. (Continued on next page)

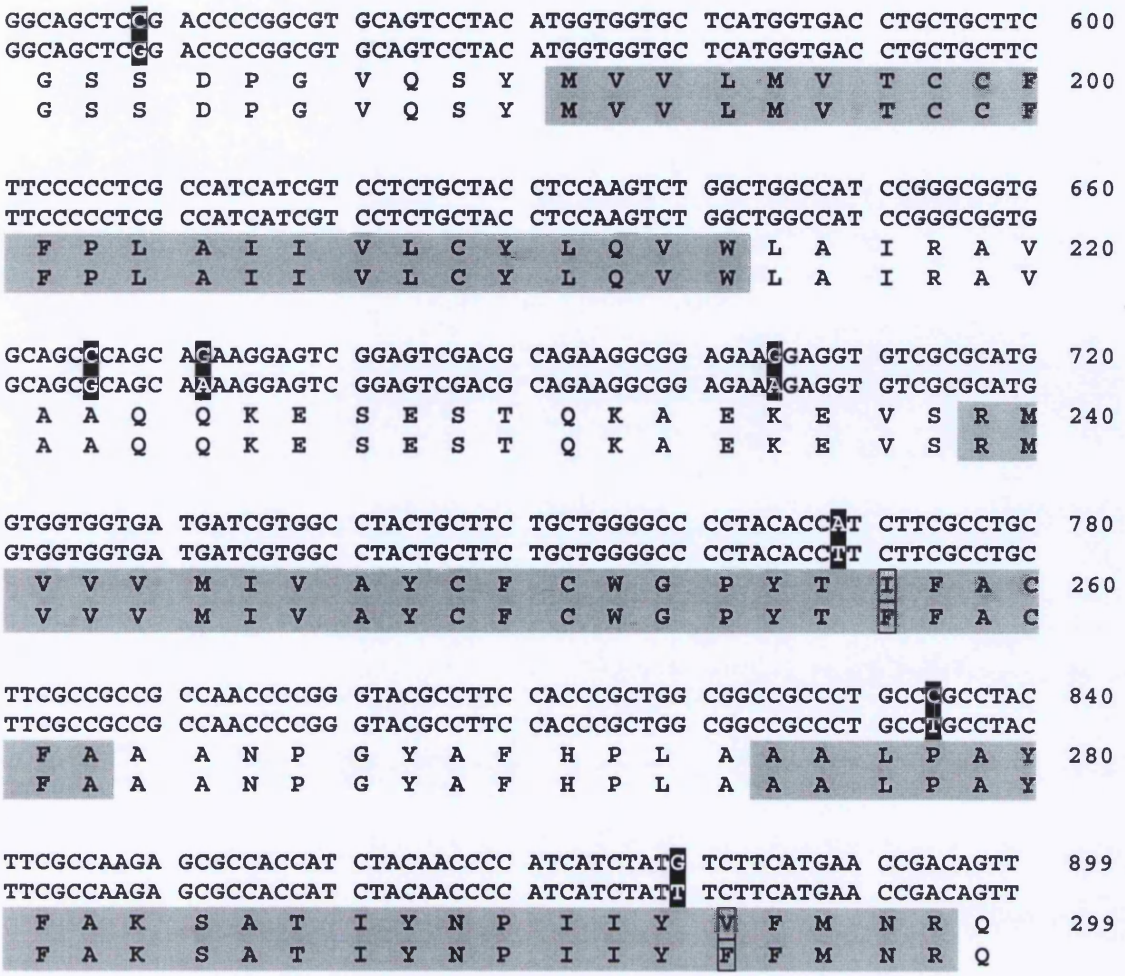
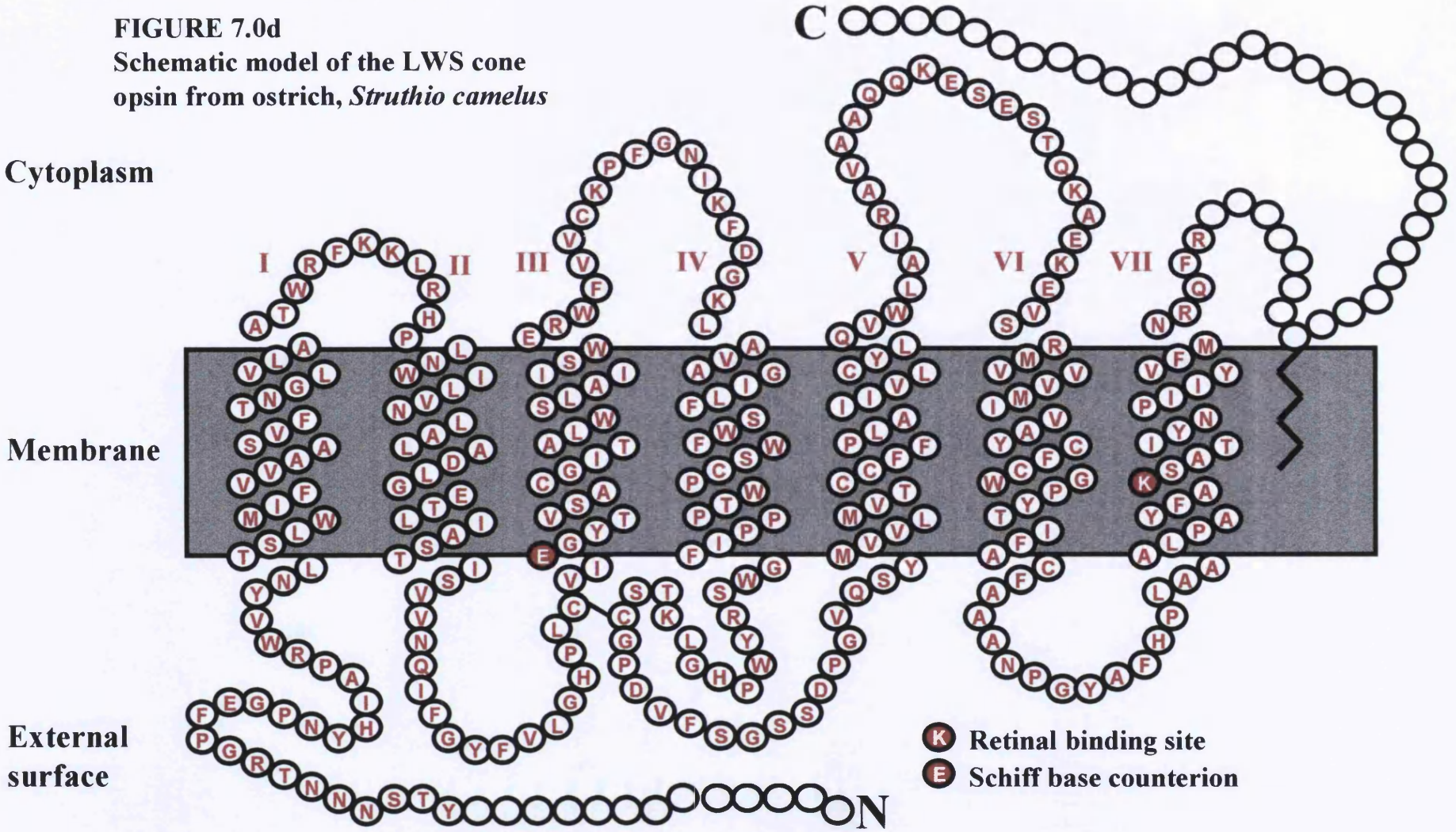


FIGURE 7.0c

Partial cDNA sequences and predicted amino acid translations of the LWS cone opsin gene from ostrich, *Struthio camelus* (upper lines) and rhea, *Rhea americana* (lower lines). Nucleotide substitutions between the two sequences are highlighted in black, amino acid substitutions are boxed and transmembrane helical regions are highlighted in grey. (Continued from previous page)

FIGURE 7.0d
Schematic model of the LWS cone opsin from ostrich, *Struthio camelus*



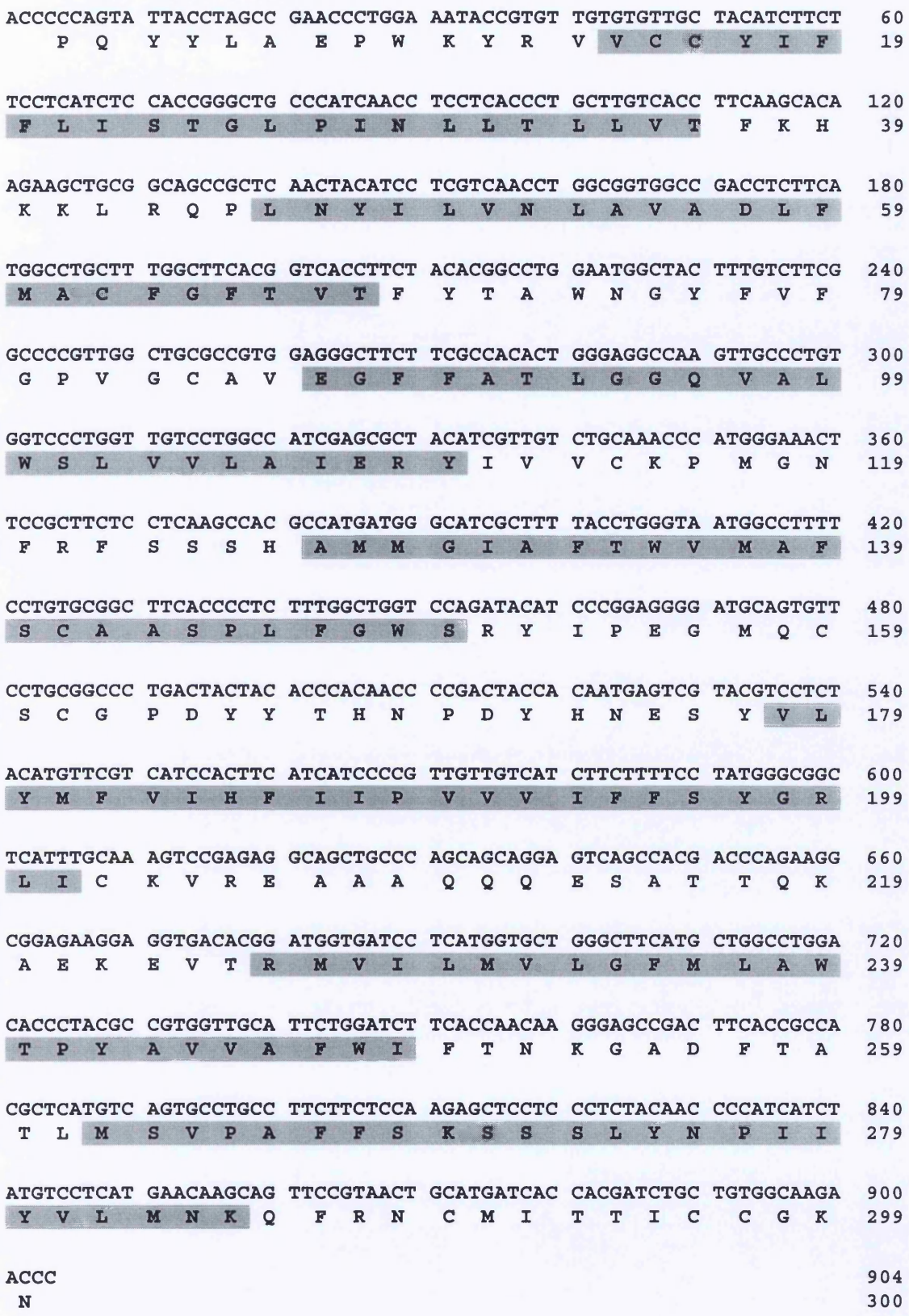
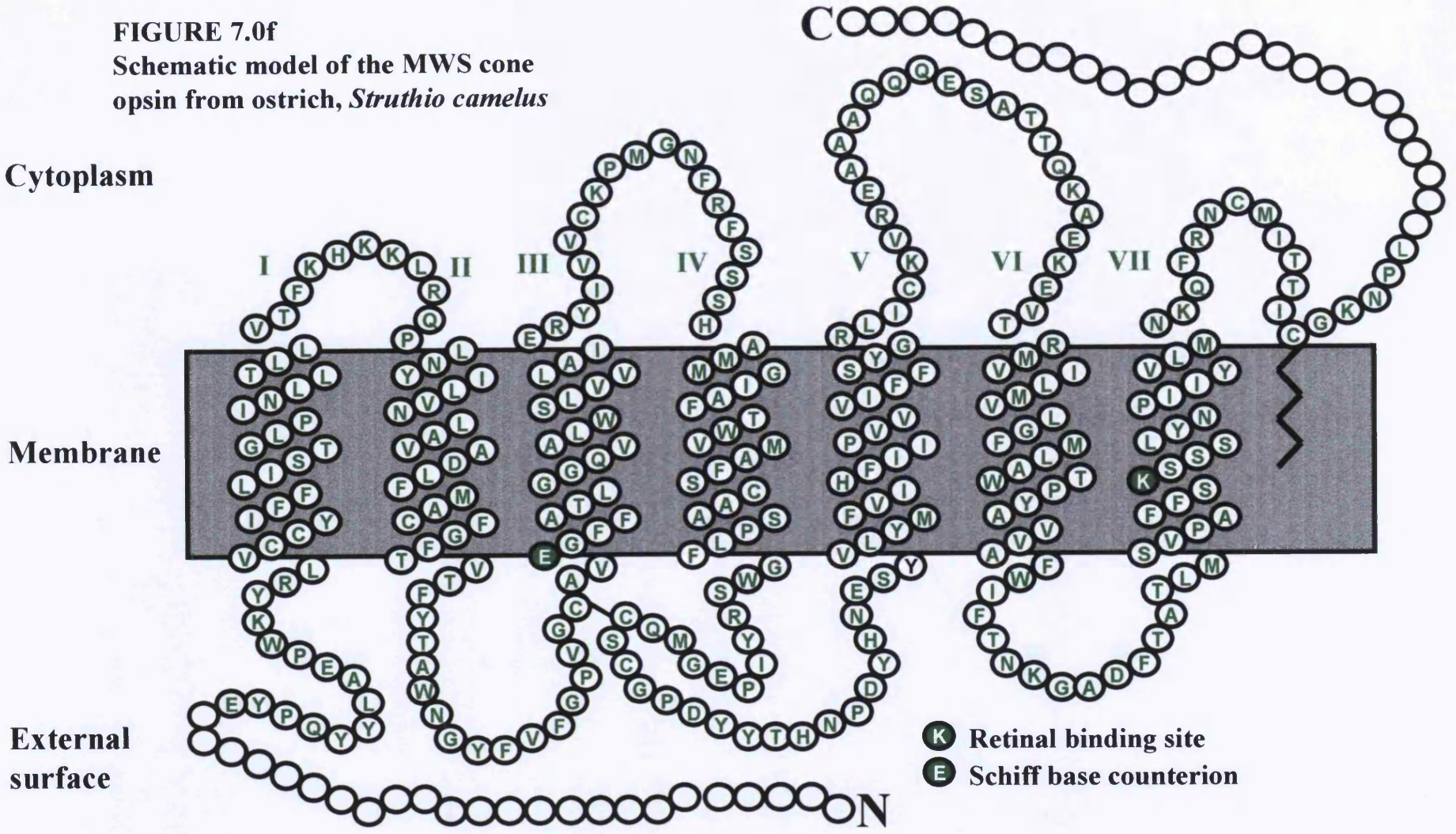


FIGURE 7.0e

Partial cDNA sequences and predicted amino acid translations of the MWS cone opsin gene from ostrich, *Struthio camelus*. Transmembrane helical regions are highlighted in grey.

FIGURE 7.0f
Schematic model of the MWS cone opsin from ostrich, *Struthio camelus*



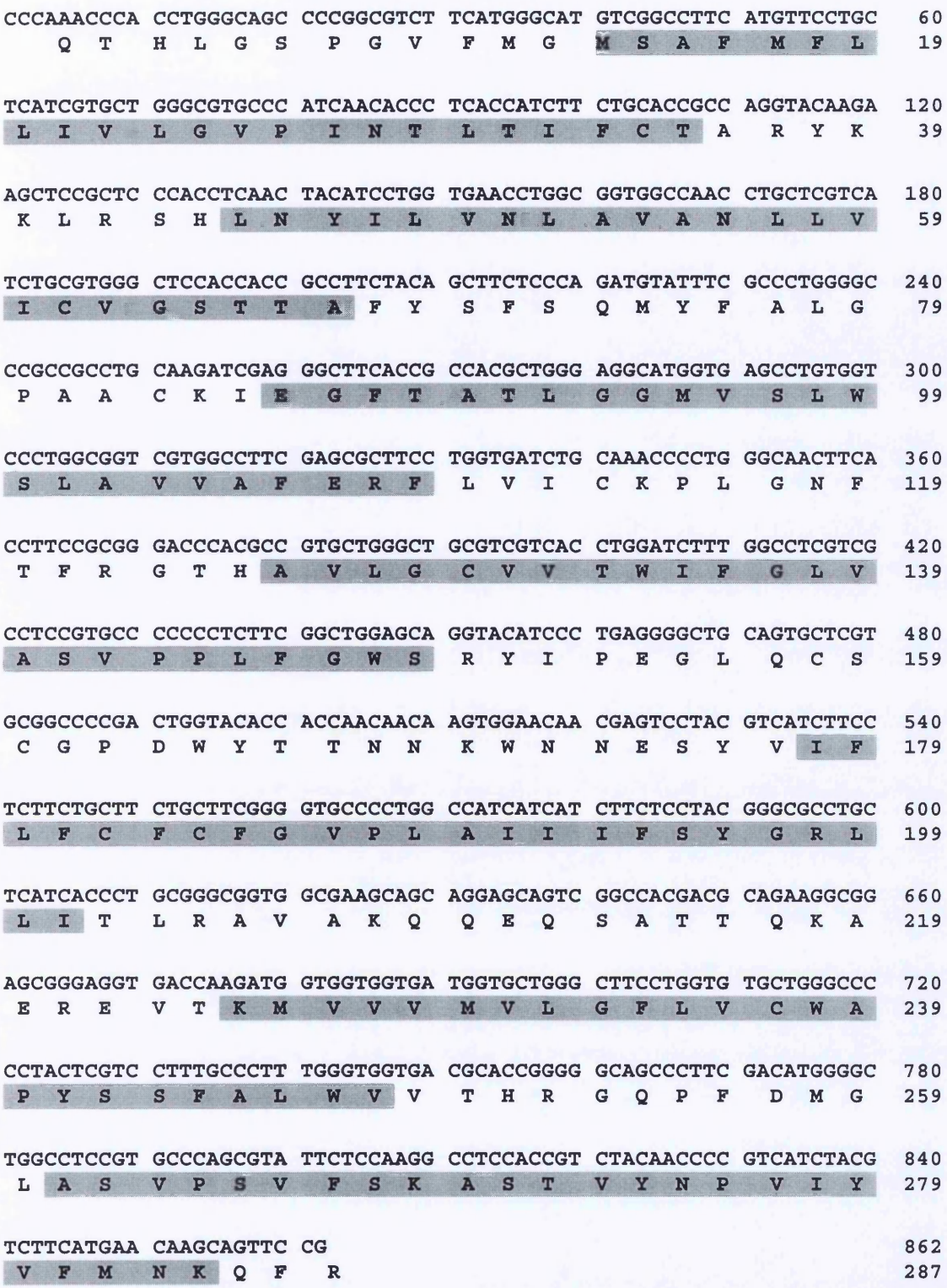
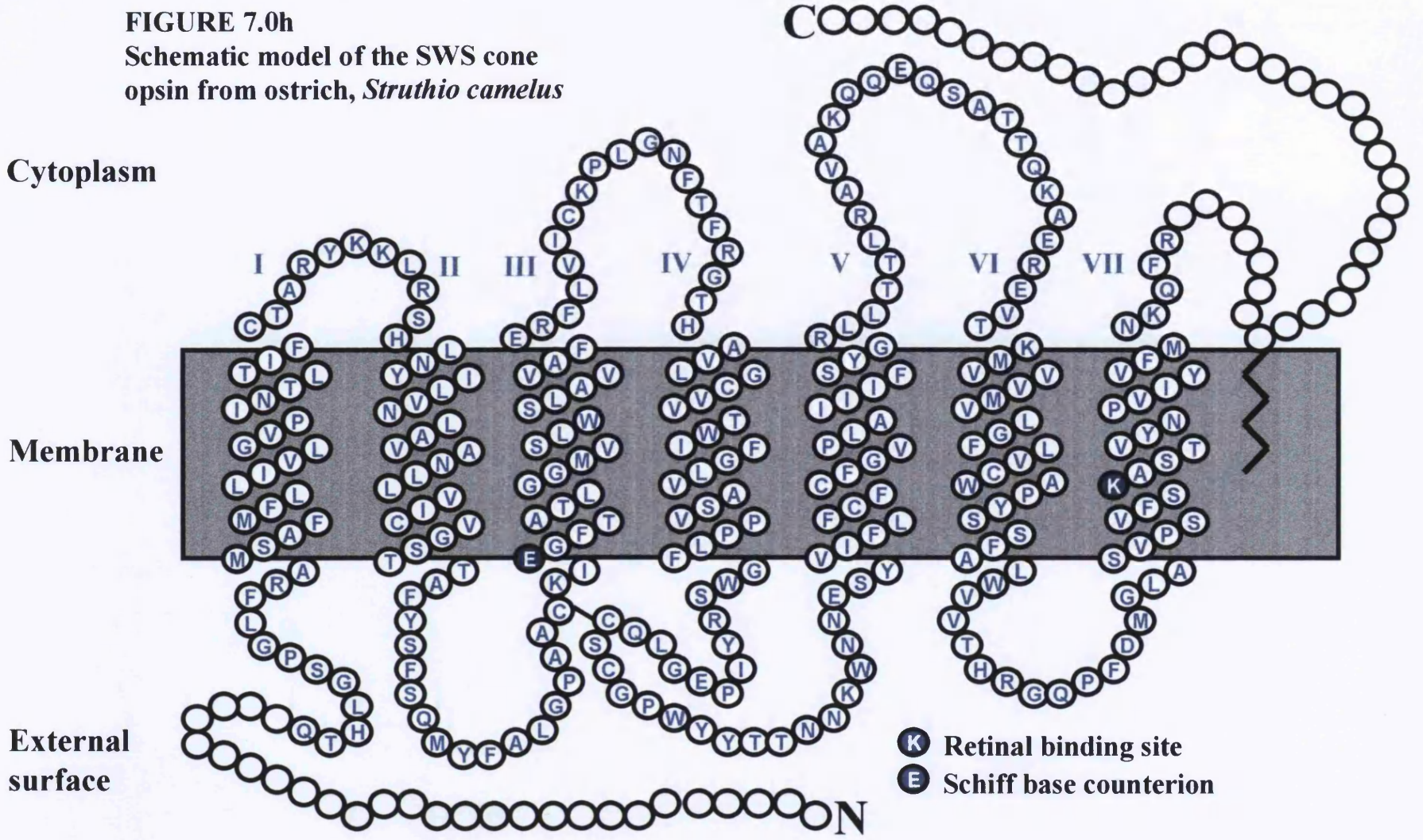


FIGURE 7.0g

Partial cDNA sequences and predicted amino acid translations of the SWS cone opsin gene from ostrich, *Struthio camelus*. Transmembrane helical regions are highlighted in grey.

FIGURE 7.0h
Schematic model of the SWS cone opsin from ostrich, *Struthio camelus*



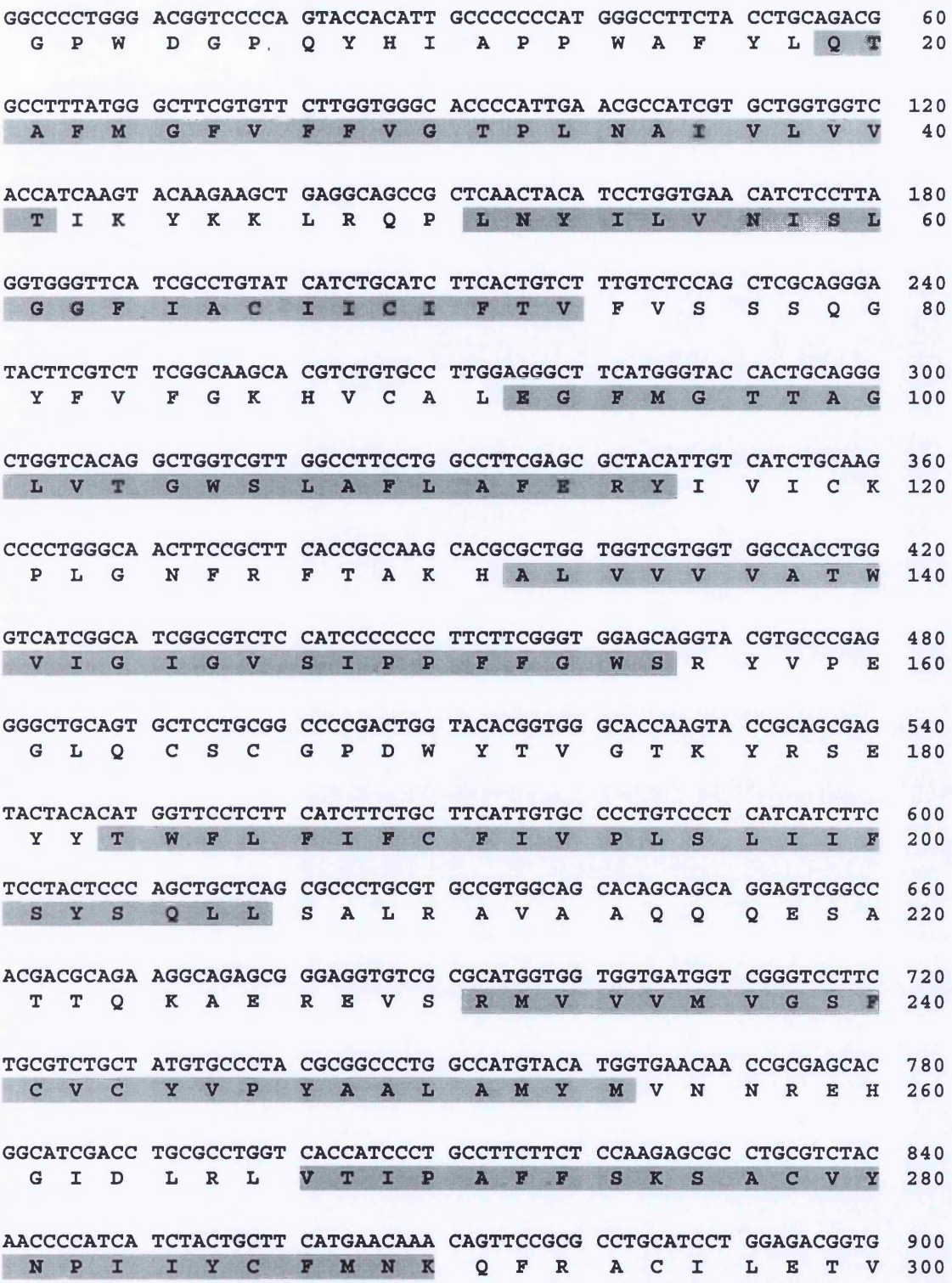


FIGURE 7.0i

Partial cDNA sequences and predicted amino acid translations of the VS cone opsin gene from ostrich, *Struthio camelus*. Transmembrane helical regions are highlighted in grey. (Continued on next page).

```
TGTGGGAAGC CCATGACGGA CGAGTCGGAC ATGTCCAGCT CGGCCCAGAA AACCGAGGTG  960
  C  G  K   P  M  T   D  E  S  D   M  S  S   S  A  Q   K  T  E  V  320

TCGTCCGTGT CCTCCAGCCA GGTCAGCCCC AGCTGAGGGC CCCGCCAGGA CTCCTGGGCC 1020
  S  S  V   S  S  S   Q  V  S  P   S  STOP                        331

CCGCGGGGCT GGCGGCGGGG CCCTAGGCCA GCCTAGGACG TAGGCGCCAG GAGGCAATAA 1080

AGCACCGCCG CGGCGTGACT GCAAAAAAAA AAAAAAA
```

FIGURE 7.0i

Partial cDNA sequences and predicted amino acid translations of the VS cone opsin gene from ostrich, *Struthio camelus*. Transmembrane helical regions are highlighted in grey. (Continued from previous page).

FIGURE 7.0j
Schematic model of the VS cone opsin from ostrich, *Struthio camelus*

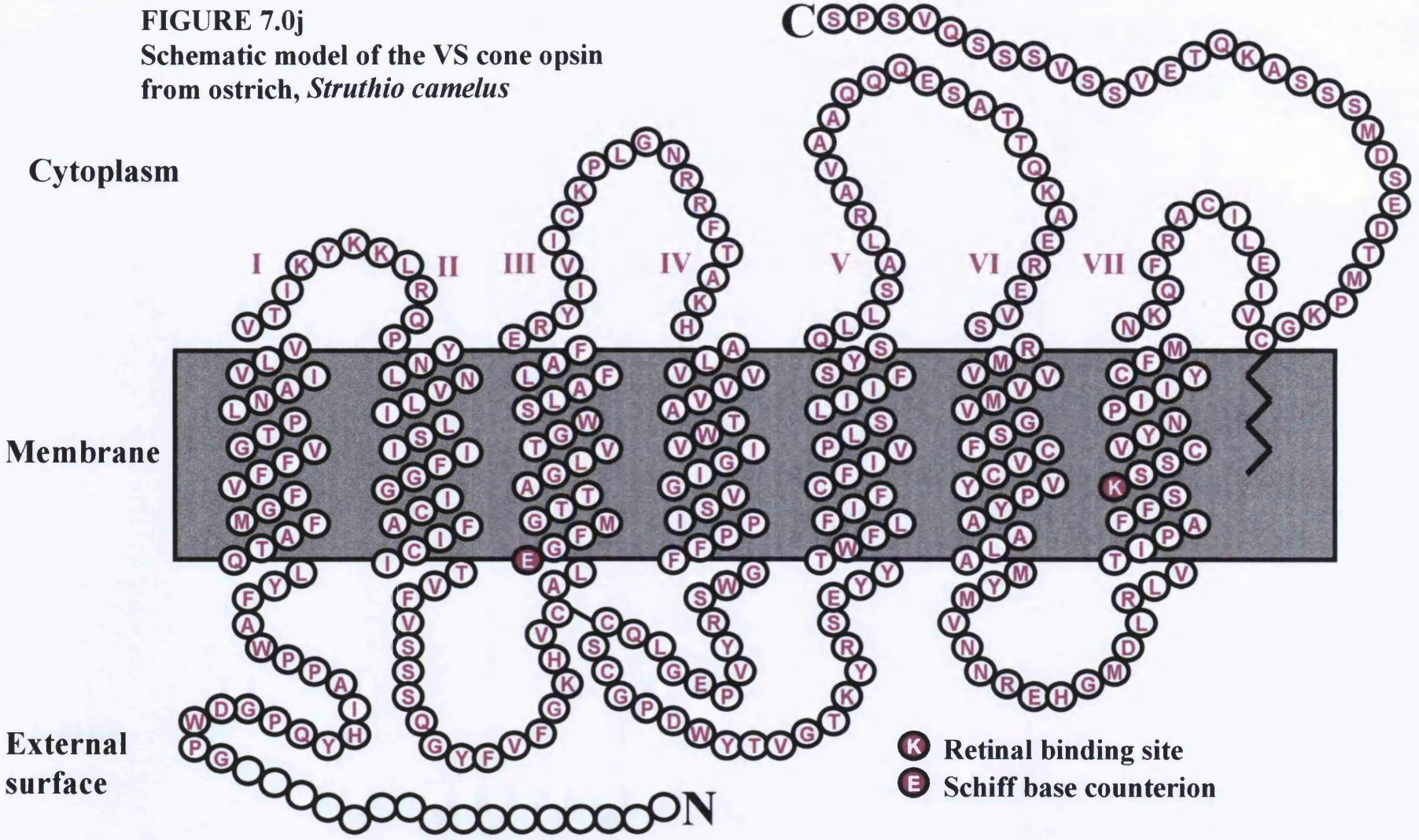


TABLE 7.1a

List of abbreviations and references used to construct the helical tables, tuning site tables and phylogenetic tree. The three digit number that forms part of each abbreviation is the λ_{max} (nm) that has been elucidated for that pigment. Red, black, green, blue and magenta colouration represents L, M_{Rd}, M_C, S_B and S_V opsin classes respectively.

Ost 570	ostrich (*Struthio camelus*)	present thesis
Rh 570	rhea (*Rhea americana*)	present thesis
Ck 569	chicken (*Gallus gallus*)	Kuwata *et al.*, 1990; Bowmaker *et al.*, 1997
Ca 569	canary (*Serinus canaria*)	Das *et al.*, 1999
Gf 614	goldfish *(Carassius auratus*)	Johnson *et al.*, 1993; Hárosi & MacNichol, 1974
Hu 558	human (*Homo sapiens*)	Dartnall *et al.*, 1983; Nathans *et al.*, 1986
Dp 524	dolphin (*Tursiops truncatus*)	Fasick *et al.*, 1998
Ost 504	ostrich (*Struthio camelus*)	present thesis
Rh 504	rhea (*Rhea americana*)	present thesis
Ck 509	chicken (*Gallus gallus*)	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
Ca 506	canary (*Serinus canaria*)	Das *et al.*, 1999
Dk 505`	duck (*Anas platyrhynchos*)	Heath *et al.*, 1998
Bu 509	budgie (*Melopsittacus undulatus*)	Heath *et al.*, 1998
Cr 501	crocodile (*Alligator mississipiensis*)	Smith *et al.*, 1995; Sillman *et al.*, 1991
Fr 502	frog (*Rana pip*iens)	Liebman & Entine, 1968; Fyhrquist *et al.*, 1998
Gf 527	goldfish (*Carassius auratus*)	Johnson *et al.*, 1993; Hárosi & MacNichol, 1974
Hu 496	human (*Homo sapiens*)	Dartnall *et al.*, 1983; Nathans & Hogness, 1984
Dp 488	dolphin (*Tursiops truncatus*)	Fasick & Robinson, 1998
Ost 505	ostrich (*Struthio camelus*)	present thesis
Ck 507	chicken (*Gallus gallus*)	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
Ca 505	canary (*Serinus canaria*)	Das *et al.*, 1999
Dk 502	duck (*Anas platyrhynchos*)	Heath *et al.*, 1998; Bowmaker *et al.*, 1997
Bu 508	budgie (*Melopsittacus undulatus*)	Heath *et al.*, 1998; Bowmaker *et al.*, 1997
Gf 532	goldfish (*Carassius auratus*)	Johnson *et al.*, 1993; Hárosi & MacNichol, 1974

TABLE 7.1a (cont..d)
List of abbreviations and references used to construct the helical tables, tuning site tables and phylogenetic tree. The three digit number that forms part of each abbreviation is the λ_{max} (nm) that has been elucidated for that pigment. Red, black, green, blue and magenta colouration represents L, M_{Rd}, M_C, S_B and S_V opsin classes respectively.

Ost 444	ostrich (*Struthio camelus*)	present thesis
Ck 455	chicken (*Gallus gallus*)	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
Ca 442	canary (*Serinus canaria*)	Das *et al.*, 1999
Bu 444	budgie (*Melopsittacus undulatus*)	Wilkie *et al.*, 1998; Bowmaker *et al.*, 1997
Gf 452	goldfish (*Carassius auratus*)	Johnson *et al.*, 1993
Ck 418	chicken (*Gallus gallus*)	Okano *et al.*, 1992; Bowmaker *et al.*, 1997
Hu 419	human (*Homo sapiens*)	Dartnall *et al.*, 1983; Nathans *et al.*, 1986
Ost 405	ostrich (*Struthio camelus*)	present thesis
Rh ?	rhea (*Rhea americana*)	present thesis
Pg 409	pigeon (*Columba livia*)	Wilkie 1999 ; Bowmaker *et al.*, 1997
Ca 366	canary (*Serinus canaria*)	Das *et al.*, 1999
Bu 371	budgie (*Melopsittacus undulatus*)	Wilkie *et al.*, 1998; Bowmaker *et al.*, 1997
Gf 360	goldfish (*Carassius auratus*)	Hisatomi *et al.*, 1996; Bowmaker *et al.*, 1991
Ms 360	mouse (*Mus musculus*)	Jacobs *et al.*, 1991; Chiu *et al.*, 1994
D.m. Rh3	fruitfly (*Drosophila melanogaster*)	O'Tousa *et al.*, 1985

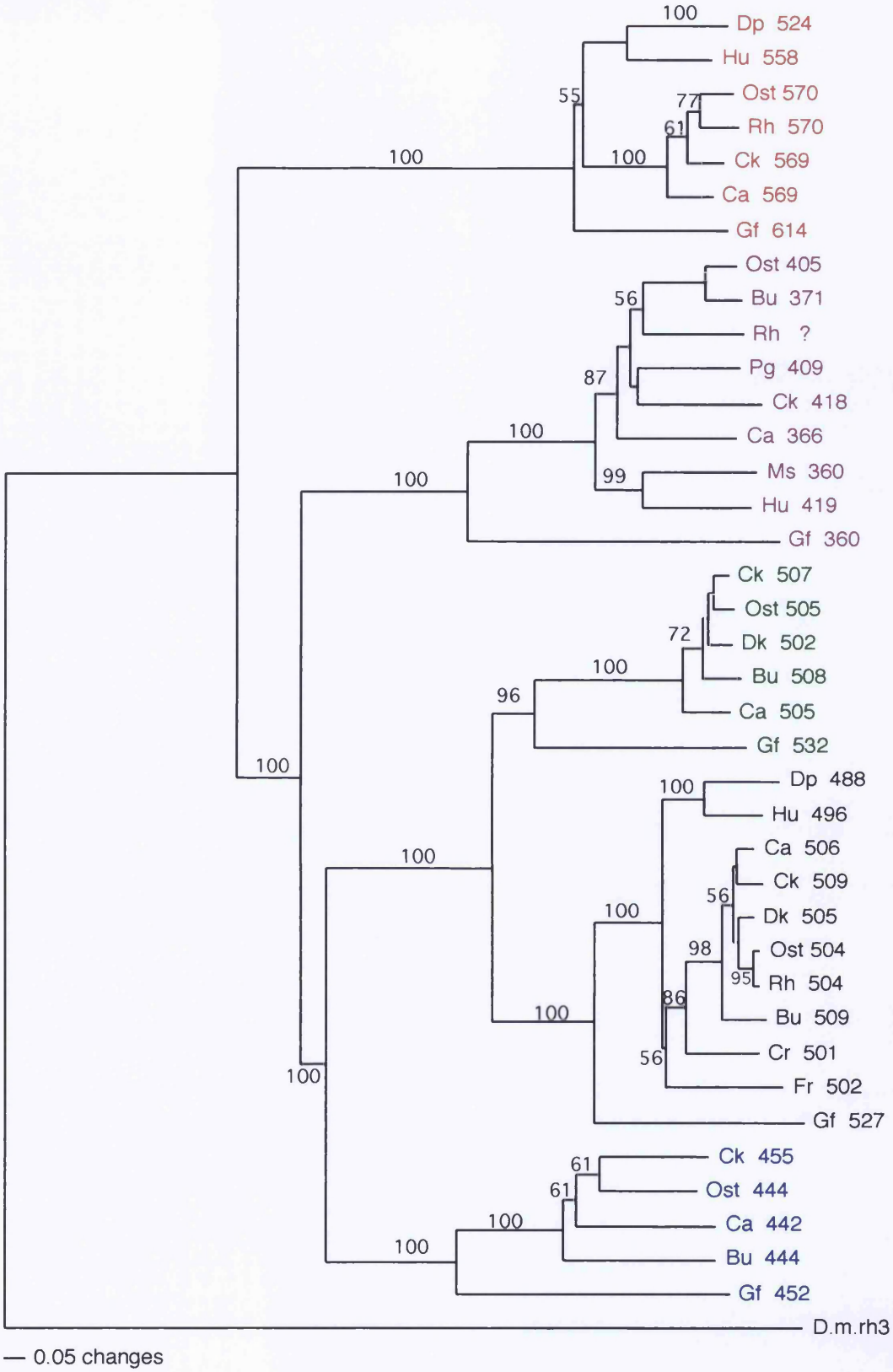


FIGURE 7.1b

Phylogenetic tree of vertebrate opsins based upon amino acid similarity.

The above phylogram was generated using PAUP* version 4.0 from the frequency of amino acid substitutions between sequences, using the Drosophila melanogaster Rh3 opsin as an outgroup. Bootstrap confidence values below 50 are not shown.

TABLE 7.1b

Percentage of identical amino acids per site between the ostrich opsin sequences and those found in rhea (*Rhea americana*), chicken (*Gallus gallus*), canary (*Serinus canaria*), budgerigar (*Melopsittacus undulatus*), duck (Anas platyrynchos) and pigeon (Colomba livia). Each percentage given is the approximate % identity to the putative ostrich opsin in that class. Colouration denotes the opsin class with black, red, green, blue and violet representing the rod, LWS, MWS, SWS and UV/VS opsin classes respectively.

	ROD	LWS	MWS	SWS	UV/VS
Ostrich	100%	100%	100%	100%	100%
Rhea	99%	96%	-	-	89%
Chicken	96%	94%	97%	88%	85%
Canary	97%	95%	95%	86%	79%
Budgerigar	95%	-	-	83%	88%
Duck	97%	-	97%	-	-
Pigeon	-	-	-	-	89%

TABLE 7.2a

The specific residues contained within the seven transmembrane helical regions of ostrich and rhea opsins are shown in comparison to those found in the same helices of other vertebrate opsins. It consists of an allignment of 38 vertebrate opsins, including all known avian opsin sequences and all the opsins of the goldfish (*Carassius auratus*). Colours have been used to highlight each opsin class with black, red, green, blue and violet corresponding to the rod, LWS, MWS, SWS and UV/VS opsin classes respectively. An asterix in the left-hand margin indicates that the site is thought to face into the retinal binding pocket (Baldwin, 1993). Residues that are conserved across all the vertebrate opsins are underlined. aa posn refers to the position of a specific residue within that helix, whereas aa no. refers to its position within the opsin (bovine rod opsin numbering). This table is shown over the next 14 pages.

HELIX I

			LWS OPSINS							ROD OPSINS											MWS OPSINS					
aa posn	aa no.		Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Ost 504	Rh 504	Ck 509	Ca 506	Dk 505	Bu 509	Cr 501	Fr 502	Gf 527	Hu 496	Dp 488	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508	Gf 532
3	40		T	T	T	T	A	T	T	L	L	L	L	L	L	L	L	L	L	L	V	V	V	V	V	L
4	41		S	S	S	S	T	S	S	A	A	A	A	A	A	A	A	A	A	A	C	C	C	C	C	A
5	42		L	L	L	L	L	V	V	A	A	A	A	A	A	A	A	A	A	A	C	C	C	C	C	V
6	43		W	W	W	W	W	W	W	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
7	44	*	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	I	I	I	I	I	M
8	45		I	I	I	I	F	I	V	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
9	46		F	F	F	F	F	F	F	M	M	M	M	M	M	M	L	F	L	L	F	F	F	F	F	F
10	47		V	V	V	V	V	V	V	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
11	48		V	V	V	V	V	V	L	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
12	49		A	A	A	V	V	T	I	L	L	L	L	L	L	I	L	I	V	V	S	S	S	S	S	C
13	50		A	A	A	A	A	A	A	L	L	L	L	L	L	L	L	T	L	L	T	T	T	T	T	L
14	51	*	S	S	S	S	S	S	S	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
15	52		V	V	V	V	T	V	I	F	F	F	F	F	F	F	F	F	F	F	L	L	F	L	L	L
16	53		F	F	F	F	F	F	F	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
17	54		T	T	T	T	T	T	T	V	V	I	I	I	I	I	I	V	I	I	I	I	I	I	I	I
18	55		N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
19	56		G	G	G	G	G	G	G	F	F	F	F	F	F	F	F	F	F	F	L	L	F	L	L	G
20	57		L	L	L	L	L	L	P	L	L	L	L	L	L	L	M	L	L	L	L	L	L	L	L	L
21	58	*	V	V	V	V	V	V	V	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
22	59		L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
23	60		A	A	A	A	V	A	A	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	L	L	L	L	L	I
24	61		A	A	A	A	A	A	A	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	C
25	62		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T

HELIX I

			SWS OPSINS					UV/VS OPSINS								
aa posn	aa no.		Ost 444	Ck 455	Ca 442	Bu 444	Gf 452	Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
3	40		M	M	M	M	M	Q	Q	Q	Q	Q	Q	Q	Q	Q
4	41		S	A	A	A	S	T	A	T	T	T	T	T	A	A
5	42		A	A	A	A	V	A	A	A	A	A	I	A	A	A
6	43		F	F	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	M	M
8	45		F	F	F	A	F	G	G	G	G	G	G	G	G	G
9	46		L	L	L	A	F	I	T	F	I	F	L	F	F	F
10	47		L	L	L	L	I	V	V	V	V	V	V	V	V	V
11	48		I	I	V	V	F	F	F	F	F	F	F	F	F	F
12	49		V	A	V	A	I	A	L	F	F	L	V	M	F	F
13	50		L	L	L	L	G	V	I	V	A	V	A	V	V	V
14	51	*	G	G	G	G	G	G	G	G	G	G	G	G	G	G
15	52		V	V	V	V	A	T	F	T	T	T	T	T	T	T
16	53		P	P	P	P	S	P	P	P	P	P	P	P	P	P
17	54		I	I	I	V	I	L	L	L	L	F	L	L	L	L
18	55		N	N	N	N	N	N	N	N	N	N	N	N	N	N
19	56		T	T	A	A	I	A	A	A	A	A	A	A	A	A
20	57		L	L	L	L	L	V	M	I	I	I	I	I	I	I
21	58	*	T	T	T	T	T	V	V	V	V	V	V	V	V	V
22	59		I	I	V	V	I	L	L	L	L	L	L	L	L	L
23	60		F	F	V	A	L	W	V	V	I	V	I	V	F	V
24	61		C	C	C	C	C	V	A	V	V	V	V	V	V	A
25	62		T	T	T	T	T	T	T	T	T	T	T	T	T	T

HELIX II

			LWS OPSINS							ROD OPSINS											MWS OPSINS					
aa posn	aa no.		Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Ost 504	Rh 504	Ck 509	Ca 506	Dk 505	Bu 509	Cr 501	Fr 502	Gf 527	Hu 496	Dp 488	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508	Gf 532
3	72		L	L	L	L	L	V	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	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	N	N
5	74		W	W	W	W	W	W	W	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	F
6	75	*	I	I	I	I	I	I	M	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
7	76	*	L	L	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	L	L	L	L	L	L	L	L	L	L	L	V	V	V	V	V	V
9	78		N	N	N	K	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
10	79	*	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
11	80		A	A	A	S	A	A	A	A	A	V	A	A	A	A	A	A	A	A	A	A	A	A	A	A
12	81		L	V	V	V	V	V	V	V	V	V	V	V	V	V	F	I	V	V	V	V	V	V	V	V
13	82	*	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	S	A	A	A	A	A	A	A	A
14	83	*	D	D	D	E	D	D	D	D	D	D	D	D	D	D	N	D	D	N	D	D	D	D	D	G
15	84		L	L	L	L	L	L	L	L	L	L	L	L	L	L	H	L	L	L	L	L	L	L	L	A
16	85		G	G	G	G	A	A	A	F	F	F	F	F	F	F	F	F	F	F	F	F	C	F	F	I
17	86	*	E	E	E	E	E	E	E	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
18	87		T	T	T	T	T	T	T	V	V	V	V	V	V	V	V	V	V	V	A	A	A	A	A	V
19	88		V	L	V	V	L	V	V	F	F	F	F	F	F	L	L	F	L	F	C	C	C	C	C	C
20	89		I	I	I	I	L	I	I	G	G	G	G	G	G	G	C	G	G	G	F	F	F	F	F	F
21	90	*	A	A	A	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
22	91		S	T	S	S	S	S	S	T	T	T	T	T	T	F	F	F	F	F	F	F	F	F	F	F
23	92		T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
24	93		I	I	I	I	I	I	I	T	T	T	T	T	T	T	I	T	S	T	V	V	V	V	V	V
25	94		S	S	S	S	S	S	S	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T

HELIX II

			SWS OPSINS					UV/VS OPSINS								
aa posn	aa no.		Ost 444	Ck 455	Ca 442	Bu 444	Gf 452	Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
3	72		L	L	L	L	L	L	L	L	L	L	L	L	L	L
4	73		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
6	75	*	I	I	I	I	I	I	I	I	I	I	I	I	I	I
7	76	*	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
9	78		N	N	N	N	N	N	N	N	N	N	N	N	N	N
10	79	*	L	L	L	L	L	I	V	I	I	I	I	I	I	V
11	80		A	A	A	A	A	S	S	S	S	S	S	S	S	S
12	81		V	L	V	V	V	A	F	L	L	F	V	F	L	L
13	82	*	A	A	A	A	A	S	G	G	G	S	S	C	G	G
14	83	*	N	N	N	N	N	G	G	G	G	G	G	G	G	G
15	84		L	L	L	L	L	F	F	F	F	F	L	F	F	F
16	85		L	L	L	L	L	V	L	I	I	I	M	L	I	L
17	86	*	V	V	V	V	V	S	L	F	F	S	C	A	F	F
18	87		I	I	V	L	L	C	C	C	C	C	C	C	D	C
19	88		C	L	C	L	L	V	I	I	F	I	V	I	T	I
20	89		G	G	G	G	G	F	F	I	F	F	F	I	F	F
21	90	*	V	V	V	V	V	S	S	C	C	S	C	C	S	S
22	91		S	S	S	S	S	V	V	I	V	V	I	I	V	V
23	92		T	T	T	T	T	F	F	F	F	F	F	F	S	F
24	93		T	T	T	T	T	V	P	I	M	T	T	T	Q	T
25	94		A	A	A	A	A	V	V	V	V	V	V	V	V	V

HELIX III

			LWS OPSINS							ROD OPSINS											MWS OPSINS					
aa posn	aa no.		Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Ost 504	Rh 504	Ck 509	Ca 506	Dk 505	Bu 509	Cr 501	Fr 502	Gf 527	Hu 496	Dp 488	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508	Gf 532
3	113	*	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E
4	114	*	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
5	115		Y	Y	Y	Y	F	Y	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
6	116		T	T	T	T	T	T	T	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	M
7	117	*	V	V	V	V	V	V	V	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
8	118	*	S	S	S	S	S	S	S	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
9	119		A	A	A	A	V	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
10	120		C	C	C	C	C	C	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
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	G
12	122	*	I	I	I	I	I	I	I	E	E	E	E	E	E	E	E	E	E	E	Q	Q	Q	Q	Q	E
13	123		T	T	T	T	A	T	T	I	I	I	I	I	I	V	I	M	I	I	V	V	V	V	V	V
14	124	*	A	A	A	A	G	G	G	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A
15	125	*	L	L	L	L	L	L	L	L	L	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	W
17	127		S	S	S	S	S	S	S	S	S	S	S	S	S	C	S	S	S	S	S	S	S	S	S	S
18	128	*	L	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	I	A	A	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
20	130		I	I	I	I	V	I	I	V	V	V	V	V	V	V	A	V	V	V	V	V	V	V	V	V
21	131	*	I	I	I	I	I	I	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
22	132	*	S	S	S	S	S	S	S	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
23	133		W	W	W	W	W	W	W	I	I	V	I	I	I	I	V	F	I	I	I	I	I	I	I	I
24	134		E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E
25	135		R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

HELIX III

			SWS OPSINS					UV/VS OPSINS								
aa posn	aa no.		Ost 444	Ck 455	Ca 442	Bu 444	Gf 452	Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
3	113	✶	E	E	E	E	E	E	E	E	E	E	E	E	E	E
4	114	✶	G	G	G	G	G	A	G	G	G	A	G	G	A	A
5	115		F	F	F	F	F	F	F	F	F	F	F	F	A	F
6	116		T	A	T	T	L	M	L	M	T	V	A	M	M	L
7	117	✶	A	A	A	A	A	G	G	G	G	G	G	G	G	G
8	118	✶	T	T	T	T	T	T	T	S	S	A	A	A	S	S
9	119		L	L	L	L	L	H	V	T	T	T	T	T	I	V
10	120		G	G	G	G	G	G	A	A	A	G	G	A	A	A
11	121	✶	G	G	G	G	G	G	G	G	G	G	G	G	G	G
12	122	✶	M	M	M	M	M	L	L	L	L	L	M	L	L	L
13	123		V	V	V	V	V	V	V	V	V	V	V	V	V	V
14	124	✶	S	S	S	S	G	T	T	T	T	T	T	T	T	T
15	125	✶	L	L	L	L	L	G	G	G	G	G	G	G	G	G
16	126		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
18	128	✶	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	A	A
20	130		V	V	V	V	V	F	F	F	F	F	F	F	V	F
21	131	✶	V	V	V	V	V	L	L	L	L	L	L	L	L	L
22	132	✶	A	A	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	F	F
24	134		E	E	E	E	E	E	E	E	E	E	E	E	E	E
25	135		R	R	R	R	R	R	R	R	R	R	R	R	R	R

HELIX IV

			LWS OPSINS							ROD OPSINS											MWS OPSINS					
aa posn	aa no.		Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Ost 504	Rh 504	Ck 509	Ca 506	Dk 505	Bu 509	Cr 501	Fr 502	Gf 527	Hu 496	Dp 488	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508	Gf 532
3	153		A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
4	154		V	V	V	V	S	I	I	I	I	I	I	I	I	I	M	I	I	I	M	M	M	M	M	S
5	155		A	A	A	A	A	V	A	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	A
6	156		G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
7	157	*	I	I	I	V	I	I	I	V	V	V	V	V	V	V	V	V	V	L	I	I	I	I	I	I
8	158		L	L	L	L	I	A	A	A	A	A	A	V	V	V	A	V	A	A	A	A	A	A	A	A
9	159		F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	L	F	F	F	F	F	F
10	160		S	S	S	S	S	S	S	T	T	S	S	T	T	T	T	T	T	T	T	T	T	T	T	T
11	161		W	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		F	L	L	I	V	I	I	I	I	I	I	I	I	I	I	F	V	I	V	V	V	I	I	V
13	163		W	W	W	W	W	W	W	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
14	164	*	S	S	S	S	S	S	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
15	165		C	C	C	C	A	A	A	L	L	M	L	L	L	L	L	C	L	M	F	F	I	F	F	M
16	166		P	P	A	A	I	V	V	A	A	A	A	A	A	T	A	T	A	A	S	S	S	S	S	A
17	167		W	W	W	W	W	W	W	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
18	168	*	T	T	T	T	C	T	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
19	169		P	P	A	A	A	A	A	A	A	A	A	A	A	A	V	V	A	A	A	A	A	A	A	A
20	170		P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A	S	P	P	P	P	P
21	171	*	P	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		I	I	I	I	I	I	I	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
23	173		F	F	F	F	F	F	F	F	F	F	F	F	F	V	F	V	A	V	F	F	F	F	F	V
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	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	W

HELIX IV

			SWS OPSINS					UV/VS OPSINS								
aa posn	aa no.		Ost 444	Ck 455	Ca 442	Bu 444	Gf 452	Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
3	153		A	A	A	A	A	A	A	A	A	A	A	A	A	A
4	154		V	V	V	V	I	L	L	L	L	L	L	L	L	L
5	155		L	L	L	L	A	L	T	V	V	M	L	V	G	M
6	156		G	G	G	G	G	V	V	V	V	A	V	V	A	V
7	157	*	C	C	C	C	C	V	V	V	V	V	A	V	V	V
8	158		V	V	A	A	I	V	L	V	A	V	V	V	A	L
9	159		V	A	I	V	L	A	A	A	A	A	A	A	L	A
10	160		T	T	T	T	P	T	T	T	T	T	T	T	T	T
11	161		W	W	W	W	W	W	W	W	W	W	W	W	W	W
12	162		I	V	I	V	I	L	T	V	V	V	I	V	I	I
13	163		F	L	F	C	S	I	I	I	I	I	I	I	I	I
14	164	*	G	G	G	G	A	G	G	G	G	G	G	G	G	G
15	165		L	F	L	L	L	V	I	I	I	L	V	I	I	I
16	166		V	V	I	A	A	G	G	G	G	G	G	G	G	G
17	167		A	A	A	A	A	V	V	V	V	V	V	V	C	V
18	168	*	S	S	S	A	S	G	S	S	S	A	A	A	A	S
19	169		V	A	V	T	L	L	I	I	I	L	I	I	T	I
20	170		P	P	P	P	P	P	P	P	P	P	P	P	P	P
21	171	*	P	P	P	P	P	P	P	P	P	P	P	P	P	P
22	172		L	L	L	L	L	F	F	F	F	W	F	F	F	F
23	173		F	F	F	L	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
25	175		W	W	W	W	W	W	W	W	W	W	W	W	W	W

HELIX V

			LWS OPSINS							ROD OPSINS											MWS OPSINS					
aa posn	aa no.		Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Ost 504	Rh 504	Ck 509	Ca 506	Dk 505	Bu 509	Cr 501	Fr 502	Gf 522	Hu 496	Dp 488	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508	Gf 532
3	204		M	M	M	M	M	M	M	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
4	205		V	V	V	V	I	I	I	I	I	I	I	I	I	I	I	I	I	I	L	L	L	L	L	L
5	206		V	V	V	V	V	V	T	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
6	207		L	L	L	L	L	L	L	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
7	208	*	M	M	M	M	M	M	M	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
8	209		V	V	V	V	I	V	I	V	V	V	V	V	V	V	V	I	V	V	V	V	V	I	I	I
9	210		T	T	T	T	T	T	T	V	V	V	V	V	V	V	V	V	V	V	I	I	I	I	T	C
10	211	*	C	C	C	C	C	C	C	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
11	212	*	C	C	C	C	C	C	C	F	F	F	F	F	F	F	F	F	F	F	F	F	S	F	F	F
12	213		F	F	F	F	I	I	F	M	M	M	M	T	M	A	L	I	T	T	I	I	I	I	I	I
13	214		F	F	F	F	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	L
14	215	*	P	P	P	P	P	P	P	P	P	P	P	P	R	P	P	P	P	P	P	P	P	P	P	P
15	216	*	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	M	L	V	V	V	V	V	V
16	217		A	A	A	A	A	A	S	M	M	A	M	L	M	A	I	I	I	V	V	V	V	V	V	T
17	218		I	I	I	V	I	I	V	V	V	V	I	V	I	V	I	V	I	I	V	V	I	V	V	I
18	219	*	I	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		V	V	I	I	I	M	V	F	F	F	F	F	F	F	T	F	F	F	F	F	F	F	F	F
20	221		L	L	L	F	L	L	L	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
21	222	*	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	S	S	S	S	S	T
22	223	*	Y	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		L	L	L	L	I	L	L	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
24	225		Q	Q	Q	Q	A	Q	Q	N	N	N	N	N	N	R	R	R	Q	Q	R	R	R	R	R	R
25	226	*	V	V	V	V	V	V	V	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L

HELIX V

			SWS OPSINS					UV/VS OPSINS								
aa posn	aa no.		Ost 444	Ck 455	Ca 442	Bu 444	Gf 452	Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
3	204		V	V	L	F	V	T	T	T	T	T	T	T	T	T
4	205		I	L	I	L	M	W	W	W	W	W	W	W	Y	W
5	206		F	F	F	F	F	F	F	F	F	F	F	F	F	F
6	207		L	L	S	L	L	L	L	L	L	L	L	L	L	L
7	208	*	F	F	S	F	F	F	F	F	F	F	F	F	L	F
8	209		C	T	C	L	C	I	I	I	I	Y	I	I	V	I
9	210		F	F	F	F	F	F	F	F	F	F	F	F	S	F
10	211	*	C	C	C	C	C	C	C	C	C	C	C	C	C	C
11	212	*	F	F	F	F	F	F	F	F	F	F	F	F	F	F
12	213		G	G	G	G	A	I	I	I	I	I	I	I	M	I
13	214		V	V	F	V	V	V	V	V	V	V	V	V	M	I
14	215	*	P	P	P	P	P	P	P	P	P	P	P	P	P	P
15	216	*	L	L	L	L	F	L	L	L	L	L	L	L	I	L
16	217		A	A	S	S	G	S	S	S	S	S	S	S	M	S
17	218		I	I	V	V	T	L	L	L	L	L	L	L	I	L
18	219	*	I	I	I	I	I	I	I	I	I	I	I	I	I	I
19	220		I	V	V	V	V	I	C	I	I	I	I	I	T	C
20	221		F	F	F	V	F	F	F	F	F	F	F	F	F	F
21	222	*	S	S	S	C	C	S	S	S	S	S	S	S	S	S
22	223	*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
23	224		G	G	G	G	G	S	T	S	S	S	S	S	S	S
24	225		R	R	R	R	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
25	226	*	L	L	L	L	L	L	L	L	L	L	L	L	L	L

HELIX VI

			LWS OPSINS							ROD OPSINS											MWS OPSINS					
aa posn	aa no.		Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Ost 504	Rh 504	Ck 509	Ca 506	Dk 505	Bu 509	Cr 501	Fr 502	Gf 527	Hu 496	Dp 488	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508	Gf 532
3	252		R	R	R	R	R	R	R	R	R	R	R	R	R	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	M
5	254	*	V	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	V	M	I	I	I	I	I	I	I	I	V	I	I	I	I	I	I	I	V
7	256		V	V	V	V	V	V	V	I	I	I	I	I	I	I	I	I	I	I	L	L	L	L	L	V
8	257	*	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
9	258	*	I	I	I	I	I	I	I	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
10	259		V	V	V	L	F	F	F	I	I	I	I	I	I	V	I	I	I	V	L	L	L	L	L	L
11	260		A	A	A	A	A	A	A	A	A	A	S	A	A	S	F	G	A	A	G	G	G	G	G	A
12	261	*	Y	Y	Y	Y	Y	Y	Y	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
13	262		C	C	C	C	C	C	C	L	L	L	L	L	L	L	L	L	L	L	M	M	M	M	M	C
14	263		F	F	F	F	F	V	L	I	I	I	I	I	I	I	I	I	I	I	L	L	L	L	L	F
15	264	*	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	A	A	A	A	A	C
16	265	*	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
17	266		G	G	G	G	G	G	G	V	V	V	V	V	G	V	V	I	V	V	T	T	T	T	T	G
18	267		P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
19	268	*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
20	269	*	T	T	T	T	T	T	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
21	270		I	F	F	I	F	F	F	S	S	S	S	S	S	S	Y	S	S	S	V	V	V	V	V	F
22	271	*	F	F	F	F	C	F	F	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	F
23	272		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A
24	273		C	C	C	S	C	C	C	F	F	F	F	F	F	F	F	W	F	F	F	F	F	F	F	C
25	274		F	F	F	F	F	F	F	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	W	W	W	W	W	F

HELIX VI

			SWS OPSINS					UV/VS OPSINS								
aa posn	aa no.		Ost 444	Ck 455	Ca 442	Bu 444	Gf 452	Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
3	252		K	K	K	R	K	R	R	R	R	R	R	R	R	H
4	253		M	M	M	M	M	M	M	M	M	M	M	M	M	M
5	254	✶	V	V	V	V	V	V	V	V	V	V	V	V	V	V
6	255		V	V	V	V	V	V	V	V	V	V	V	V	V	V
7	256		V	V	V	V	V	V	V	V	V	V	V	V	V	V
8	257	✶	M	M	M	M	M	M	M	M	M	M	M	M	M	M
9	258	✶	V	V	V	V	V	V	V	V	V	V	V	V	V	V
10	259		L	L	L	L	L	G	G	G	G	G	G	G	G	G
11	260		G	G	G	G	G	S	S	S	S	S	S	S	S	S
12	261	✶	F	F	F	F	F	F	F	F	F	F	F	F	F	F
13	262		L	L	L	L	L	C	C	C	C	C	C	C	V	C
14	263		V	V	V	I	V	L	V	V	V	L	M	V	V	L
15	264	✶	C	C	C	C	C	C	C	C	W	C	C	C	C	C
16	265	✶	W	W	W	W	W	Y	Y	Y	Y	Y	Y	Y	Y	Y
17	266		A	A	L	L	A	V	V	V	V	V	V	V	G	V
18	267		P	P	P	P	P	P	P	P	P	P	P	P	P	P
19	268	✶	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
20	269	✶	S	T	C	E	A	A	A	A	A	A	A	A	A	A
21	270		S	A	S	T	S	A	A	A	A	A	A	A	I	A
22	271	✶	F	F	F	F	F	L	F	L	L	L	L	L	T	L
23	272		A	A	A	A	S	A	A	A	A	A	A	A	A	A
24	273		L	L	L	F	L	M	M	M	M	M	M	M	L	M
25	274		W	W	W	W	W	Y	Y	Y	Y	Y	Y	Y	Y	Y

HELIX VII

			LWS OPSINS							ROD OPSINS											MWS OPSINS					
aa posn	aa no.		Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Ost 504	Rh 504	Ck 509	Ca 506	Dk 505	Bu 509	Cr 501	Fr 502	Gf 527	Hu 496	Dp 488	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508	Gf 532
3	288		A	A	A	A	A	A	A	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
4	289		A	A	A	A	A	A	A	T	T	T	T	T	T	T	T	T	T	T	S	A	A	S	S	A
5	290		L	L	L	L	M	L	L	I	I	I	I	I	I	I	V	L	I	I	V	V	V	V	V	I
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	P
7	292	*	A	A	A	A	A	A	S	A	A	A	A	A	A	A	A	A	A	S	A	A	A	A	A	A
8	293		Y	Y	Y	F	Y	F	Y	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
9	294		F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
10	295		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	S	S	S	S	S	S
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	K
12	297		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	T	S	S	S	S	S	S	S	T
13	298		A	A	A	A	A	A	A	S	S	S	S	S	S	S	S	A	A	S	S	S	S	S	S	S
14	299	*	Y	Y	Y	Y	T	I	T	A	A	A	A	A	S	A	A	A	A	S	S	S	S	S	S	A
15	300		I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	V	I	I	L	L	L	L	L	L
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	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	N
18	303	*	P	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	I	I	I	I	V	I	V	V	V	V	V	V	V	V	C	V	V	I	I	I	I	V	V
20	305		I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
21	306	*	Y	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	*	V	F	V	F	V	V	V	I	I	I	I	I	I	I	I	I	I	I	V	V	V	V	V	V
23	308		F	F	F	F	F	F	F	A	A	V	V	V	V	V	M	C	M	M	L	L	L	L	L	L
24	309		M	M	M	M	M	M	M	M	M	M	M	M	M	M	L	M	M	M	M	M	M	M	M	L
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	N

HELIX VII

			SWS OPSINS					UV/VS OPSINS								
aa posn	aa no.		Ost 444	Ck 455	Ca 442	Bu 444	Gf 452	Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
3	288		A	A	A	A	A	V	V	V	V	V	V	V	V	V
4	289		S	S	S	S	T	T	T	T	T	T	T	T	A	T
5	290		V	I	I	V	I	I	I	I	I	I	I	I	I	I
6	291	*	P	P	P	P	P	P	P	P	P	P	P	P	P	P
7	292	*	S	S	S	S	S	A	S	A	A	A	A	A	S	A
8	293		V	V	V	V	C	F	F	F	F	F	F	F	L	F
9	294		F	F	F	F	L	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
11	296	*	K	K	K	K	K	K	K	K	K	K	K	K	K	K
12	297		A	S	A	A	A	S	S	S	S	S	S	S	S	S
13	298		S	S	S	S	S	A	A	S	A	S	S	A	S	S
14	299	*	T	T	T	T	T	C	C	C	C	C	C	C	C	C
15	300		V	V	V	V	V	V	I	V	V	V	V	V	V	V
16	301		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
18	303	*	P	P	P	P	P	P	P	P	P	P	P	P	P	P
19	304		V	V	V	I	V	I	I	I	I	I	I	I	L	I
20	305		I	I	I	I	I	I	I	I	I	I	I	I	I	I
21	306	*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
22	307	*	V	V	V	V	V	C	C	C	C	C	C	C	A	C
23	308		F	L	F	L	L	F	F	F	F	F	F	F	F	F
24	309		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

TABLE 7.2b

A comparison of eight sites from the M_{Rd} (rod opsins- shown in black) and M_C (MWS cone opsins - shown in green) opsins of five avian species. Only those sites that are thought to face the retinal binding pocket (Baldwin, 1993) and are conserved within each opsin class but differ between the two classes are shown. Sites 222 and 295 have been suggested as possible tuning sites for avian MWS opsins (Okano *et al.*, 1992; Heath *et al.*, 1997; Das *et al.*, 1999). Numbering of amino acid sites is as per bovine rod opsin.

aa no.	Ost 504	Ck 509	Ca 506	Dk 505	Bu 509	Ost 505	Ck 507	Ca 505	Dk 502	Bu 508
44	M	M	M	M	M	I	I	I	I	I
122	E	E	E	E	E	Q	Q	Q	Q	Q
157	V	V	V	V	V	I	I	I	I	I
216	L	L	L	L	L	V	V	V	V	V
222	C	C	C	C	C	S	S	S	S	S
264	C	C	C	C	C	A	A	A	A	A
295	A	A	A	A	A	S	S	S	S	S
307	I	I	I	I	I	V	V	V	V	V

FIGURE 7.2a
The relative position of eight sites possibly important in tuning M_R and M_C opsins.

It has been shown that the retinal binding pocket in which the chromophore resides is created by the seven transmembrane helices of an opsin forming a bundle within the membrane, thus creating a hollow cavity on the extra-cellular surface (Baldwin, 1993; Schertler *et al.*, 1983; Unger *et al.*, 1997). This diagram illustrates a schematic three-dimensional opsin structure that provides some indication of the position of the α-helices relative to eachother and the shape of the retinal binding pocket. Each helix is shown as three disks, with the central disk representing a theoretical section through the centre of that helix with the accompanying darker and paler disks respectively representing a section at +3.0Å and -3.0Å relative to the centre disk. The retinal chromophore has been schematically placed within the retinal binding pocket and its site of attachment symbolised by K296 with the Schiff's base counterion shown as E113.

The eight proposed tuning sites from **Table 7.2b** have been added to the opsin diagram. Each of the M_R opsin residues at these sites (circled in black) have been placed in their approximate position, and adjacent to each is the residue found in M_C opsins (circled in green). Also shown is the position of the histidine residue at site 211 (H211) which is exclusive to M_R and M_C opsins.

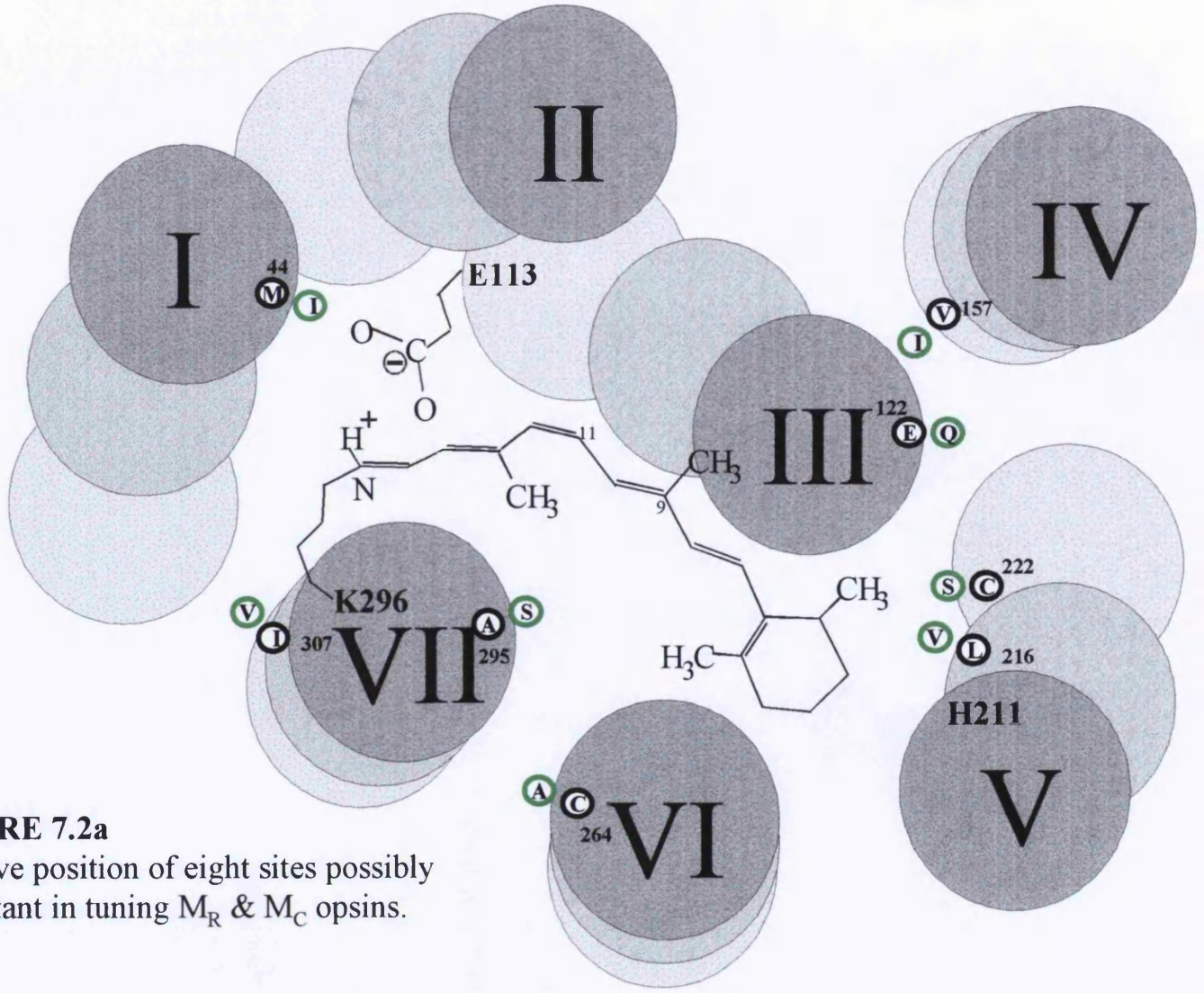


FIGURE 7.2a
Relative position of eight sites possibly important in tuning M_R & M_C opsins.

TABLE 7.2c

Ten sites thought to be important in the spectral tuning of L opsins. Seven of these sites were identified by Asenjo *et al.* (1994) in a comparison of human L_G and L_R opsins. Site 292 is another suggested tuning site, identified by studies of mouse 'green' opsin (Sun *et* al, 1997) and dolphin 'red' opsin (Fasick & Robinson, 1998; Fasick *et al.*, 1999). Sites 181 and 184 have been suggested to face the chloride binding pocket and are found in the second intra-cellular loop (Wang *et al.*, 1993). Numbering of amino acid sites is as per bovine rod opsin.

aa no.	Ost 570	Rh 570	Ck 569	Ca 569	Gf 614	Hu 558	Dp 524	Hu 532	Ms 508
100	S	S	S	S	S	S	S	Y	S
164	S	S	S	S	S	S	A	A	A
181	H	H	H	H	H	H	H	H	Y
184	K	K	K	K	K	K	K	K	K
214	F	F	F	F	I	I	I	T	F
217	A	A	A	A	A	A	S	S	S
261	Y	Y	Y	Y	Y	Y	Y	F	Y
269	T	T	T	T	T	T	T	A	T
292	A	A	A	A	A	A	S	A	S
293	Y	Y	Y	F	Y	F	Y	F	Y

FIGURE 7.2b
The relative position of seven helical sites thought to be important in tuning L opsins..

It has been shown that the retinal binding pocket in which the chromophore resides is created by the seven transmembrane helices of an opsin forming a bundle within the membrane, thus creating a hollow cavity on the extra-cellular surface (Baldwin, 1993; Schertler *et al.*, 1983; Unger *et al.*, 1997). This diagram illustrates a schematic three-dimensional opsin structure that provides some indication of the position of the α-helices relative to eachother and the shape of the retinal binding pocket. Each helix is shown as three disks, with the central disk representing a theoretical section through the centre of that helix with the accompanying darker and paler disks respectively representing a section at +3.0Å and -3.0Å relative to the centre disk. The retinal chromophore has been schematically placed within the retinal binding pocket and its site of attachment symbolised by K296 with the Schiff's base counterion shown as E113.

Only seven of the ten sites shown in **Table 7.2c** have been added to the opsin diagram. Sites 100, 181 and 184 are found within the connecting loops of opsin and so are not within this helical diagram. The approximate position of the other seven sites within the helices are indicated by their amino acid number (circled in red) as per bovine rod opsin.

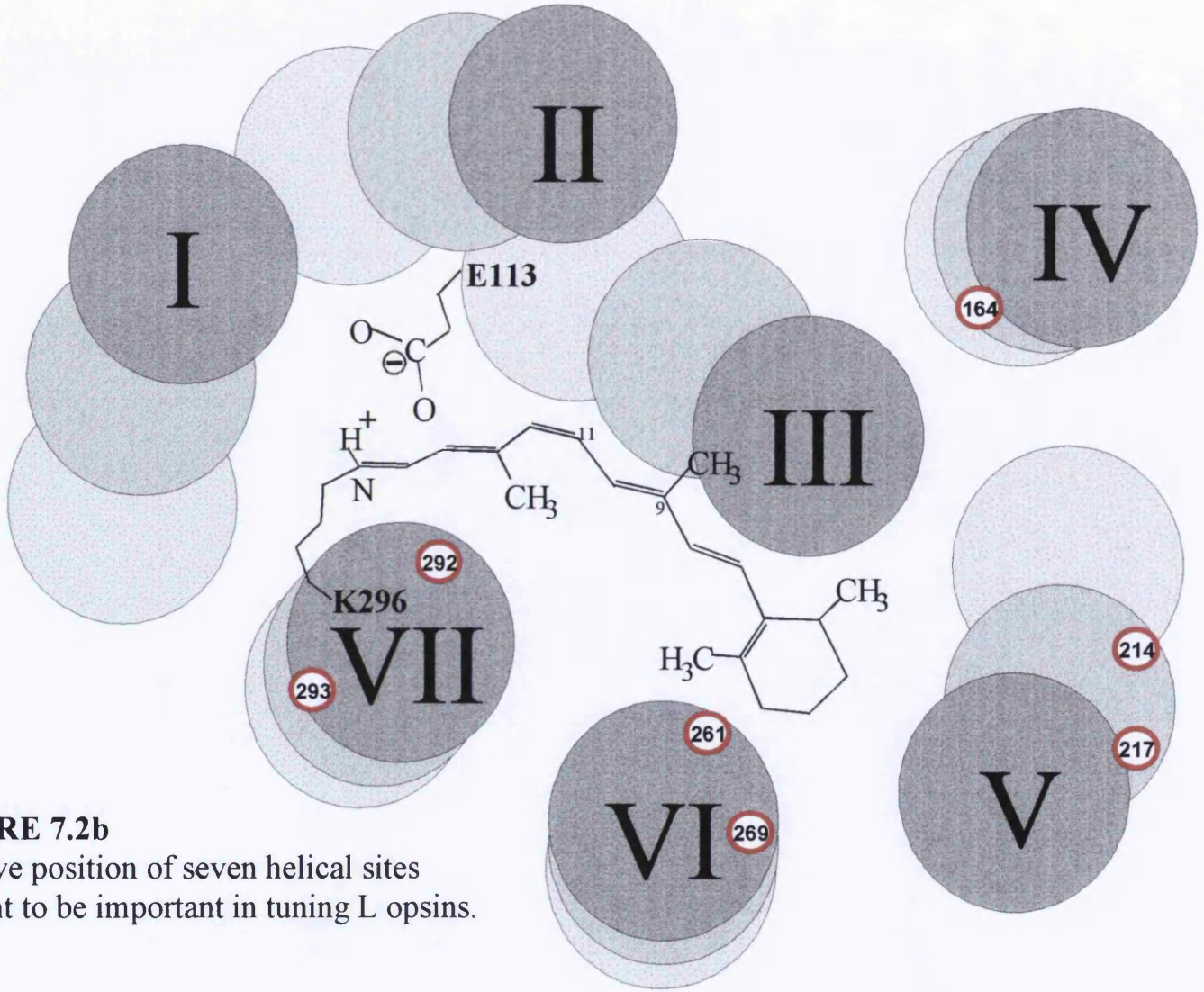


FIGURE 7.2b
Relative position of seven helical sites thought to be important in tuning L opsins.

TABLE 7.2d

Twelve sites that are possibly involved in the tuning of S_B opsins. Sites 124, 289, 292 and 307 were proposed as possible tuning sites by Chang *et al.* (1995). Sites 208 and 269 have also been suggested as possible sites for tuning among the S_B opsins (Das *et al.*, 1999). The other sites shown contain residues that are highly conserved across S_B opsins and have polar/charged residues that face into the retinal binding pocket. Charged/polar residues are underlined and those residues that are conserved only in S_B opsins are shown in black. Numbering of amino acid sites is as per bovine rod opsin.

aa no.	Ost 444	Ck 455	Ca 442	Bu 444	Gf 452
58	T	T	T	T	T
83	N	N	N	N	N
118	T	T	T	T	T
124	S	S	S	S	G
168	S	S	S	A	S
208	C	T	C	L	C
222	S	S	S	C	C
269	S	T	C	E	A
289	S	S	S	S	T
292	S	S	S	S	S
299	T	T	T	T	T
307	V	V	V	V	V

FIGURE 7.2c
The relative position of ten sites thought to be important in tuning S_B opsins..

It has been shown that the retinal binding pocket in which the chromophore resides is created by the seven transmembrane helices of an opsin forming a bundle within the membrane, thus creating a hollow cavity on the extra-cellular surface (Baldwin, 1993; Schertler *et al.*, 1983; Unger *et al.*, 1997). This diagram illustrates a schematic three-dimensional opsin structure that provides some indication of the position of the α-helices relative to eachother and the shape of the retinal binding pocket. Each helix is shown as three disks, with the central disk representing a theoretical section through the centre of that helix with the accompanying darker and paler disks respectively representing a section at +3.0Å and -3.0Å relative to the centre disk. The retinal chromophore has been schematically placed within the retinal binding pocket and its site of attachment symbolised by K296 with the Schiff's base counterion shown as E113.

The ten sites shown in **Table 7.2d** have been added to the opsin diagram. The approximate position of these sites within the helices are indicated by their amino acid number (circled in blue) as per bovine rod opsin.

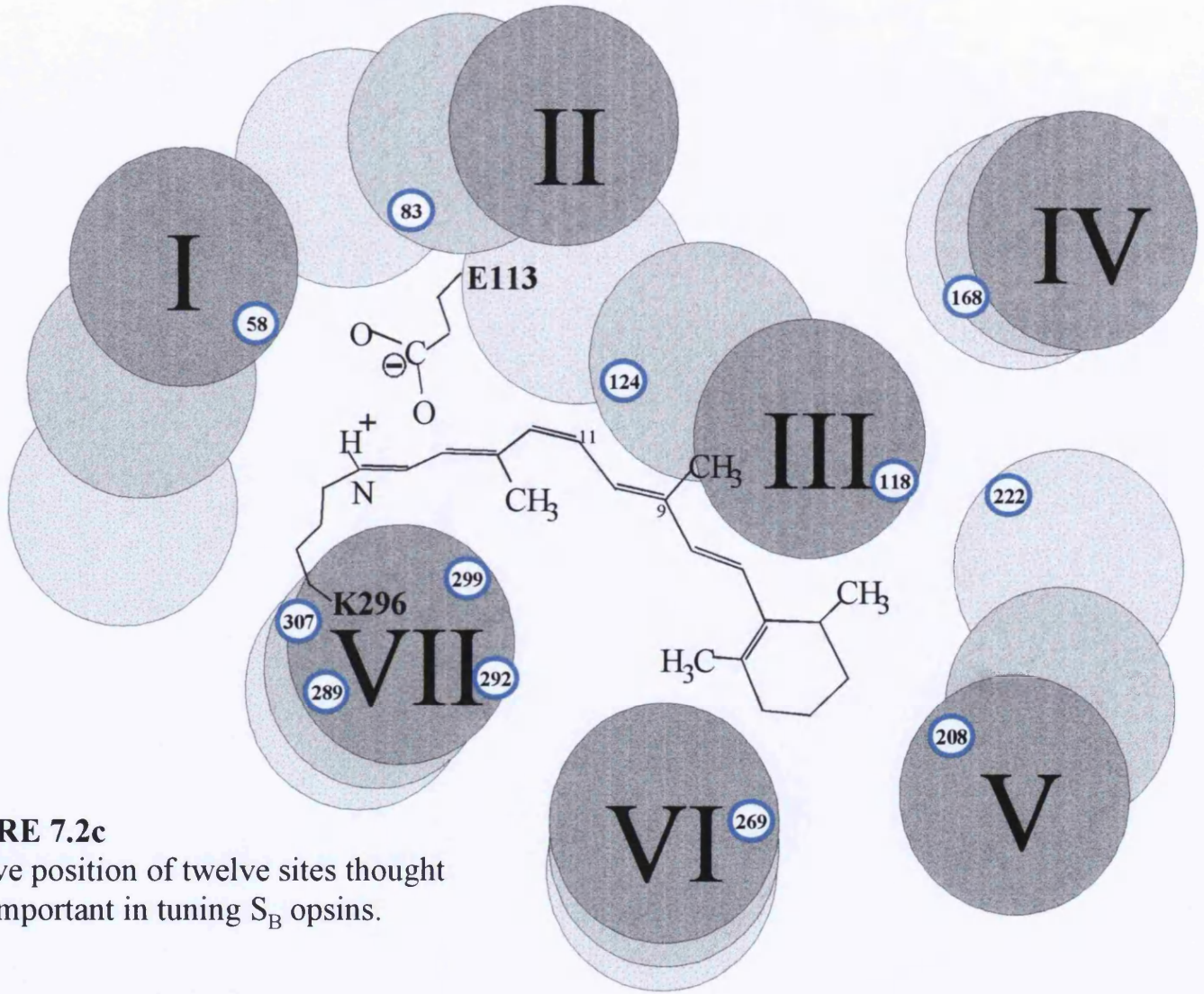


FIGURE 7.2c
Relative position of twelve sites thought to be important in tuning S_B opsins.

TABLE 7.2e

Conserved sites in the S_V opsins. The 17 sites shown in the table below are all highly conserved amongst the S_V opsins and have not been identified in the other vertebrate opsin classes. All of these sites are found in the transmembrane helical regions. Stars indicate those sites thought to face into the retinal binding pocket (Baldwin, 1993). Amino acid numbering is as per bovine rod opsin. Charged/polar residues are underlined.

aa no.	Previously sited by:		Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
40			Q	Q	Q	Q	Q	Q	Q	Q	Q
45	4, 5		G	G	G	G	G	G	G	G	G
117	6	*	G	G	G	G	G	G	G	G	G
124	2, 6	*	T	T	T	T	T	T	T	T	T
125	1,4,5	*	G	G	G	G	G	G	G	G	G
131		*	L	L	L	L	L	L	L	L	L
167	3		V	V	V	V	V	V	V	C	V
204	3,5		T	T	T	T	T	T	T	T	T
205	5		W	W	W	W	W	W	W	Y	W
218			L	L	L	L	L	L	L	I	L
224	3		S	T	S	S	S	S	S	S	S
259	4		G	G	G	G	G	G	G	G	G
265	1,3,4,5,6	*	Y	Y	Y	Y	Y	Y	Y	Y	Y
288			V	V	V	V	V	V	V	V	V
289	2		T	T	T	T	T	T	T	A	T
299	4,5,6	*	C	C	C	C	C	C	C	C	C
307	2,5	*	C	C	C	C	C	C	C	A	C

1 – Yokoyama (1994); **2** – Chang *et al.*, 1985; **3** – Yokoyama (1985); **4** - Hisatomi *et al.*, 1996; **5** – Kawamura & Yokoyama, 1986; **6** – Lin *et al.*, 1998.

FIGURE 7.2d
The relative position of seventeen sites conserved to the S_V opsins.

It has been shown that the retinal binding pocket in which the chromophore resides is created by the seven transmembrane helices of an opsin forming a bundle within the membrane, thus creating a hollow cavity on the extra-cellular surface (Baldwin, 1993; Schertler *et al.*, 1983; Unger *et al.*, 1997). This diagram illustrates a schematic three-dimensional opsin structure that provides some indication of the position of the α-helices relative to eachother and the shape of the retinal binding pocket. Each helix is shown as three disks, with the central disk representing a theoretical section through the centre of that helix with the accompanying darker and paler disks respectively representing a section at +3.0Å and -3.0Å relative to the centre disk. The retinal chromophore has been schematically placed within the retinal binding pocket and its site of attachment symbolised by K296 with the Schiff's base counterion shown as E113.

The seventeen sites shown in **Table 7.2e** have been added to the opsin diagram. The approximate position of these sites within the helices are indicated by their amino acid number (circled in violet) as per bovine rod opsin.

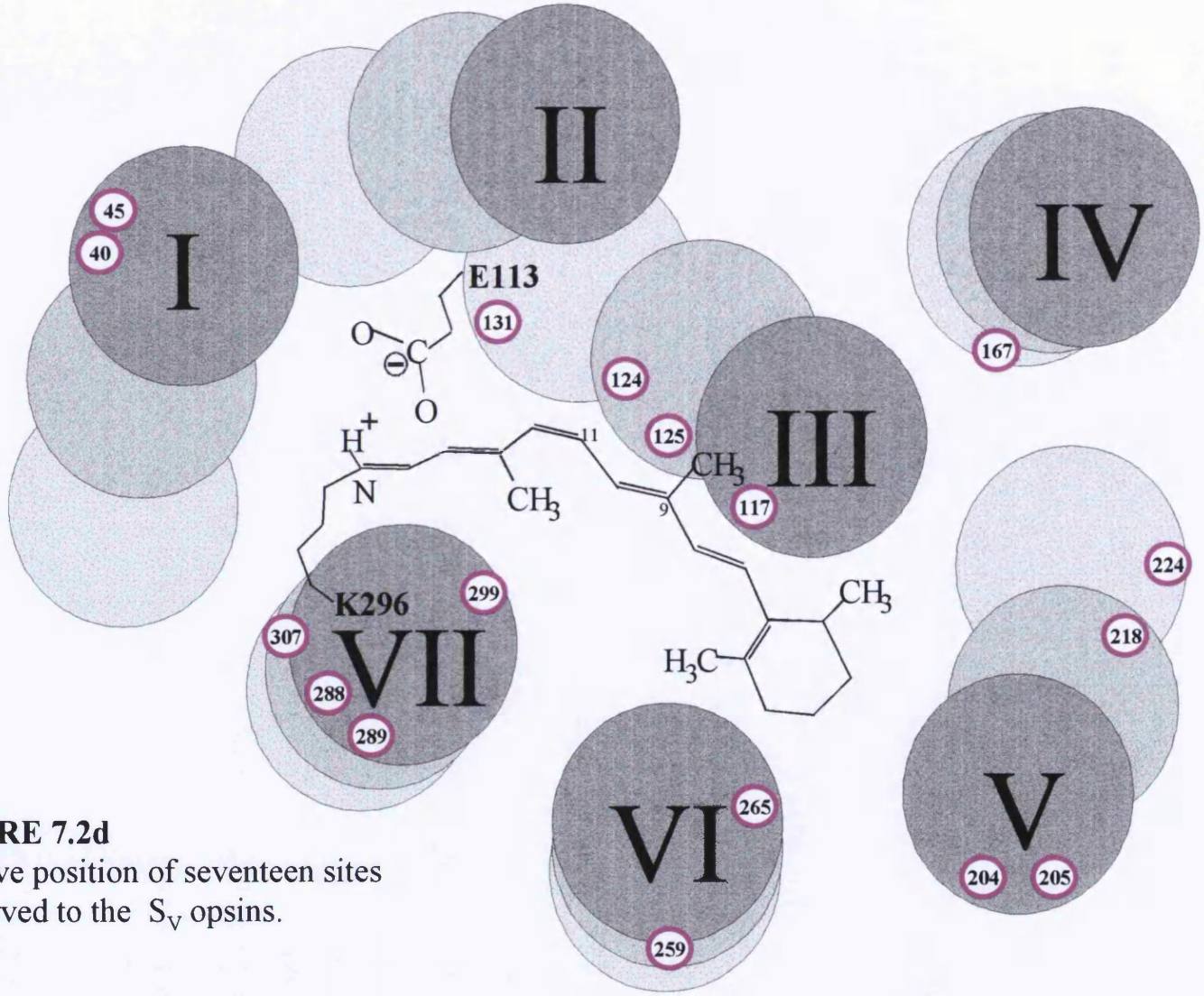


FIGURE 7.2d
Relative position of seventeen sites conserved to the S_V opsins.

TABLE 7.2f

The 8 sites shown in the table have all been proposed as possible tuning sites for S_V opsins. All of these sites are found in the transmembrane helical regions. Stars indicate those sites thought to face into the retinal binding pocket (Baldwin, 1993). Amino acid numbering is as per bovine rod opsin. Charged/polar residues are underlined.

aa no.	Previously sited by:		Ck 418	Hu 419	Ost 405	Rh ?	Pg 409	Ca 366	Bu 371	Gf 360	Ms 360
86	3,4,5	*	S	L	F	F	S	C	A	F	F
90	2,3,5	*	S	S	C	C	S	C	C	S	S
93	4, 5		V	P	I	M	T	T	T	Q	T
118	4,5	*	T	T	S	S	A	A	A	S	S
119	4		H	V	T	T	T	T	T	I	V
168	6	*	G	S	S	S	A	A	A	A	S
292	1,3	*	A	S	A	A	A	A	A	S	A
298	4,5		A	A	S	A	S	S	A	S	S

1 – Chang *et al.*, 1985; **2** – Yokoyama (1985); **3** – Lin *et al.*, 1998; **4** – Wilkie *et al*, 1998; **5** – Das *et al.*, 1999; **6** – this thesis.

FIGURE 7.2e
The relative position of eight sites possibly important in spectral tuning among the S_V opsins.

It has been shown that the retinal binding pocket in which the chromophore resides is created by the seven transmembrane helices of an opsin forming a bundle within the membrane, thus creating a hollow cavity on the extra-cellular surface (Baldwin, 1993; Schertler *et al.*, 1983; Unger *et al.*, 1997). This diagram illustrates a schematic three-dimensional opsin structure that provides some indication of the position of the α-helices relative to eachother and the shape of the retinal binding pocket. Each helix is shown as three disks, with the central disk representing a theoretical section through the centre of that helix with the accompanying darker and paler disks respectively representing a section at +3.0Å and -3.0Å relative to the centre disk. The retinal chromophore has been schematically placed within the retinal binding pocket and its site of attachment symbolised by K296 with the Schiff's base counterion shown as E113.

The eight sites shown in **Table 7.2f** have been added to the opsin diagram. The approximate position of these sites within the helices are indicated by their amino acid number (circled in violet) as per bovine rod opsin.

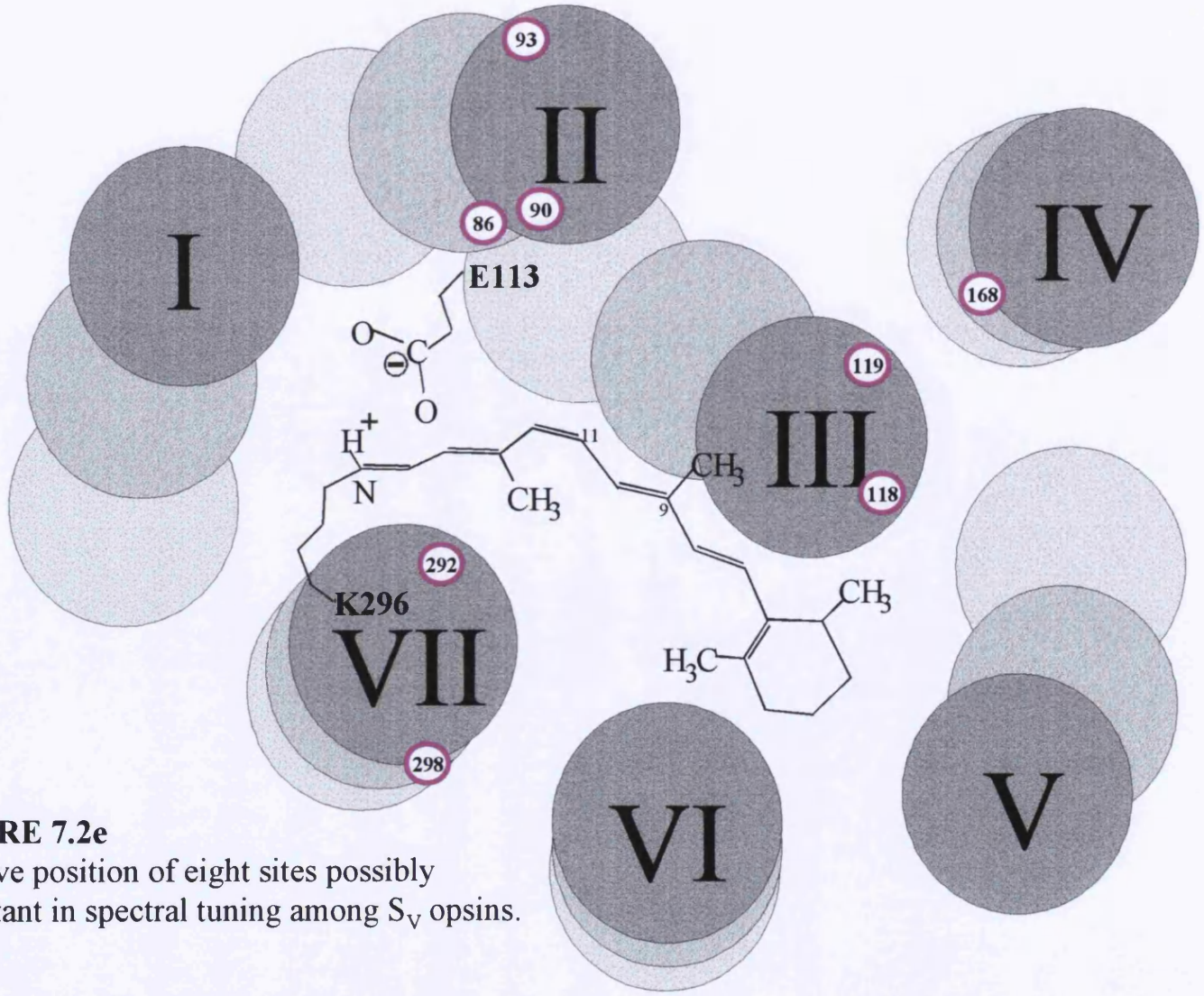


FIGURE 7.2e
Relative position of eight sites possibly important in spectral tuning among S_V opsins.

DISCUSSION

7.3 *Opsin Sequences*

The nucleotide sequences of the seven opsins elucidated for this study and the deduced amino acid translation (**Figures 7.0a – 7.0j**) each contain the characteristic seven helical regions that are typical of G-protein receptors such as opsins. All five opsin classes have been identified for the ostrich and two of these sequences, LWS and rod opsin, can be compared to those identified in the rhea (**Figures 7.0a & 7.0c**). This comparison reveals fifty nine base pair substitutions (93.5% identity) and only eight amino acid substitutions (97.2% identity) between the two paleognathous LWS cone opsins. Furthermore, the two rod opsins differ by only nineteen nucleotides (98.1% identity) and just one amino acid (99.4% identity). The degree of identity between the LWS opsins of ostrich and rhea indicates that these two opsins are highly homologous. However, such levels of identity are standard among avian LWS opsins, for instance the amino acid identity between canary and chicken LWS opsins is 93% (Das *et al.*, 1999). Avian rod opsins are generally more conserved than the other opsin types, for instance there is a 97.5% amino acid identity between canary and chicken rod opsins (Das *et al.*, 1999), however, the identities observed between the two paleognathous rod opsins are much higher. The ostrich and rhea belong to very closely related sister groups (e.g. Härlid *et al.*, 1997, 1998; Groth & Barrowclough, 1999) and so it could be suggested that the intense similarity in the rod opsins of these two species reflects this close relationship. Perhaps, if the rod opsins of two other avian species that are as closely related were compared the data yielded would be comparable.

The 3' end of the ostrich VS cone opsin has been identified (**Figures 7.0i & 7.0j**). A poly (A) addition signal (AATAAA) is present within the 3' untranslated region, 21 base pairs upstream of the poly (A) tail. This is identical to the position of the poly addition signal in canary (Das, 1997).

There are several key features that are ubiquitous to opsins as described in section 3.3, these are shown briefly below:

- Lys-296, which is the site of attachment of chromophore to the Schiff's base.

- Glu-113, the Schiff's base counterion.
- Glycosylation sites (Asn residues) near the N-terminal
- Palmitoylated Cys residues in the C-terminal, which anchor the tail to form a fourth cytoplasmic loop.
- Cys-110 & Cys-187, which form a disulphide bridge to ensure the correct folding and formation of the opsin.
- Ser & Thr residues in the C-terminal, thought to be sites of phosphorylation for opsin kinase.

Where the necessary sequence is available for comparison (mainly helical regions), the seven opsins identified in this study (**Figures 7.0a – 7.0j**) possess all the proposed ubiquitous opsin features from the list above. Ostrich and rhea opsins possess only one Cys residue (rather than two) at the palmitoylation site in the C-terminal, however, this has also been observed in other avian sequences (e.g. canary cone opsins, Das *et al.*, 1999) and this single residue probably still acts to anchor the C-terminal segment to the membrane. All of the opsins also possess Glu-134 and Arg-135, which are thought to interact directly with the G-protein transducin (Franke *et al.*, 1990).

7.4 *Sequence Comparison*

The inclusion of ostrich and rhea opsins in a phylogenetic tree of vertebrate opsins (**Figure 7.1b**) clearly provides evidence that these opsins are most closely related to other avian opsins. Each paleognathous opsin class distinctly clusters around the same five classes identified for neognathous birds (Bowmaker & Hunt, 1997), these being L, M_R, M_C, S_B & S_V opsins. For instance, the ostrich and rhea rod opsins are in the same clade (M_R opsins) as the rod opsins of the chicken and canary, but distinct from other avian L, M_C, S_B & S_V opsin classes. Within each opsin class the paleognathous opsins appear to be most closely related to each other, then to the other avian opsins and finally to other vertebrate opsins in that class e.g. goldfish (*Carassius auratus*). This is corroborated by evidence of the identity of paleognathous opsins in comparison to the canary and chicken opsins (**Table 7.1b**), in which each opsin class for ostrich and rhea is highly homologous to those found in these neognathous birds, suggesting a close relationship between these groups.

If the paleognathous opsins had formed their own clades that were clearly distinct from those of neognathous opsins, this would have provided evidence for the phylogeny of paleognathous birds as the first offshoot from the main avian stem. Instead, the clear grouping of ostrich and rhea opsins together with other bird opsins observed in this study only suggests that the paleognathous birds are a distinct, closely related group within class Aves.

7.5 *L Opsins*

As discussed in section 3.5, chloride (anion) sensitivity is ubiquitous to LWS cone visual pigments. Wang *et al.* (1993) determined that sites 181 and 184 (bovine rod numbering) were important in LWS opsins for binding Cl^- ions. His-181 and Lys-184 are conserved across most vertebrate LWS opsins including those of ostrich and rhea (**Table 7.2c**). Contrary to most other L opsins the mouse (*Mus musculus*) 'green' pigment possesses Tyr-181 and this substitution is reflected in its λ_{max} (508 nm) which is substantially blue-shifted (Sun *et al.*, 1997), however the loss of the chloride shift does not fully account for all of this spectral shift. Recent studies of the 'green' opsin of mouse (Sun *et al.*, 1997) and the 'red' opsin of the bottle-nosed dolphin, *Tursiops truncatus* (Fasick & Robinson, 1998; Fasick *et al.*, 1999) suggest that site 292 (which is thought to face the retinal binding pocket; **Figure 7.2b**) is an important site in spectrally tuning L opsins. All vertebrate L opsins, including those of ostrich and rhea, possess an alanine residue rather than the serine found in the blue-shifted mouse and dolphin L opsins at site 292 (**Table 7.2c**).

Other tuning sites for L opsins have been elucidated by comparison of human L_G and L_R opsins. For instance Asenjo *et al.* (1994) identified seven further amino acids that are involved in spectrally tuning human red and green pigments, these were sites 100, 164, 214, 217, 261, 269 and 293. At each of these sites the ostrich and rhea typically possess those residues identified for chicken and canary L opsins (**Table 7.2c**). The similarity between the avian L opsins at the proposed tuning sites most likely reflects the close relationship and the near identical λ_{max} values (all around 570 nm; **Table 6.4**) of these pigments. This is corroborated by evidence that paleognathous LWS opsins have around 95% amino acid identity with neognathous LWS opsins (**Table 7.1**). However, the avian opsins are not conserved across all the sites proposed by

Asenjo *et al.* (1994). At site 293, most of the avian LWS opsins possess a polar Tyr residue whereas the canary LWS opsin has the non-polar Phe residue that is also found in human red and green opsins. This can probably be explained by the position of this residue, which is placed on the outer edge of helix V facing away from the retinal binding pocket (**Figure 7.2b**). It must also be remembered that these proposed tuning sites are for human red and green opsins and that avian LWS opsins may not use all the same sites in their spectral tuning.

Whilst this comparison of avian LWS spectral tuning is obviously limited to those birds for which LWS opsin sequences are available, the spectral sensitivities measured by MSP in other bird species suggest that this is not the complete picture. The tawny owl (*Strix aluco*) and penguin (*Spheniscus humboldti*) LWS cones have been measured with λ_{max}'s of 555 nm and 543 nm respectively (Bowmaker & Martin, 1978, 1985). These pigments are substantially blue-shifted compared to those of other avian species and must possess differences in their overall tuning mechanism.

It is interesting that the three spectral locations observed in avian LWS pigments, around 570, 555 and 543 nm (**Table 6.4**) are very similar to the 563, 556 and 543 nm measurements observed for the three allelic variants of the single polymorphic middle- to longwave pigment in certain New World primates (Bowmaker *et al.*, 1984, 1985; Jacobs, 1984; Travis *et al.*, 1988; Neitz *et al.*, 1991; Williams *et al.*, 1992; Ibbotson *et al.*, 1992). It would seem that the LWS pigments from these different Orders are clustering around the same three spectral locations. Dartnall & Lythgoe (1965) suggested that maximal sensitivities of visual pigments do tend to cluster around specific regions of the spectrum. This may be due to constraints within the opsin structure as to where specific amino acids can be located. If the New World primates and avian LWS pigments possess the same spectral tuning sites, then it is likely that the structural constraints of opsins are determining the spectral locations of these spectral clusters. However, primate and avian L opsins do not vary at the same sites.

7.6 *M_{Rd} & M_C Opsins*

The two paleognathous rod opsins share a 96-98 % amino acid identity with neognathous avian rod opsins (**Table 7.1**). Avian rod opsins are also closely related to the MWS avian opsins sharing around 70 % homology (data not shown) and the phylogeny of vertebrate opsins suggests that rod and MWS avian opsins probably occupy 'sister' clades (**Figure 7.1b**). The λ_{max} values for the rod (503 –509 nm) and MWS (502 – 508 nm) pigments of both paleognathous and neognathous birds are strikingly similar (**Table 6.4**). The similarity in both the sequence identity and spectral sensitivity of these two avian opsin groups suggests that they may possess similar mechanisms for spectral tuning. Indeed, at most of the sites that face the retinal binding pocket (**Figure 7.2a**) vertebrate MWS and rod opsins possess the same residues. There are however, a few exceptions (**Table 7.2b** reveals a comparison of eight sites where this is the case). Ostrich and rhea rod and MWS opsins possess the identical residues to those found in neognathous opsins at these sites.

Site 122 is thought to be involved in the rate of regeneration and metarhodopsin II decay of rod opsins (Weitz & Nathans, 1993). The E122Q substitutions between rod and MWS opsins probably partially explain the differential kinetics of these processes in rod and cone opsins (Imai *et al.*, 1997). However, substitutions between charged and uncharged amino acids at this site would be expected to produce a 15-20 nm hypsochromic shift in MWS opsins as compared to rod opsins (evidence from site-directed mutagenesis of human and bovine rod opsin, Sakmar et al, 1989; Nakayama & Khorana, 1990). Therefore, compensatory red-shifting tuning sites are required in the MWS opsins and sites 222 and 295 have been proposed as possibilities for this role (Okano *et al.*, 1992; Heath *et al.*, 1997; Das *et al.*, 1999) since they contain non-conservative substitutions, C222S and A295S, that face the retinal binding pocket (**Figure 7.2a**). Consistent with this hypothesis, ostrich and rhea MWS and rod opsins contain these same substitutions (**Table 7.2b**). His-211 is conserved across all avian MWS and rod opsins (including those of ostrich and rhea) but is replaced by Cys-211 in other opsin pigments; this site is thought to be involved in stabilizing the metarhodopsin II conformation of bovine rod opsin (Weitz & Nathans, 1992).

7.7 *S_B Opsins*

Mechanisms for blue-shifting visual pigments are discussed in section 3.7. It is suggested that polar amino acids around the protonated Schiff's base increase the overall stability of the chromophore and thus increase the energy differential between ground and excited states thereby producing a blue-shift in the opsin. Sites 124, 289, 292 and 307 were all proposed as potential spectral tuning sites for the SWS opsins by Chang *et al.* (1995). The SWS pigments from neognathous birds and ostrich all have the same conserved residues at these sites (**Table 7.2d**). From comparison of the helices of the vertebrate opsins (**Table 7.2a**), two further tuning sites can be suggested. Sites 83 and 299 possess polar residues that are only conserved across the SWS opsins (**Table 7.2a**) and face the retinal binding pocket (**Figure 7.2c**) thus making them potential tuning sites for this opsin class. Site-directed mutagenesis in bovine rod opsin, in which the native Asp residue at site 83 was substituted for an Asn residue (which is found at this site in all bird SWS opsins) produced a blue-shift of around 8.5 nm (Nathans, 1990).

The spectral sensitivity of the avian SWS pigments sequenced so far is highly conserved, each having a λ_{max} around 442-444 nm, with the only significant exception being chicken at around 455 nm (**Table 6.4**). Das *et al.* (1999) suggested two possible sites with substitutions (S208F & C269T) that could be responsible for the spectral shift in the chicken SWS pigment. Ostrich and budgerigar, like the chicken, both possess polar residues at site 269 (**Table 7.2d**) and so this implies that this site does not have a short-wave tuning function. Site 208 however, has non-polar residues in the ostrich and other avian SWS opsins compared to the polar residue in chicken, making this therefore a good candidate as a tuning site. The polar threonine residue at this site is ideally positioned to have an effect on the spectral sensitivity of the visual pigment since it faces the conjugated side chain and ring of the chromophore (**Figure 7.2c**). Chan *et al.* (1992) suggested that polar residues along the conjugated side chain of the chromophore can increase delocalisation (thereby reducing the energy differential) and promote a shift to longer wavelengths, this could explain the red-shifted chicken SWS pigment.

7.8 *S_V Opsins*

The λ_{max} of opsins in this group ranges from around 365 – 420 nm (**Table 6.4**). A number of sites have been proposed for tuning visual pigments down to violet wavelengths (**Table 7.2e**), although any site which contains a residue that faces the retinal binding pocket and is conserved only to S_V opsins is a potential tuning site. A good example is site 265, at which all other opsin groups possess a Trp residue but all SV opsins have a Tyr instead. A W265Y mutant of bovine rod opsin creates a 15 nm shift to shorter wavelengths in this pigment (Nakayama & Khorana, 1991). Another option when analysing avian UV/VS opsin sequence comparisons (**Table 7.2a**) is to look for sites facing the chromophore pocket that are generally conserved across other opsin groups but possess non-conservative substitutions within that opsin group.

A number of sites have been suggested that are believed to spectrally tune S_V opsins within the UV to violet wavelengths of the spectrum (**Table 7.2f**). Within this range the S_V opsins appear to be clustered around certain spectral locations, these are around 360-370, 400-410 and around 420 nm (**Table 6.4**). Such clustering suggests that there are numerous sites ubiquitous to these opsins that are involved in determining the maximal sensitivity of the S_V opsin in each species.

MSP evidence suggests that ostriches possess a fourth cone class that lies within the 400 – 410 nm cluster of pigments (**Figure 6.0e**). The 405 nm measurement of λ_{max} of this pigment is only from one cell and must be taken with some caution, however, measurements of the absorbance of the lens in the ostrich eye indicates that it is similar to that of the duck in substantially filtering UV wavelength light (**Figure 6.2**). The ostrich is very unlikely therefore to possess a pigment that clusters within the 360 – 370 nm bracket.

Of the tuning sites shown in **Table 7.2f**, ostrich and rhea data does not support sites 86, 90, 292 and 298 as possible tuning sites but does corroborate sites 93 and 118. Another site suggested by this study, is site 168. This site faces the retinal binding pocket and contains a polar residue (Ser) in ostrich and rhea VS opsins with all other avian SV opsins possessing a non-polar residue at this position. It must be remembered that individual tuning sites must never be thought of in isolation. The

spectral tuning of a specific opsin is influenced by the entire amino acid make-up of that opsin. Site-directed mutations that produce a large spectral shift in one opsin may produce an entirely different response in another opsin. The only way to gain a purposeful indication of the effect of these sites in the ostrich is to produce site-directed mutants of the ostrich VS opsin and express them.

An assumption that has been made in analysing these results is that the ostrich and rhea VS opsins have a similar λ_{max}. This is a logical assumption to make, since these two species are closely related and all other data accrued in this study for these two species has been almost identical.

CONCLUSIONS

8.0 CONCLUSIONS

Microspectrophotometry (MSP) was used to determine the absorbance spectra of both rod and cone visual pigments and oil droplets from the retinae of the ostrich (*Struthio camelus*) and rhea (*Rhea americana*). The retinal photoreceptor complement found in the ostrich and rhea is remarkably similar to that found in most neognathous birds. In both birds, the retina comprises rods, double cones and four classes of single cone and have the potential for tetrachromatic colour vision.

The rods have λ_{max} at about 505-nm, whereas three cone pigments were recorded with λ_{max} at about 570, 505 and 445 nm. The 570-nm pigment is located in both members of the double cones and in a class of single cone containing a red (R-type) oil droplet with λ_{cut} at about 560-nm. The 510-nm and 445-nm cone pigments were found in populations of single cones containing yellow (Y-type) and clear (C-type) oil droplets with λ_{cut} at about 500 and 420-nm respectively. Double cones possess a pale (P-type) droplet in the principal member and a small droplet containing low concentrations of carotenoid (A-type) in the accessory member. The fourth class of single cone contains a transparent (T-type) droplet and in the ostrich a 405-nm pigment has been characterised.

This research represents the first comprehensive analysis of the retinal photoreceptor complement of paleognathous birds. The most striking feature of the data from the ostrich and rhea is their great similarity to each other in terms of all aspects of their retinal photoreceptors. This may reflect both their close phylogenetic relationship and the similarity of their environment and visual behaviour. The λ_{max} of each visual pigment is similar to those identified for other avian species, and the type of associated oil droplet found in each cone class is also typical.

Since the arrangement of paleognathous retinae is so similar to most neognathous birds, the observed pattern may simply reflect a fundamental avian retinal system that is conserved across species, but modified in species such as nocturnal birds and penguins occupying 'extreme' photic environments.

Light and fluorescence microscopy of whole fresh tissue mounts was used to determine the relative numbers of oil droplets in the retinae. The relative percentage of each cone class is similar between paleognathous and neognathous birds. Across avian species it appears that when the fourth cone class of a bird is VS, rather than UVS, then the cones in this class tend to make up a lower percentage of the total photoreceptor population.

Nucleotide sequences from opsin genes of both the ostrich and rhea have been identified. Approximately 1Kb of partial cDNA sequence of the rod and LWS opsins for both species and the MWS, SWS and VS cone opsins of the ostrich was cloned and sequenced. All of the opsins from these two birds can be classified into typical vertebrate opsin classes.

Comparison of the α-helical sequences of the LWS and rod opsins from ostrich and rhea give further indication of the close relationship between these two species. The two paleognathous rod opsins share a 99% amino acid identity with eachother compared to 96-98% with neognathous avian rod opsins. The ostrich and rhea belong to very closely related sister groups and so it could be suggested that the intense similarity in the rod opsins of these two species reflects this close relationship. A comparison of the deduced amino acid translations for all the identified opsin sequences in this study with those identified for other avian species has been made. The paleognathous opsins are highly homologous to the opsins found for neognathous avian species. In particular the galliforms, an avian group which most molecular studies indicate are the closest relative to the paleognathous birds.

It is suggested that site-directed mutagenesis studies on the ostrich VS opsin of the spectral tuning sites previously identified for avian UV/VS opsins are required to elucidate the correct tuning mechanism of this opsin type.

In conclusion, this study indicates that the retinal photoreceptor complement of paleognathous birds is not significantly different from that found in most neognathous birds.

APPENDICES

9.0 ABBREVIATONS FOR AMINO ACIDS

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

9.1 RECIPES FOR BUFFERS AND OTHER SOLUTIONS

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

Loading buffer for Agarose Gels:

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

NE buffer:

	150 mM	sodium chloride
	25 mM	EDTA

TAE buffer:

For 500 ml 50X:	121 g	Tris (0.04 M)
	28.6 ml	glacial acetic acid
	9.3 g	EDTA pH 8.0 (0.001 M)
	made up to 500 ml in dd H_2O.	

TBE buffer:

For 1 litre 10X:	108 g	Tris (0.045M)
	55 g	boric acid
	9.5 g	EDTA (0.001 M)
	made up to 1 litre in dd H_2O.	

TE buffer:

For 1 litre 10X:	12.11 g	Tris (0.01 M)
	3.7 g	EDTA (0.001 M)
	made up to 1 litre in dd H_2O.	

Denaturing Solution:

	43.83 g	sodium chloride (1.5 M)
	10 g	sodium hydroxide (0.5 M)
	made up to 500 ml in dd H_2O.	

Neutralising Solution:

43.83 g	sodium chloride	(1.5 M)
30.28 g	Tris pH 7.2	(0.5 M)
186 g	EDTA	(0.001 M)

made up to 500 ml in dd H_2O.

Ampicillin:

A 50 mg/ml stock solution of ampicillin was made in ddH_2O, filter sterilised through a 0.22-micron filter 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	sodium hydroxide (1M)

made up to 1 litre with dd H_2O.

For LB & ampicillin:

A final antibiotic concentration of 100 μg/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.

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10.0 REFERENCE LIST

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